Identification of Two Functionally Different Classes of Exocellulases†

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ABSTRACT: There are two classes of synergism in cellulase mixtures: synergism between endocellulases and exocellulases, and synergism between certain exocellulases. Exocellulases have been defined traditionally as releasing cellobiose from the nonreducing ends of cellulose, but this definition is inadequate to explain exo/exo synergism. Several recent reports indicate that some exocellulases are capable of hydrolyzing cellulose from the reducing end. The existence of two exocellulase classes with different specificities could provide an explanation for exo/exo synergism. In this paper, we report the substrate specificity of three *Thermomonospora fusca* (E3, E4, and E6) and two *Trichoderma reesei* (CBH I and CBH II) exocellulases on labeled cellooligosaccharides. We describe a new nonradioactive technique for determining substrate specificity, in which ion-spray mass spectrometry was used to analyze the products of enzymatic digests of cellopentaose labeled with ^{18}O at the reducing end. Exocellulase reactivity was also investigated on cellopentaose labeled at the nonreducing end with ^{14}C, and cellooligosaccharides reduced with $NaBH_4$. The distribution of label in the reaction products supports the existence of two functional classes of exocellulases. One class (containing CBH I, E4, and E6) preferentially cleaves cellooligosaccharides from the reducing end, while the other (containing E3 and CBH II) preferentially cleaves from the nonreducing end. This classification of exocellulases is consistent with exo/exo synergism experiments, and with published cellulase crystallographic data.

Cellulases are enzymes that hydrolyze cellulose into small, soluble glucose oligomers, primarily cellobiose (CB).[1] They have been isolated from a wide range of microorganisms (Henrissat & Bairoch, 1993), with each microorganism producing a mixture of functionally distinct enzymes. Cellulases traditionally are grouped into two broad classes according to how they degrade cellulose: endocellulases (EC 3.2.1.4) cleave cellulose at various sites along cellulose chains, whereas exocellulases (EC 3.2.1.91) cleave cellobiose units from the ends of cellulose chains. It has been shown (Irwin et al., 1993; Wood et al., 1989) that maximal rates of cellulose degradation come from mixtures of three or more cellulases, not from enzymes acting alone. Overall, enzymes in a cellulase mixture engage in a complex series of interactions, and the results of these interactions directly affect the ability of the mixture to degrade cellulose.

Some cellulases exhibit synergism: i.e., mixtures have higher activity than the sum of the activities of the enzymes acting alone. Cellulase synergism has been studied for enzymes from several fungal and bacterial systems [referenced in Eriksson et al. (1990) and Irwin et al. (1993)]. Also, cross-synergism between cellulases from different microorganisms has been demonstrated. This suggests that there are several distinct activities needed to degrade cellulose efficiently, and that different organisms each produce cellulases that have some or all of these activities. Irwin et al. (1993) investigated synergism in mixtures of six purified bacterial cellulases (E1−E6) from *Thermomonospora fusca*, and two purified fungal cellulases [cellobiohydrolase (CBH) I and II] from *Trichoderma reesei*. Their data divide cellulase synergism into two broad classes: synergism between endocellulases and exocellulases, and synergism between certain exocellulases.

By conventional definition, exocellulases release cellobiose from cellulose by hydrolysis from the nonreducing ends of cellulose chains (Schomburg & Salzmann, 1991). Such a model does not explain exo/exo synergism (first observed by Fägerstam & Pettersson, 1980), and implies that the exocellulases in a mixture would compete for a limited number of hydrolysis sites (the nonreducing chain ends), instead of cooperating to give synergistic hydrolysis. By acting only at the nonreducing ends, enzymatic hydrolysis in this manner is inefficient, since half the chain ends are unused. Recent developments have begun to question this traditional model. A report describing two exocellulases from *Aspergillus aculeatus* (Arai et al., 1989) provides evidence that one of the enzymes attacks cellulose reducing ends, while the other attacks nonreducing ends. *T. reesei* CBH II (Biely et al., 1993) prefers to attack the nonreducing end of cellooligosaccharides (COligos), while *T. reesei* CBH

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[1] Abbreviations: CB, cellobiose; CBH, cellobiohydrolase; COligo, cellooligosaccharide; CTri, cellotriose; CTet, cellotetraose; CP, cellopentaose; CBol, reduced cellobiose; CTriol, reduced cellotriose; CTetol, reduced cellotetraose; CPol, reduced cellopentaose; ^{18}O-CP, cellopentaose labeled with ^{18}O at C-1 of the reducing end; ^{14}C-CP, cellopentaose labeled with ^{14}C in the nonreducing end glucose; TLC, thin-layer chromatography; IS-MS, ion-spray mass spectrometry; CMC, (carboxymethyl)cellulose; DNS, dinitrosalicylic acid reagent.

