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Quantitative Analysis of Cellulose-Reducing Ends

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Abstract

Methods for the quantification of total and accessible reducing ends on traditional cellulose substrates have been evaluated because of their relevance to enzyme-catalyzed cellulose saccharificaion. For example, quantification of accessible reducing ends is likely to be the most direct measure of substrate concentration for the exo-acting, reducing end-preferring cellobiohydrolases. Two colorimetric assays (dinitrosalicylic acid [DNS] and bicinchoninic acid [BCA] assay) and a radioisotope approach (NaB^3H_4 labeling) were evaluated for this application. Cellulose substrates included microcrystalline celluloses, bacterial celluloses, and filter paper. Estimates of the number of reducing ends per unit mass cellulose were found to be dependent on the assay system (i.e. the DNS and BCA assays gave strikingly different results). DNS-based values were several-fold higher than those obtained using the BCA assay, with fold-differences being substrate specific. Sodium borohydride reduction of celluloses, using cold or radiolabeled reagent under relatively mild conditions, was used to assess the number of surface (solvent-accessible) reducing ends. The results indicate that 30–40% of the reducing ends on traditional cellulose substrates are not solvent accessible; that is, they are buried in the interior of cellulose structures and thus not available to exo-acting enzymes.

Index Entries: Cellulose; cellobiohydrolase; reducing sugar assays; insoluble reducing ends; solvent-accessible reducing ends.

Introduction

Typical kinetic analyses of enzyme-substrate systems are based on observed rates of enzyme-catalyzed reactions over a range of enzyme and substrate concentrations. Analyses of this type are commonplace for soluble enzyme-soluble substrate systems in which measures of substrate and

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enzyme concentrations are rather straightforward. However, the biological world is replete with soluble enzyme-insoluble substrate systems for which measurements of substrate concentration can be rather ambiguous. Systems of this type include lipases, proteases, and many of the glycosyl hydrolases that act on polysaccharides. In this article, we address the concern of how to best measure the substrate concentration in cellulase-cellulose systems. More specifically, we focus on the substrate parameters that are of most relevance to the activity of exo-acting cellulases, hereafter referred to as cellobiohydrolases (CBHs).

Fungal cellulase enzyme systems capable of efficiently catalyzing the hydrolytic degradation of crystalline cellulose are typically composed of endo-acting cellulases (EGs), exo-acting cellulases (CBHs), and at least one cellobiase *(1–6)*. The CBHs are typically the predominant enzymes, on a mole fraction basis, in such systems *(7)*. Consequently, the CBHs have been the focus of many studies *(8)*. The three-dimensional structure of prototypical CBHs is known *(9–12)* and their specificities are, in general, well characterized *(13,14)*. However, mechanism-based kinetic analyses of CBH-catalyzed cellulose saccharification are rather limited *(15,16)*. Studies of this latter type are particularly difficult owing to the inherent complexity of native cellulose substrates.

A fundamental dilemma that must be addressed when analyzing CBH-cellulose reaction mixtures is how to express substrate concentrations. Substrate concentrations for cellulase-cellulose reaction mixtures in general are most commonly expressed in units of mass (such as milligrams of cellulose per reaction mixture) or enzyme-accessible surface area *(17)*. The latter values are typically obtained from solute exclusion experiments *(17–19)*. Expressing substrate concentrations in terms of surface area is theoretically appealing in that it seems to better represent the amount of substrate actually available to the enzymes *(20,21)*. The same reasoning suggests that it would be beneficial, at least for some analyses, if measures of substrate concentration for reaction mixtures containing only exo-acting cellulases were based on cellulose chain ends.

In this article, we address the issue of determining substrate concentrations based on cellulose chain ends. Typical insoluble cellulose substrates were evaluated. Our study focused on the analysis of reducing ends, rather than non reducing ends, because of the relative ease with which reducing ends can be detected and because of their importance with respect to reducing end-specific CBHs. The results are expected to be of relevance to those considering factors that dictate rates of CBH activity on typical cellulose substrates.

Materials and Methods

Preparation of Cellulose Substrate

Microcrystalline cellulose (MCC) was obtained commercially (Avicel, PH 101, FMC, Philadelphia, PA). Amorphous microcrystalline cellulose

(AMCC) was produced from MCC by the method of Isogai and Atalla *(22)* using an SO_2-diethylamine-dimethylsulfoxide (DMSO) solvent for cellulose dissolution. Phosphoric acid swollen cellulose (PSC) was prepared according to Ståhlberg et al. *(23)*. Bacterial microcrystalline cellulose (BMCC) was prepared from cultures of *Acetobacter xylinum* (ATCC #23769). Cultures were incubated in nutrient medium–containing trays for 10 d at 30°C without shaking *(24)*. Surface layers of cellulose were harvested and purified by the method of Gilkes et al. *(25)*. Amorphous bacterial microcrystalline cellulose (ABMCC) was prepared by first soaking BMCC in DMSO for 2 h, followed by centrifugation and then two additional washes with DMSO. The BMCC was then treated as in the preparation of amorphous MCC, first being dissolved in the SO_2-diethylamine-DMSO solvent *(22)* and then precipitated, and subsequently washed, with cold water. The AMCC, BMCC, and ABMCC preparations were kept in water containing 0.02% sodium azide until used. Amorphous celluloses, including PSC, were prepared on a minimum of two occasions to ensure reproducibility. Filter paper, obtained commercially (Whatman no. 1; Maidstone, England), was added to reaction mixtures as 7 = mm diameter, 3.5-mg disks.

Colorimetric Reducing End Assays

All samples were analyzed in triplicate on at least two separate occasions.

Soluble-Phase Reducing Groups

The DNS (DNS and DNS_{sg}) were performed as described by Ghose *(26)*. Assays including "supplemental glucose," 100 µg, are herein referred to as DNS_{sg}. The Nelson-Somogyi copper-based assays were performed as described by Nelson *(27)* and Robyt and Whelan *(28)*. The traditional assay results in a final reaction mixture volume, prior to measuring absorbance, of 10 mL. We also included a series of assays for which final reaction mixture volumes were 5 mL ($Nelson_{5mL}$). The *p*-hydroxybenzoic acid (PAHBAH) assay was done according to Lever *(29)*. The assay based on BCA chelation of cuprous ions (BCA assay) was conducted as described by Garcia et al. *(30)*.