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I (Vrsanská & Biely, 1992) and *Cellulomonas fimi* CbhB (formerly called CenE) (Shen et al., 1994) prefer the reducing end. Extending these results to the data of Irwin et al. (1993) suggests that the observed exo/exo synergism could be caused by interactions between traditional (nonreducing-end-attacking) and nontraditional (reducing-end-attacking) exocellulases.

The synergistic experiments in Irwin et al. (1993) divide exocellulases into at least two functional classes. One class contains E3 and CBH II, while the other contains CBH I. The classification of exocellulase E4 is uncertain as it gives synergism with both E3 and CBH I, as well as with some endocellulases. By preferentially producing soluble reducing sugars, E4 functions like an exocellulase. Nevertheless, it possesses a low, but real, activity on (carboxymethyl)-cellulose (50−200 fold higher activity than E3, CBH I, or CBH II), a traditional endocellulase substrate.

In this study, we investigate the substrate specificity of three *T. fusca* (E3, E4, and E6) and two *T. reesei* (CBH I and CBH II) exocellulases on labeled COligos. We describe a new technique for determining substrate specificity in which the products of enzymatic digests of cellopentaose labeled at the reducing end with ^{18}O are analyzed by ion-spray mass spectrometry (IS-MS) (Bruins et al., 1987; Covey et al., 1988). Exocellulases are also incubated with $NaBH_4$-reduced cellotetraose and cellopentaose, as well as with cellopentaose labeled with ^{14}C at the nonreducing end.

EXPERIMENTAL PROCEDURES

General. D-Glucose, D-glucitol, cellobiose (CB), cellotriose (CTri), cellotetraose (CTet), cellopentaose (CP), and $NaBH_4$ were from Sigma (St. Louis, MO). Hydroxylapatite (Hypatite C) was from Clarkson Chemical Co. (Williamsport, PA). $H_2{}^{18}O$ (99.1 atom % ^{18}O) was from Isotec, Inc. (Miamisburg, OH). Thin-layer chromatography (TLC) of COligos was performed as previously described (Jung et al., 1993). Cellulase activity assays on filter paper, acid-swollen cellulose, and (carboxymethyl)cellulose (CMC) were performed as previously described (Irwin et al., 1993) using the dinitrosalicylic acid reagent (DNS) to measure reducing sugars. Both glucose and cellobiose were used to generate DNS standard curves.

Protein Purification. CBH I and CBH II were purified from *T. reesei* crude cellulase as described (Irwin et al., 1993; Walker et al., 1993). *T. fusca* E4 was overexpressed in *Streptomyces lividans* and purified as described (Jung et al., 1993). E3 was purified from *T. fusca* crude cellulase as described (Irwin et al., 1993), with several additions. The crude cellulase was adjusted to 250 mM $(NH_4)_2SO_4$, 1 mM gluconolactone, and 5 mM potassium phosphate, pH 6.0, before being loaded on the *p*-nitrobenzyl 1-thio-β-D-cellobioside affinity column. After the Q-Sepharose column, the E3 pool was purified with the cellobiose affinity column a second time, followed by chromatography on Con A-Sepharose. The fractions containing E3 were pooled and transferred to 1 mM potassium phosphate, pH 6.0, with a PTTK 30 000 NMWL membrane (Millipore). This material was loaded on a 50 mL hydroxylapatite column, and eluted with a 1.2 L linear gradient of 1−100 mM potassium phosphate, pH 6.0. The final yield of E3 was 355 mg from a 30 L culture.

E6 was purified from *T. fusca* crude cellulase as described (Irwin et al., 1993), through the Q-Sepharose column. Fractions containing the 106 kDa protein (E6) were pooled and applied to a phenyl-Sepharose CL-4B column as described (Walker et al., 1992). After elution, the fractions containing E6 were desalted, concentrated, and transferred (as before) to a buffer containing 10% glycerol, 20 mM Bis-Tris, and 20 mM Tris-HCl, pH 8.0 (buffer A). This pool was further purified by perfusion chromatography on a 4.6 mm × 100 mm POROS II Q/M column (PerSeptive Biosystems) connected to an HPLC system (Bio-Rad) at a flow rate of 3.0 mL/min. E6 (1 mL, 2.1 mg) was injected onto the column (equilibrated with buffer A) and eluted with a linear gradient of 0−50% buffer B (buffer A + 2.0 M NaCl) over 18 min. An SDS gel of the active column fractions showed a single protein band at 106 kDa, but Western blotting with antisera against the cloned E4 protein [as in Irwin et al. (1993)] indicated that the purest E6 fractions contained a small amount of E4 degradation products. The best E6 fractions were over 98.5% pure, with activities of 3.7 μmol of CB/(min·μmol) on CMC, and 17.9 μmol of CB/(min·μmol) on acid-swollen cellulose.

Preparation of Labeled COligos. CB, CTri, CTet, and CP were reduced with an excess of $NaBH_4$ (Bhat et al., 1990; Bray & Clarke, 1990). CB (500 mg) was dissolved in 15 mL of 100 mM NH_4OH, pH 10.5; 75 mg of solid $NaBH_4$ was added, and the mixture was incubated at 25 °C for 5 h, followed by acidification with 50% acetic acid. The solution was dried under vacuum, and the resulting white solid was suspended in acidified methanol and dried under vacuum, a total of 5 times. The white, solid product contained no detectable reducing sugars (DNS assay), and was identified as reduced cellobiose (CBol) by ^{13}C-NMR (data not shown).

CTriol, CTetol, and CPol were prepared in a manner similar to that for CBol. COligos (3−5 mg) were dissolved in 500−1000 μL of water, to which was added 500 μL of 400 mM $NaBH_4$ in 200 mM NH_4OH, pH 10.5. After incubation at 25 °C for 3 h, the solution was acidified with 50% acetic acid, dried, and treated with acidified methanol as before. The reduced COligos were deionized by passage through IR-120 (H^+ form) and Dowex 1-X8 (HCO_3^- form) resins as described (Bhat et al., 1990), and stored at −20 °C.

CP was labeled with ^{18}O at the reducing end anomeric carbon by oxygen exchange with $H_2{}^{18}O$ (Mega et al., 1990; Rittenberg & Graff, 1958). In a glovebag under N_2, 5.0 mg of CP was dissolved in 220 μL ~65% $H_2{}^{18}O$, 20 mM NH_4HCO_3, pH 8.2. The sample was sealed in a 1.0 mL V-vial and incubated in a waterbath for 24−36 h at 61 °C. CP was not degraded under these conditions, as determined by TLC. After cooling, aliquots containing 160 μg of ^{18}O-CP were placed into glass vials, lyophilized, and stored under N_2 at −20 °C. ^{18}O-CB and ^{18}O-CTri were prepared in a similar manner in 60% and 50% $H_2{}^{18}O$, respectively.

Cellopentaose with a ^{14}C label in the terminal nonreducing end glucose unit (^{14}C-CP) was provided by H. J. Strobel, University of Kentucky. It was prepared by incubating [U-^{14}C]glucose-1-phosphate and CTet in the presence of *Clostridium thermocellum* cellodextrin phosphorylase, and purified by HPLC.

Action of Exocellulases on Normal and Reduced COligos. The procedure for COligo hydrolysis was used as described (Jung et al., 1993), with modifications. E3, E4, and E6 were assayed in 5 mM succinate, pH 6.0, while CBH I and CBH II were assayed in 5 mM succinate, pH 4.5. The substrate

concentrations used were 3.0 mM for CTri and 2.0 mM for CTet, CTetol, CP, and CPol. The hydrolysis products were identified by TLC using known standards.