Insoluble-Phase Reducing Groups

The BCA assay, as applied to the quantification of insoluble reducing ends, was taken from Johnston et al. *(31)*. One milliliter of BCA "working reagent," (prepared as in Garcia et al., *[30]*) was added to glass tubes containing 1 mL of test cellulose suspension. The tubes were mixed, capped with glass marbles, and incubated at 80°C for 30 min. Tubes were then cooled to room temperature by standing in water, mixed, and transferred to 2-mL microcentrifuge tubes, and the cellulose was pelleted by centrifuging at 3000 rpm for 5 min. The absorbance, at 560 nm, of the cellulose-free supernatants was then determined. Nanomoles of reducing groups per milligram of cellulose was calculated from measured color yields for standard glucose samples (0–55 µ*M*) treated per the aforementioned protocol.

The DNS assay, as applied to the quantification of insoluble reducing ends, was taken from Irwin et al. *(32)*. DNS reagent, 1 mL, was added to 0.4 mL of cellulose suspension containing 200 nmol of cellobiose (cellobiose addition being analogous to using supplemental glucose in the DNS_{sg} assay). Reaction mixtures were then mixed, capped with glass marbles, and incubated in boiling water for 15 min. Following heating, the tubes were cooled to room temperature, again mixed, transferred to 2-mL microcentrifuge tubes, and centrifuged at 3000 rpm for 5 min. The absorbance of the resulting cellulose-free supernatants was measured at 600 nm. Nanomoles of reducing groups per milligrams of cellulose were calculated from measured color yields for standard cellobiose solutions (0–800 nmol/assay).

Solvent Accessible Reducing Ends

All samples were analyzed in triplicate on at least two separate occasions. Sodium borohydride solutions were prepared just prior to initiating experiments.

Sodium Borohydride Reduction

The "mild" $NaBH_4$ reduction was done at 22°C (room temperature) and pH 8.0 The slightly alkaline conditions were necessary to maintain reasonable stability for the $NaBH_4$ reagent. Sodium borohydride, 0.1 mL of 0.25 *M* $NaBH_4$ in 0.1 *M* NaOH, was added to test solutions containing 1 mg of cellulose suspended in 0.785 mL of 0.1 *M* sodium phosphate, pH 8.0 Reaction mixtures were mixed and allowed to react for up to 90 min at room temperature. Reactions were terminated at selected times by the addition of 20 µL of 37% (w/v) HCl. Terminated reaction mixtures were typically allowed to stand for 30 min prior to neutralization by the addition of 95 µL of 2 *N* NaOH. Separate experiments showed that residual sodium borohydride could not be detected following the low-pH 30-min incubation period.

Sequential Sodium Borohydride Treatments

MCC was used as a representative cellulose. Cellulose was initially reduced as described above. The terminated, neutralized reaction mixture was then centrifuged at 3000 rpm for 5 min and the supernatant removed. To the resulting cellulose pellet was added 0.785 mL of reaction buffer and 0.1 mL of fresh sodium borohydride (all concentrations as given above). The cellulose was suspended by mixing and the reaction allowed to proceed for another 90 min, at which time the reaction was terminated, and neutralized, and the cellulose was again separated via centrifugation. The same cycle was repeated a third time. In total, test celluloses received three sequential $NaBH_4$ treatments, each treatment using fresh reagents.

The "mild" reduction protocol, see previous section, was modified to simulate reaction conditions used in the colorimetric assays for reducing ends. This test included three treatments: (1) the "mild" protocol in 0.1 *M* sodium phosphate, pH 8.0, and 22°C; (2) the "high pH" treatment in 0.25 *M*

sodium carbonate/bicarbonate, pH 10.0, and 22°C; and (3) the "high-pH/high-temperature" treatment in 0.25 *M* sodium carbonate/bicarbonate, pH 10.0, and 80°C. As in the previous section, reactions were initiated by adding 0.1 mL of 0.25 *M* $NaBH_4$ in 0.1 *M* NaOH to 0.785 mL of cellulose suspension (containing 1 mg of cellulose). Reactions were allowed to continue for 90 min. The cellulose was then separated from the soluble phase, as just described, and the treatment was repeated. Samples received a maximum of five sequential $NaBH_4$ treatments (up to 450 min). Following the last treatment, reactions were terminated by the addition of HCl and subsequently neutralized with NaOH.

Reductions with Sodium Borotritiide

All reductions and subsequent treatments involving tritium, up to the scintillation counting, were done in a hood using 20-mL scintillation vials as reaction vessels. The NaB^3H_4-containing reducing solution was 0.2505 *M* $NaBH_4/NaB^3H_4$ in 0.1 *M* NaOH with a specific activity of 0.738 mCi/mmol of sodium borohydride. Thus, 100 µL of reducing solution contained 50 nmole of original NaB^3H_4 preparation (specific activity of 370 mCi/mmol; obtained from ICN, Irvine, CA) and 25 µmol of cold $NaBH_4$. Reductions were initiated by adding a 100-µL aliquot of NaB^3H_4-containing reducing solution to a 20-mL scintillation vial containing cellulose (0.1–1.0 mg of cellulose) suspended in 0.88 mL of 0.1 *M* sodium phosphate buffer, pH 8.0 Initiated reaction mixtures were mixed and allowed to proceed at 22°C for the desired reaction time. Reactions were terminated by the addition of 20 µL of 37% HCl. The acidified reaction mixture was allowed to stand open for a minimum of 30 min. Reaction mixtures were then evaporated to dryness on a hot plate at 50°C (approx 1 h). The evaporation step was necessary to drive off residual labeled $^3H_2$ gas *(33)*. The solid residue remaining was then resuspended in 1 mL of phosphate buffer and to that suspension was added 10 mL of scintillation cocktail (Scintisafe Gel; Fisher, Pittsburgh, PA). Samples were counted in a Beckman LS 6500 Scintillation System (Fullerton, CA) at 20 min per vial. Blanks containing no reducing sugar and "zero-time" samples to which the acid was added prior to the addition of reducing agent were included in all experiments. Corrected counts per minute were converted to micromoles of reducing ends per gram of cellulose using calibration curves, 0–40 nmol, with glucose as the calibration standard (tests showed that results based on cellobiose as the calibration standard gave equivalent results). As for previous experiments, all samples were done in triplicate on a minimum of two occasions.