Action of Exocellulases on ^{18}O-CP. All assays were performed in duplicate. One vial (160 μg) of ^{18}O-CP was dissolved in 100 μL of 1 mM succinate, pH 6.0 (net 1.9 mM ^{18}O-CP). After a 5 min preincubation at 50 °C, enzyme was added, and the mixture was incubated at 50 °C. The reaction mixture was then frozen on dry ice, and a 10 μL aliquot was analyzed by TLC. If the resulting chromatogram showed that the ^{18}O-CP was ≥80% cleaved, the reaction mixture was thawed and the enzymatic reaction halted by addition of 30 μL of 2 M NH_4OH, pH 10.5, followed by lyophilization. If the ^{18}O-CP was <80% cleaved, the reaction mixture was incubated longer before workup. To limit exchange of ^{18}O with bulk solvent, sufficient enzyme was used to ensure that all assays were incubated at 50 °C for 60 min or less.

For analysis by mass spectrometry, the lyophilized ^{18}O-CP digests were dissolved to 1 mg/mL in water (pH 7), and diluted 1:1 with acetonitrile. Spectra were recorded on a PE-Sciex TAGA 6000E, upgraded to an API 3, using continuous-infusion ion-spray mass spectrometry (Bruins et al., 1987; Covey et al., 1988). Initially, positive-ion spectra were recorded by scanning Q1 from 100 *m/z* to 1100 *m/z* every 0.5 amu, with a 3 ms dwell time and averaging over 16 scans. The speed of sample infusion was 2.0 μL/min, with an applied declustering potential of 60 V. For each spectra, regions of interest were rescanned with similar parameters at 0.1 amu resolution. Glucose cannot be detected by IS-MS under these conditions. For each COligo, the amount of ^{18}O label was calculated from the heights of the M and (M+2) ion peaks, using the formula

$$\%\ ^{18}O = 100 \times [(M+2(corr))/((M+2(corr)) + M)]$$

with the (M+2) heights corrected for ^{13}C natural abundance.

CBH I and E6 Hydrolysis of ^{14}C-CP. CBH I and E6 were incubated in duplicate at 50 °C in 10 μL reactions containing 1.74 μM enzyme and 2.0 mM ^{14}C-CP (1800 cpm/μg) in 10 mM succinate, pH 4.5 (CBH I) or pH 6.0 (E6); 4 μL aliquots (12 000 cpm) were removed at two time points (15 and 45 min for CBH I, 30 and 60 min for E6), mixed with 2 μL 2 M NH_4OH, and frozen on dry ice. These samples were separated by TLC with three ascents of solvent, drying the TLC plate in a fume hood for 20 min between ascents. After staining for COligos, the resulting spots were scraped from the TLC plate and transferred to 5 mL scintillation vials. Water (900 μL) was added to each vial, followed by vortexing for 30 s and addition of 3 mL of ACS counting fluid (Amersham) (Chirico & Brown, 1985). The ^{14}C present in each COligo was determined with a Beckman LS 7000 liquid scintillation counter. The efficiency of recovery of counts from the silica gel was at least 75%.

RESULTS

Synergistic Behavior of E6 in Exo/Exo Mixtures. To determine whether E6 could give synergism with other exocellulases, we determined its activity on filter paper in equimolar mixtures with E3 and CBH I. The synergistic activity of E6 with endocellulases has previously been investigated (Irwin et al., 1993). However, the E6 used in that study was contaminated with E4, so synergism with other exocellulases could not be tested. The E6 used in this study had less than 1/15 the CMC activity of the E6 used previously, and the CMC activity [3.7 μmol of CB/(min·μmol)] was only slightly higher than that of other purified exocellulases [0.5−2.1 μmol of CB/(min·μmol)]. The data in Table 1 show that E6 gave synergism in mixtures with E3, but not in mixtures with CBH I. The E3 + CBH I mixture also gave synergism, as previously reported (Irwin et al., 1993). The cloning of E6 and its subsequent expression in a noncellulolytic host (currently in progress in our laboratory) will allow it to be purified in the absence of contaminating cellulase activities. However, we believe that the synergistic behavior of E6 will not qualitatively change upon further purification, since the E6 + CBH I mixture did not give synergism even though the E6 used was contaminated with a small amount of E4.