Quantification of Reducing Ends on $NaBH_4$-Treated Celluloses

Reduced and neutralized cellulose preparations were assayed using the BCA assay as described for insoluble reducing ends using glucose as the calibration standard.

Table 1
Detection Limit, Linear Range and Working Range for DNS, DNS_{sg}, Nelson-Somogyi (Nelson 10 mL), Modified Nelson-Somogyi (Nelson 5 mL), PAHBAH,[a] and BCA Assays

Assay	Detection limit (µg glu)	Associated SE	Linear range upper limit (µg glu)	Working range (µg glu) [b]
DNS	55.0	0.058	>4000	80–257
DNS_{sg}	1.9	0.157	>4000	25–187
Nelson 10 mL	1.1	0.050	~400	22–170
Nelson 5 mL	0.66	0.024	~200	12–86
PAHBAH	0.22	0.024	>20 [a]	2–16
BCA	0.12	0.007	~10	1–8

[a] The PAHBAH calibration curve is not linear over a wide range, but for small ranges appears linear (with slope changes). The range reported here was used for determination of sensitivity and detection limit.

[b] Based on linear regression parameters, slope and intercept.

Results and Discussion

The present study was initiated in response to the desire to know the number of chain ends available to exo-acting enzymes in typical cellulose/cellulase reaction mixtures. Several laboratories have published relevant data. In each case the number of reducing ends associated with a particular cellulose preparation was estimated by adapting a colorimetric reducing sugar assay traditionally used with aqueous solutions. Colorimetric assays adapted in this way include those based on DNS *(32)*, PAHBAH *(34)*, Cu-arsenomolybdate (Nelson-Somogyi; *[23]*), and Cu-BCA *(31)*. Published studies give little indication of how results obtained with the different assays compare, making assay selection and data comparisons difficult. Thus, an objective of the present study was to provide such information, along with estimates of the number of reducing ends associated with traditional cellulose substrates.

Soluble-Phase Reducing Ends

Initial experiments were designed to obtain analytical parameters for common colorimetric reducing sugar assays when used to quantify soluble reducing sugars generated as a consequence of cellulose saccharification. The assays were run as published, with the exception of the more concentrated Nelson (5 mL) assay. The results are presented in Tables 1–3. Detection limits for all of the assays are near 1 µg (although values in this range differ by nearly 16-fold), with the exception of the traditional DNS assay, which is known to consume a fixed amount of analyte *(26)*.

Table 2
Calibration Sensitivity, Analytical Sensitivity, and SEs for DNS, DNS_{sg}, Nelson-Somogyi (Nelson 10mL), Modified Nelson-Somogyi (Nelson 5mL), PAHBAH, and BCA assays

	Calibration sensitivity (Abs/µg glu)		Analytical sensitivity (DAbs/µg glu) [a]			
Assay	Calibration sensitivity ($\times 10^{-3}$)	Associated SE ($\times 10^{-3}$)	Analytical sensitivity	Associated SE ($\times 10^{-2}$)	SD ($\times 10^{-3}$) [b]	Reference concentration [c]
DNS	3.95	0.042	1.780	1.91	2.22	90
DNS_{sg}	4.31	0.067	0.896	1.60	4.81	90
Nelson 10 ml	4.73	0.023	0.783	1.12	6.04	90
Nelson 5 mL	9.37	0.030	1.770	3.03	5.33	50
PAHBAH	50.40	0.880	3.880	7.38	13.40	12
BCA	106.00	1.070	15.400	40.33	6.87	5

[a] Signal change is defined as absorbance change for the assays.
[b] Pooled SD from different experiments.
[c] The concentration at which replicate measurements were analyzed to obtain pooled SDs.

Table 3
Cellodextrin Reponses for DNS, Nelson-Somogyi (Nelson), and BCA Assays

Assay [a]	Similar response of equimolar amounts of G, G2, G3	G		G2		G3	
		m_{ratio} [b]	b_{ratio} [c]	m_{ratio}	b_{ratio}	m_{ratio}	b_{ratio}
DNS	No	1.0	1.0	1.5	1.0	1.8	0.9
Nelson	Yes	1.0	1.0	1.0	1.0	1.0	1.0
BCA	Yes	1.0	1.0	1.0	1.0	1.0	1.0

[a] Not determined for PAHBAH assay.

[b] Calculated as the ratio of the calibration sensitivities (or slope m) of the different cellodextrin standard curves to the glucose standard curve. Thus, a ratio of 1 is expected, theoretically, for a true reducing sugar assay, which has the same molar color yield for a series of saccharides.

[c] Calculated as the ratio of the intercepts of the different cellodextrin standard curves to the glucose standard curve. Thus, a ratio of 1 indicates agreement in the intercepts between the cellodextrin and the glucose calibration curves.

The combined data provide a ready means by which to compare and select appropriate assays for application in cellulase-catalyzed cellulose saccharification experiments. Products in such experiments are expected to include glucose; cellobiose; and, potentially, some cellooligosaccharides. Optimum reducing sugar assays would have equivalent molar color yields for these soluble products. As shown in Table 3, this optimum situation only applies to the two copper-based assays (Nelson, BCA). Because of their importance with respect to the analysis of insoluble cellulose (discussed next), calibration curves reflecting the molar color yields for the DNS and BCA assays are presented in Fig. 1.

Insoluble-Phase Reducing Ends

The DNS- and BCA-based assays are frequently used for the quantification of insoluble reducing ends in cellulose/cellulase systems *(31, 32, 35–40)*. The relative merit of using the assays for this determination was evaluated by applying the assays, as published, to traditional cellulose substrates (Table 4). The results from both assays had relative errors of approx 3%. Figures 2–5, from which the values in Table 4 were calculated, illustrate the linear relationship between mass of cellulose and number of reducing ends for both assays. The linear relationships suggest that either method may be used to determine relative numbers of reducing ends for a given cellulose preparation (e.g., relative values for MCC-derived substrates). The similar values obtained for MCC, AMCC, and PSC, and for BMCC and ABMCC, further support this conclusion. The similarity in the results for the MCCs and the corresponding amorphous preparations demonstrates that the solid-state structure of the cellulose, typically expressed in terms of relative crystallinity *(41)*, had little effect on color yield. This conclusion is based on the premise that the method used for the prepa-

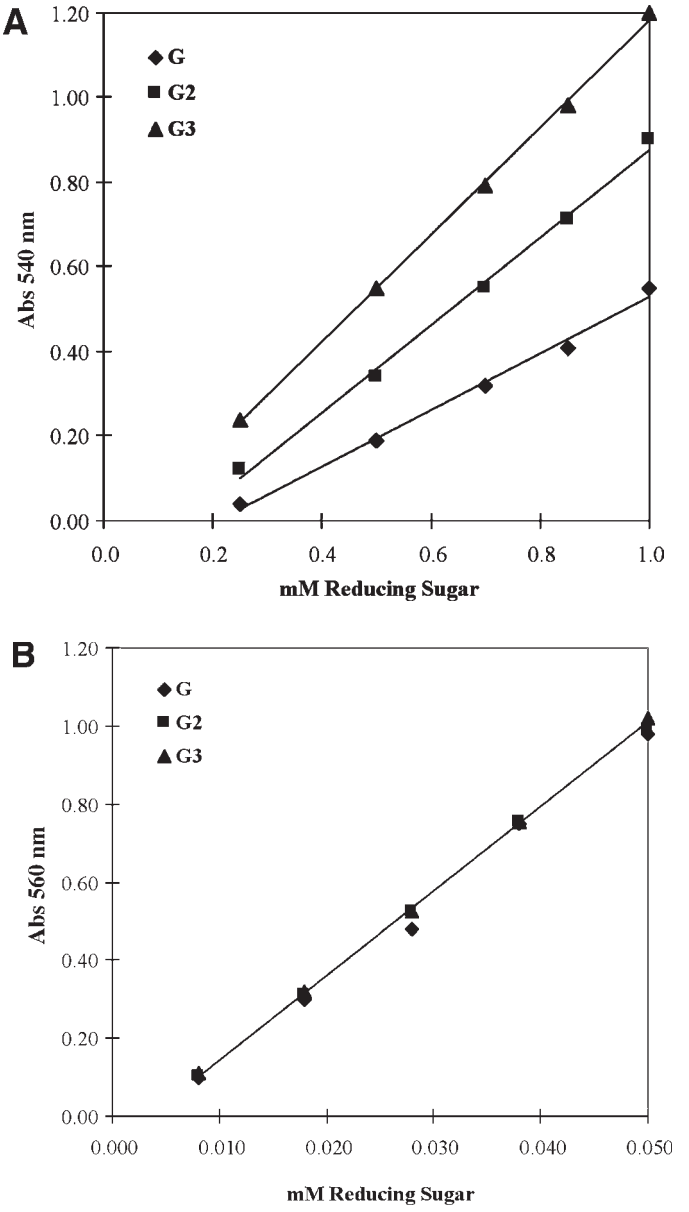

Fig. 1. Absorbance response of equimolar amounts of soluble cellulose saccharification products glucose (G), cellobiose (G2), and cellotriose (G3) by **(A)** DNS assay and **(B)** BCA assay.

ration of the amorphous celluloses did not result in significant depolymerization and concomitant production of new reducing ends (as shown by Isogai and Attala *[22]*). The ordinates of the BCA- and DNS-based plots in Figs. 2–5 are in units of glucose and cellobiose equivalents, respectively, owing to the use of the different sugars for the preparation of standard curves (*see* Materials and Methods).

Table 4
Reducing Ends Per Amount Cellulose as Determined by BCA and DNS Assays

Cellulose preparation	Amount (mg)	Reducing ends (μmol/g) SE	
		BCA[a]	DNS[b]
MCC	0.1 – 1.0	32.13 (0.38)	188.36 (3.27)
AMCC	0.1 – 1.0	30.40 (0.68)	197.39 (8.80)
PSC	0.1 – 1.0	30.02 (0.77)	179.27 (5.80)
BMCC	0.1 – 1.0	18.26 (0.25)	60.98 (1.00)
ABMCC	0.1 – 1.0	20.51 (1.93)	67.73 (1.12)
Filter Paper	3.5 – 17.50	1.25 (0.03)	10.50 (0.43)

[a] Glucose equivalents.
[b] Cellobiose equivalents.

An obvious conclusion from the data in Table 4 is that the DNS assay, compared with the BCA assay, consistently gives higher values for equivalent cellulose preparations. The fold-difference between the two assays was dependent on the source of the cellulose. Values for the MCC-based substrates (MCC, PSC, and AMCC) differed approximately six-fold. Those for filter paper differed approx eight-fold. The DNS values for the bacterial cellulose–based preparations (BMCC, ABMCC) were just over three-fold higher than the corresponding BCA values. The high DNS-obtained values, relative to the BCA-obtained values, are likely attributable to overconsumption of the DNS reagent as a result of the generation of reactive side products during the assay *(42)*. These reactive side products are likely to be a result of cellulose degradation under the relatively harsh conditions used for the DNS assay (100°C, pH 13.0, 15 min). The conditions for the BCA assay, by comparison, were relatively mild (80°C, pH 10.0, 30 min).