Table 1: Filter Paper Activity of Cellulases and Cellulase Mixtures[a]

cellulase(s)	act. [μmol of CB/(min·μmol)]	sum of individual act.	synergistic effect[c]
E6	0.51[b]		
E3	0.14[b]		
CBH I	0.40[b]		
E6 + E3	1.07	0.65	1.6
E6 + CBH I	0.81	0.91	0.9
E3 + CBH I	1.90	0.54	3.5

[a] Determined (in triplicate) on one disk (3.4 mg) of Whatman no. 1 filter paper in 50 mM sodium acetate, pH 5.5, for 16.5 h at 50 °C. [b] Target digestion of 5.2% was not achieved; activity calculated from digestion achieved by 0.6 nmol of enzyme. [c] Synergistic effect = activity/sum of individual activities.

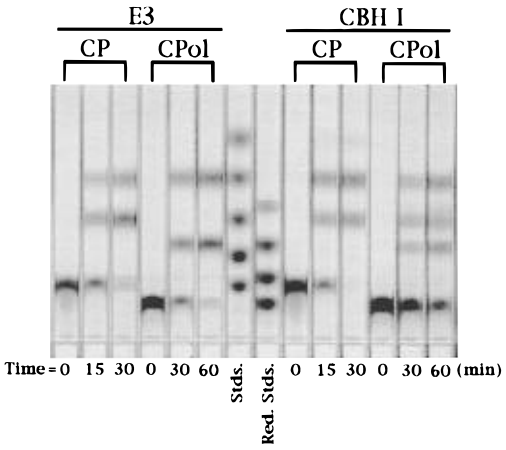

FIGURE 1: TLC of the hydrolysis of CP and CPol by E3 and CBH I (1.66 μM). Enzymes (0.29 μM) were incubated with 2.0 mM CP or CPol. Aliquots were removed at several time points and the products of hydrolysis separated by TLC as described. Standards (listed from top to bottom): glucose, CB, CTri, CTet, CP. Reduced standards: CBol, CTriol, CTetol, CPol.

Exocellulase Hydrolysis of Normal and Reduced COligos. To determine substrate specificity, we investigated how the various exocellulases hydrolyzed labeled COligos. Preliminary experiments were designed to characterize qualitatively the reactivities of the enzymes with nonlabeled COligos, and also with COligos reduced with $NaBH_4$. $NaBH_4$ labels COligos specifically by reduction of the hemiacetal linkage at C-1 of the reducing end, thus opening the reducing end sugar ring. Enzymes were incubated separately with CP and CPol, and samples from the reaction mixtures were analyzed by TLC. Figure 1 presents the hydrolysis of these substrates by E3 and CBH I. Both enzymes degraded CP

to CB and CTri, while CBH I also produced a small amount of glucose. CTet (the complementary product to glucose) was not visible. These two enzymes showed a marked difference in cleavage of CPol. E3 cut CPol at only one position, producing exclusively CB and CTriol. In contrast, CBH I cleaved CPol to either CB and CTriol or CTri and CBol. Both enzymes were slightly inhibited on CPol (relative to CP), with CBH I being more strongly affected. Assays of the other exocellulases on these substrates showed that CBH II has activity like that of E3, while E4 and E6 have activity like CBH I.

The exocellulases were also incubated in the same manner with smaller COligos. All five enzymes displayed the same behavior toward CTri, cleaving it very slowly to glucose and cellobiose. In contrast, the enzymes cleaved CTet in ways that are too diverse to be unambiguously classified. Finally, all of the exocellulases except CBH I were strongly inhibited on CTetol, indicating that the chemical modification had a large effect on substrate binding to the enzymes.

Exocellulase Hydrolysis of ^{18}O-CP. Based on the preliminary data, we decided to use labeled forms of cellopentaose in subsequent specificity experiments, as this substrate was hydrolyzed well by all the cellulases studied. Also, hydrolysis of labeled CP (containing an odd number of glucose units) would permit unequivocal identification of the glycosidic bond(s) being cleaved. Numerous reports describe using fluorophoric- or chromophoric-labeled COligos as substrates in specificity experiments [for example see Claeyssens and Henrissat (1992)]. However, the results from these experiments are difficult to interpret, as it has been shown that changing the labeling group can alter the apparent mode of hydrolysis (Bhat et al., 1990). To avoid these difficulties, we labeled the hydroxyl group of the cellopentaose anomeric carbon isotopically by an oxygen exchange reaction with ^{18}O-enriched water. Replacement of ^{16}O with ^{18}O does not alter the interactions of CP with cellulases. In addition, use of the nonradioactive ^{18}O removes the technical difficulties associated with using radioactive (^{3}H-labeled) COligos [as in Bhat et al. (1990) and Claeyssens et al. (1990)].