It is not clear why the fold-differences between the DNS- and BCA-obtained values vary to the extent they do for the different celluloses. As already discussed, the fold-differences are not likely attributable to differences in the crystallinity of the substrates—or to properties thought to be associated with crystallinity, such as porosity and surface area. The differences appear to be attributable to characteristics originating with the starting material, since MCC-based substrates have similar values, BMCC-based substrates have similar values, and so on. This suggests that the substrate-specific fold-differences between the DNS and BCA assays are related to the degree of polymerization (DP) of the substrates. The average DP of the filter paper (~1640) is clearly greater than that of the microcrystalline substrates (~220) *(43)*. The average DP for the different MCC- and BMCC-derived substrates is expected to be similar, and yet the corresponding fold-differences for these substrates differ nearly twofold. This apparent discrepancy may be explained by the DP profiles of the different substrates.

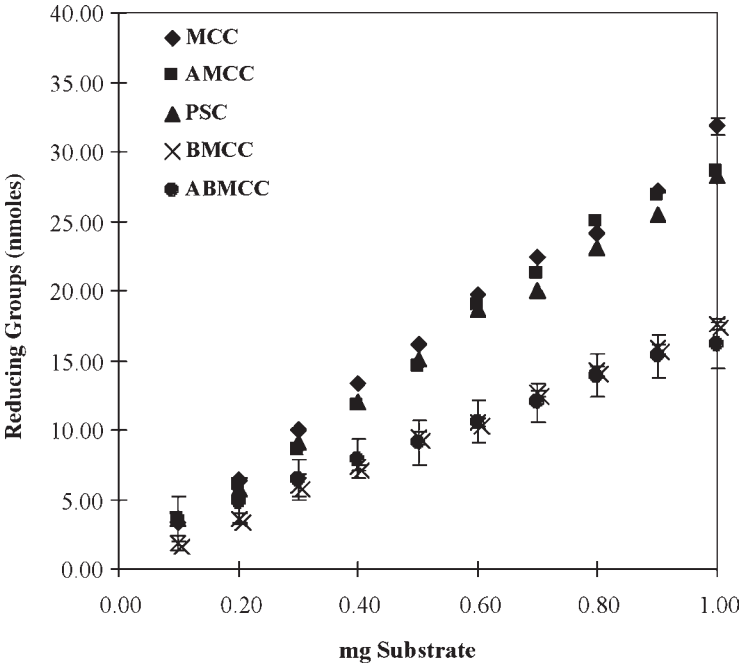

Fig. 2. Reducing groups per amount of cellulose determined by BCA assay for MCC (◆), AMCC (■), PSC (▲), BMCC, (×), and ABMCC, (●). Values represent the mean of triplicate determinations; error bars represent ± the SE. When not shown, the error bars fall within the symbols.

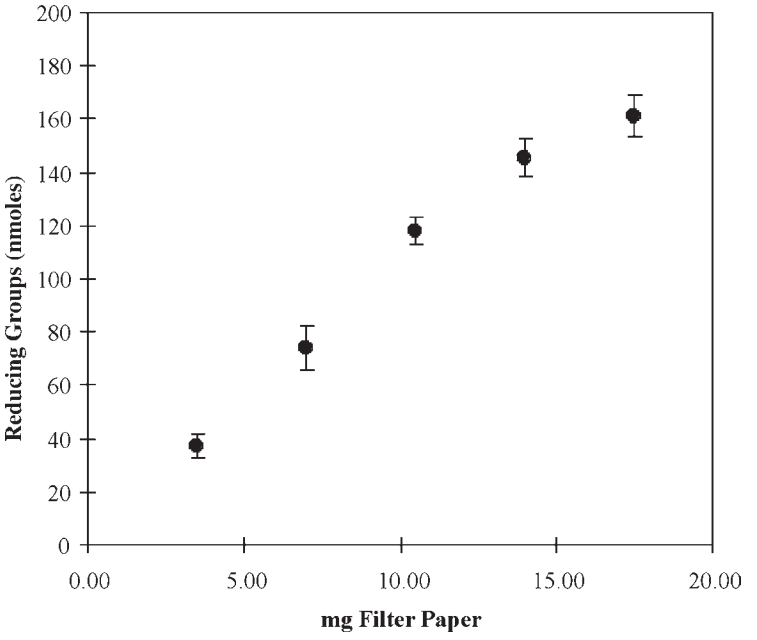

Fig. 3. Reducing groups per amount of filter paper determined by BCA assay. The statistical parameters are as in Fig. 2.

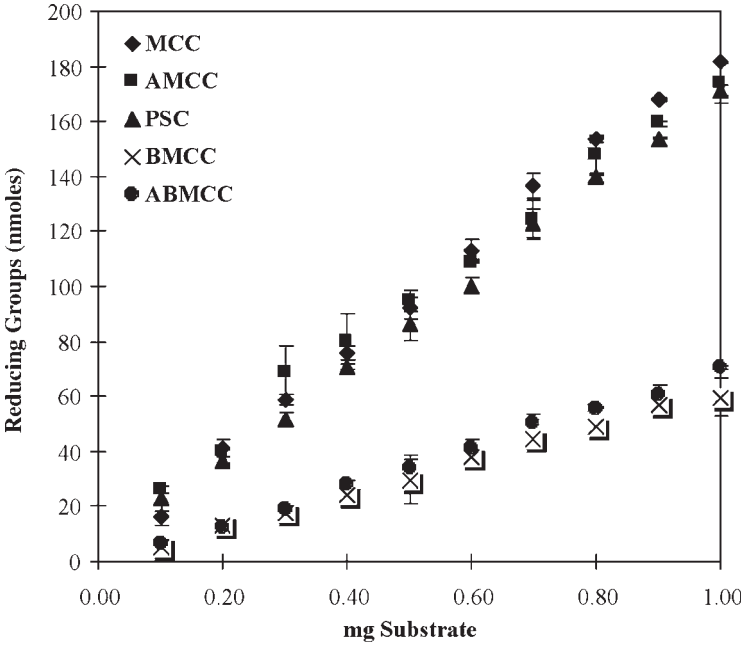

Fig. 4. Reducing groups per amount of cellulose determined by DNS assay for MCC, (◆), AMCC, (■), PSC, (▲), BMCC, (×), ABMCC, (●). The statistical parameters are as in Fig. 2.