CP was labeled with ^{18}O as described under Experimental Procedures. Incorporation of the label was detected and quantitated by IS-MS. The mass spectrum of nonlabeled CP (MW = 828.7) is shown in Figure 2A. The spectrum clearly contained a molecular ion at *m/z* 829.4 which corresponded to ($CP+H^+$), as well as peaks at *m/z* 830.4 (M+1) and *m/z* 831.4 (M+2) due to natural abundance isotopes. The low natural abundance of ^{18}O (0.204%) had a negligible contribution to the intensity at (M+2), and as a result the (M+2) signal was assumed to be due solely to ^{13}C. In addition, other singly-charged CP adducts were present at *m/z* 846.4 (M+17, $CP+NH_4^+$) and *m/z* 851.4 (M+22, $CP+Na^+$). The *m/z* values were all in good agreement with predicted values (±1 mass unit).

The mass spectrum of ^{18}O-CP (formed after incubation in $H_2{}^{18}O$) is shown in Figure 2B. This spectrum contained the same molecular ions and adducts (H^+, NH_4^+, and Na^+) as for CP in Figure 2A, but with (M+2) peaks (at *m/z* 831.4, 848.4, and 853.4) more intense than those of the corresponding parent ions. This change in intensity reflects the replacement of one atom of ^{16}O with ^{18}O in a large mole fraction of CP, with the exact fraction labeled (% ^{18}O) calculated from the peak heights as discussed under Experimental Procedures. In almost all of the spectra, independent

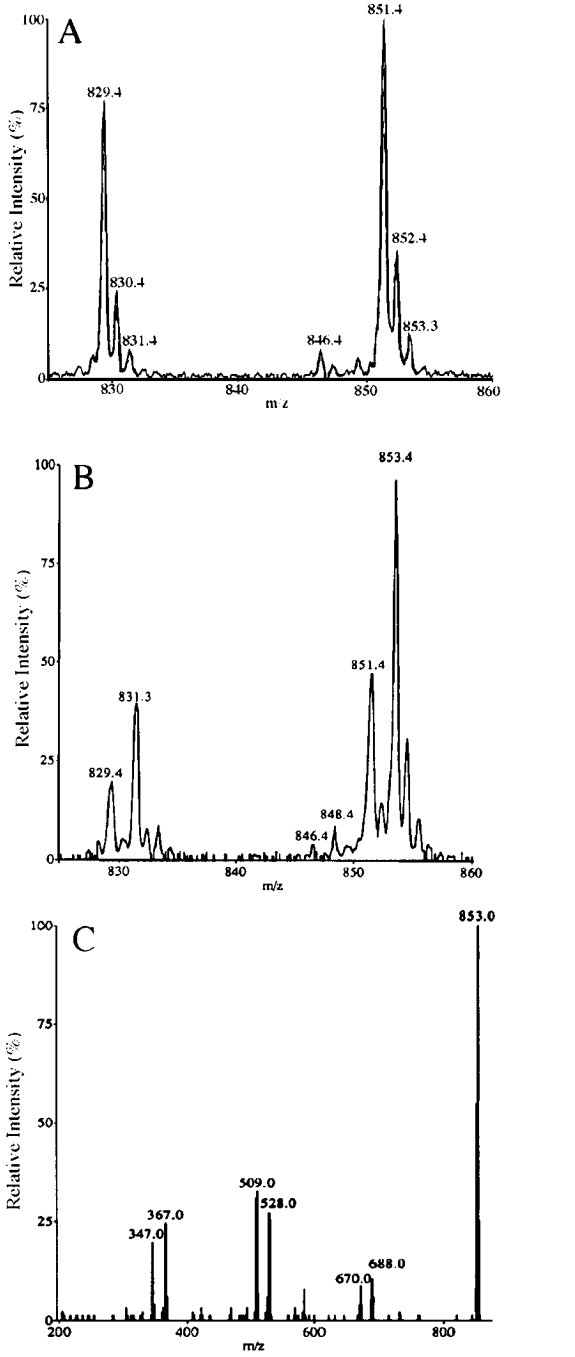

FIGURE 2: Ion-spray mass spectra of cellopentaose. (A) Spectrum of CP (nonlabeled). (B) Spectrum of CP after labeling with ^{18}O. (C) MS/MS (fragmentation) spectrum of the ^{18}O-CP parent ion at *m/z* 853.