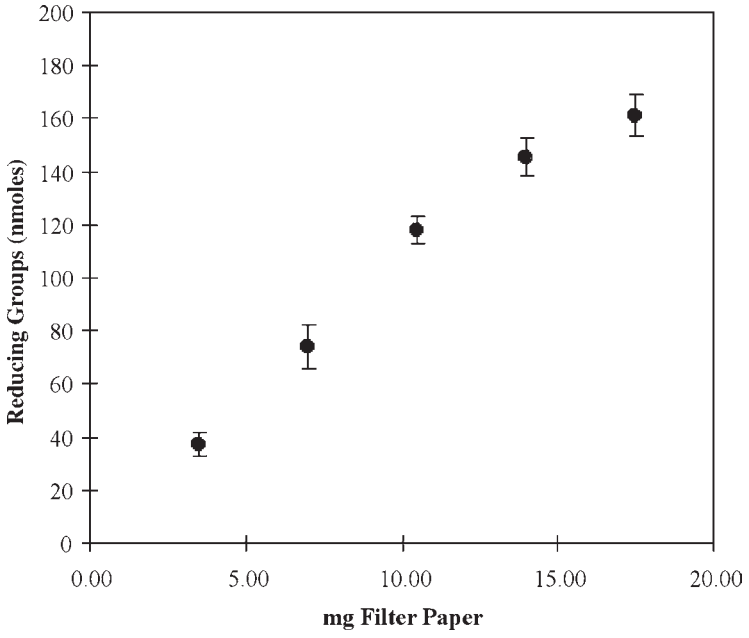

Fig. 5. Reducing groups per amount of filter paper determined. The statistical parameters are as in Fig. 2.

Stålbrand et al. *(44)* have shown that although the average DP values for an MCC- and a BMCC-derived substrate were similar, their DP profiles were significantly different. The BMCC substrate was shown to have a higher mole fraction of its constituent molecules in the low DP range. As discussed with relation to Fig. 1, the DNS assay is DP sensitive when working with cellooligosaccharides and, presumably, also with cellulose, whereas the BCA assay is not. Hence, it seems that if the observed substrate-specific fold-differences are a function of the cellulose preparations' DP, as is suspected, then that function is best considered in terms of the overall DP profiles of the substrates rather than simply average DP values.

The BCA assay results presented here for the MCC substrates are comparable with those obtained by Ståhlberg et al. *(23)* using the Nelson-Somogyi method. They reported ~37 µmol of reducing ends/g of cellulose for their microcrystalline (MCC) and phosphoric acid–regenerated cellulose (PSC) preparations. Similarly, Johnston et al. *(31)*, using the BCA assay, reported 30–35 µmol of reducing ends/g of phosphoric acid–regenerated cellulose. (The similar results for the Nelson-Somogyi and the BCA assays are expected since both are based on quantification of the cupric-to-cuprous reduction.) The DNS results for the filter paper substrate are in general agreement with those reported by Irwin et al. *(32)*. The similarity of the values obtained in our laboratory to those scattered in the literature indicates that the interlaboratory reproducibility of the assays, at least when applied to substrates for which we can make a comparison, is good.

Insoluble-Phase/Solvent-Accessible Reducing Ends

Values obtained using the DNS and BCA assays are presumably a function of the total number of reducing ends per unit weight cellulose. This is an important number for many purposes, but it may not be the most relevant value when considering enzyme-accessible reducing ends. The latter value is important when asking questions related to effective substrate concentrations for reducing end-specific exo-acting cellulases, a prototypical enzyme in this category being *Trichoderma reesei* CBH I *(10)*. An approach to obtaining these enzyme-applicable values is to determine the fraction of reducing ends exposed to solvent under conditions more conducive to enzyme activity. In the DNS and BCA assays, the cellulose is in a highly alkaline solution (pH ≥ 10.0) at elevated temperatures ($T \geq 80°C$). These conditions are conducive to cellulose swelling and thus increase solvent accessibility *(45)*, and they are clearly well outside those compatible with typical enzyme systems. The "enzyme-applicable" approach just suggested was implemented in this present work by first reducing the cellulose with $NaBH_4$ under relatively mild conditions ($NaBH_{4,\,mild}$), and then assaying the $NaBH_{4,\,mild}$-treated cellulose for remaining reducing ends (using the BCA assay as described for insoluble cellulose. The number of solvent-accessible reducing ends could then be calculated by taking the difference in the number of reducing ends associated with the $NaBH_{4,\,mild}$-treated and the untreated substrates. Results from such assays applied to the test

Table 5
Solvent-Accessible Reducing Ends per Amount of Cellulose as Determined by Combined $NaBH_4$-BCA and NaB^3H_4-Tritium Uptake Assays

Cellulose preparation[a]	Solvent-accessible reducing ends (mmol/g) $NaBH_4$-BCA[b]	NaB^3H_4-tritium uptake[b]	Solvent accessibility (% of total)[b,c]
Glucose	—	—	100
Cellobiose	—	—	100
MCC	17.12 ± 0.55	13.85 ± 0.50	60.00 ± 3.59
AMCC	23.63 ± 0.28	22.50 ± 0.64	72.37 ± 0.66
PSC	24.08 ± 0.99	20.93 ± 0.73	72.36 ± 2.03
BMCC	8.64 ± 0.46	14.55 ± 0.26	45.32 ± 2.34
ABMCC	10.29 ± 0.21	15.71 ± 0.73	49.04 ± 1.52
Filter Paper	0.773 ± 0.06	1.33 ± 0.08	59.22 ± 4.49

[a] As in Fig. 4
[b] ± SE.
[c] Calculated from BCA-obtained values in Table 4 and $NaBH_4$-BCA values in this table.

celluloses are presented in Table 5. While these values are not optimum for assessing the number of CBH-accessible reducing ends—the optimum would be an "enzyme-accessible" value rather than a "solvent-accessible" value—they are a significant improvement over the "total" reducing end values obtained by traditional colorimetric assays. A second assay was developed for our study in order to check the validity of the values obtained using the combined $NaBH_4$-BCA assay. In this second approach, the initial reduction was done using sodium borotritiide, and then the number of solvent-accessible reducing ends was determined by the extent of tritium incorporation into cellulose (*see* Materials and Methods for details). It can be seen that the results obtained using this isotope uptake approach are in general agreement with those obtained using the $NaBH_4$-BCA colorimetric approach (Table 5). The similarity in the values lends credibility to the numbers obtained from either assay.

A comparison of the relative accessibility of the different substrates' reducing ends, as determined by the combined $NaBH_4$-BCA assay, is also included in Table 5. The tabulated values were taken from the plateau region of the progress curves presented in Fig. 6. The time courses for reduction of the soluble substrates (glucose and cellobiose) show that all reducing ends are readily susceptible to $NaBH_4$ reduction under the chosen "mild" conditions. By contrast, the insoluble substrates all show a minimum of two classes of reducing ends; for simplicity, the two classes are heretofore referred to as those that are generally susceptible to $NaBH_4$ reduction and those that aren't. The percentages of reducing ends susceptible to $NaBH_4$ reduction were found to be similar for all of the celluloses, ranging from 45 (BMCC) to 72% (AMCC, PSC).

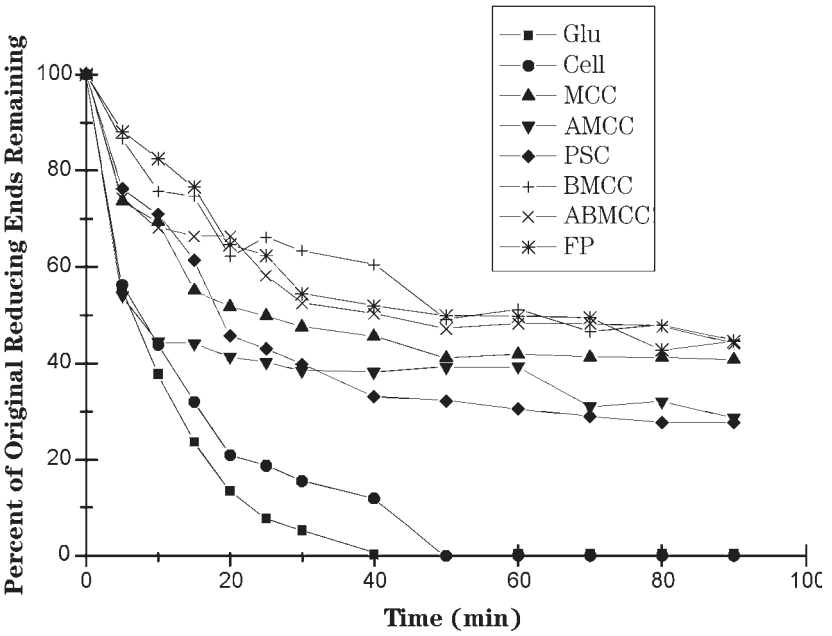

Fig. 6. Time courses of reduction of celluloses by $NaBH_4$ at pH 8.0, 22°C. Unreacted reducing ends were determined by BCA assay. The celluloses are as in Table 4. FP, filter paper.

A small increase (~20%) in solvent-accessible reducing ends was observed for the amorphous MCC-based preparations (AMCC and PSC) compared with the MCC starting material. This result can be rationalized based on the documented enhanced reactivity, presumably corresponding to a more open structure, of amorphous vs crystalline cellulose *(21,46)*. The fact that 30% of the reducing ends of the AMCC preparation are not susceptible to $NaBH_4$ reduction shows that, even with amorphous substrates, a significant fraction of the "total" reducing ends (as measured by traditional colorimetric assays) are not available as sites for initiation of reducing end–specific saccharification. The two BMCC-based substrates had essentially the same percentage of total reducing ends unavailable for reaction with $NaBH_4$. This result was not anticipated based on the presumed higher surface area associated with amorphous, vs crystalline, substrates. The somewhat unexpected lower accessibility of the reducing ends in the amorphous preparation (vs BMCC) suggests that amorphous aggregates formed during ABMCC preparation are not particularly solvent ($NaBH_4$) permeable; interestingly, they are less so than those in the analogous AMCC substrate preparation. The difference in the aggregation states of the two amorphous cellulose preparations (ABMCC and AMCC) detected, as a difference in the percentage of $NaBH_4$-susceptible reducing ends, is likely a result of complex aggregation events that occur during cellulose regeneration. The percent-

age of reducing ends accessible in the filter paper substrate was in line with that observed for the MCC and BMCC substrates.

Several experiments were conducted to evaluate the effect of reaction conditions on $NaBH_4$-accessible reducing ends. An initial concern in the pH 8.0/22°C reductions was that the observed plateaus in reduction time courses (Fig. 6) were the result of reagent decomposition. If this were the case, then the observed plateaus would reflect a lack of reagent rather than inaccessible reducing groups. The experiment depicted in Fig. 7 illustrates that this is not the case. In this experiment, the MCC preparation underwent the mild $NaBH_4$ treatment, as did the preparations depicted in Fig. 6, but at the completion of the typical time course the cellulose was washed and then again treated with fresh $NaBH_4$ reagent. Subsequent to this second $NaBH_4$ treatment the cellulose was again washed and $NaBH_4$ treated. The consequence of the three sequential $NaBH_4$ treatments was only a minimal decrease in residual BCA-detectable reducing ends when compared to the celluloses that had received only the single treatment (as in Fig. 6). This result provides strong support for the notion that the plateaus observed in Fig. 6 are owing to the $NaBH_4$-inaccessible nature of a significant fraction of the reducing ends associated with these cellulose preparations.

Time courses of $NaBH_4$ reduction of the MCC substrate under different pH/temperature conditions were compared to test the hypothesis that reaction conditions corresponding to those of the colorimetric assays are consistent with measuring "total" reducing ends (Fig. 8). Three reaction conditions were compared: (1) the "mild" conditions as employed above, (2) those analogous to the BCA assay (i.e., pH 10.0 and 80°C), and (3) those of intermediate severity. A plateau in the extent of reduction is evident in the time courses corresponding to the two milder conditions. By contrast, reductions under the more severe conditions resulted in complete depletion of BCA-detectable reducing ends. This result supports the contention that conditions commonly used for traditional colorimetric assays are consistent with measuring "total" numbers of reducing ends. The results from this experiment also demonstrate that the color generated in the BCA assay is dependent on the presence of reducing ends, since no reducing ends could be detected after exhaustive $NaBH_4$ reduction. This was also shown to be the case for color generation in the DNS assay (data not shown). This is significant in that it indicates that potentially color-yielding side products generated during the course of reducing sugar assays (as suggested above for the DNS assay) are a result of the presence of reducing ends *per se*—and not owing to side reactions occurring at sites away from the reducing end termini.

Conclusion

The colorimetric assays discussed herein are most commonly used in cellulose/cellulase studies to quantify the number of reducing sugars associated with the soluble phase (glucose, cellobiose, low-DP cellooli-

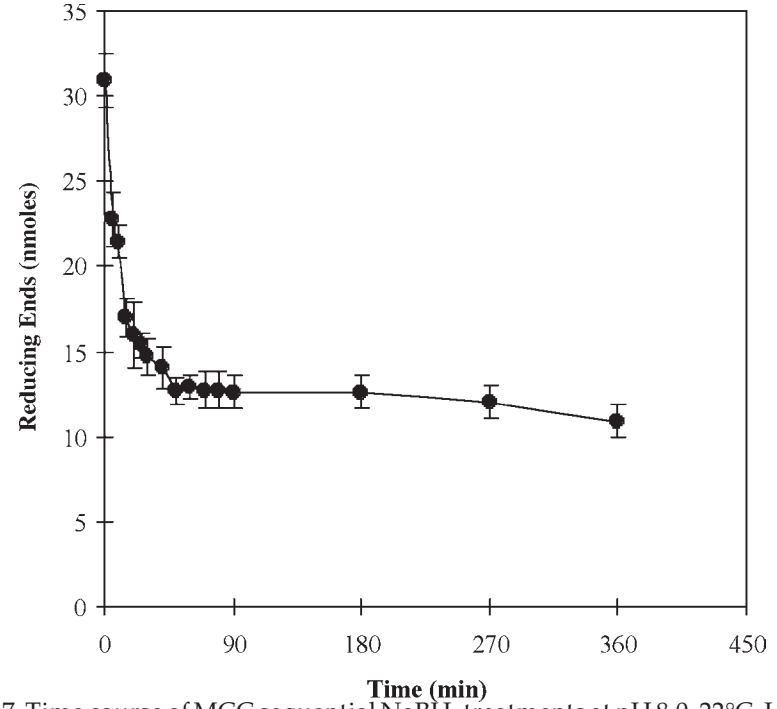

Fig. 7. Time course of MCC sequential $NaBH_4$ treatments at pH 8.0, 22°C. Unreacted reducing ends were determined by BCA assay.

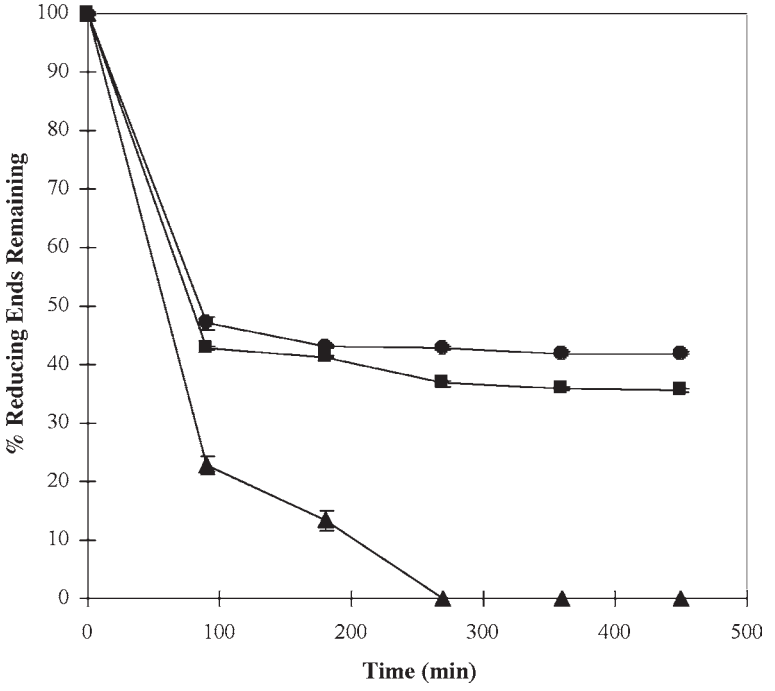

Fig. 8. Time courses of MCC reduction by $NaBH_4$ at different reaction conditions: (●), pH = 8.0, *T* = 22°C; (■), pH = 10.0, *T* = 22°C; (▲), pH = 10.0, *T* = 80°C.

gosaccharides). However, they are increasingly being used to determine the number of reducing ends associated with the insoluble phase, cellulose. The combined results from this study show the merits of the different reducing sugar assays when used with either the soluble or the insoluble substrates (detection limits, sensitivities, and so on) and suggest that the limitations of these assays regarding the analysis of celluloses *per se* should be considered. The DNS assay tends to overestimate absolute numbers of reducing ends per unit mass cellulose, and it appears likely that the degree of overestimation is a function of the cellulose's DP profile. Hence, the BCA assay apprears to be the more appropriate assay for obtaining absolute values for cellulose reducing ends. Both the BCA and the DNS assays are shown to relate to the "total" number of cellulose-associated reducing ends. One can obtain reasonable estimates of the absolute number of solvent-accessible reducing ends by using either the combined $NaBH_4$/BCA assay or the sodium borotritiide/tritium uptake assay as presented herein. Values obtained with the latter two assays will be particularly relevant in studies concerned with the number of available catalytic sites for exo-acting cellulases and for those studies examining general changes in the surface properties and/or accessibility of cellulose substrates.

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