values for % ^{18}O were calculated from each of the ion−adduct forms (H^+, NH_4^+, Na^+, or K^+). In this manner, we determined from Figure 2B that ^{18}O-CP contained 66.5 ± 1.1% ^{18}O, in good agreement with the ~65% $H_2{}^{18}O$ used in the labeling reaction. Figure 2C presents the MS/MS spectrum of the ^{18}O-CP ion peak at *m/z* 853. This parent ion fragmented to daughter ions close to the molecular weights of CB (MW = 342.3), CTri (MW = 504.45) and CTet (MW = 666.6), confirming the identity of the parent as cellopentaose.

E3, E4, CBH I, and CBH II were incubated individually with ^{18}O-CP, and the products of hydrolysis were analyzed by IS-MS. As a representative example, Figure 3 shows the results from 1.9 mM ^{18}O-CP incubated with 0.24 μM CBH

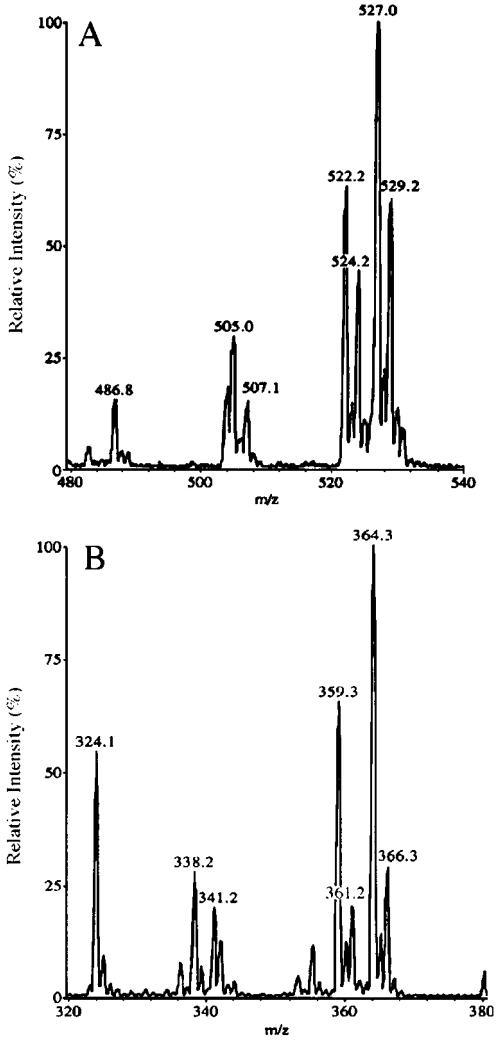

FIGURE 3: Ion-spray mass spectrum showing the products from the hydrolysis of ^{18}O-CP by CBH II. (A) Region of the spectrum corresponding to cellotriose. (B) Region of the spectrum corresponding to cellobiose.

II for 20 min. Occasionally spectra contained molecular ions that did not correlate with masses of expected COligos, and these unidentifiable peaks were not used in the calculations. The CTri (MW = 504.45) product spectrum (Figure 3A) contained ions that correspond to adducts with H^+ (M+1, *m/z* 505.0), NH_4^+ (M+17, *m/z* 522.2), and Na^+ (M+22, *m/z* 527.2). The CTri product contained ^{18}O (net 35.4 ± 4.4% ^{18}O), visible in (M+2) ion peaks at *m/z* 507.1, 524.2, and 529.2. It also contained a molecular ion at *m/z* 486.8 (M−17), which corresponds to CTri that has fragmented and lost an OH^-. This ion did not have a large (M+2) peak at *m/z* 488.8, which indicated that the missing hydroxyl group contained the ^{18}O. The CB (MW = 342.3) region of the product spectrum (Figure 3B) had a similar molecular ion at *m/z* 324.1 (M−17), that also corresponds to loss of $^{18}OH^-$. Just as with CTri, CB adducts with NH_4^+ (M+17, *m/z* 359.3) and Na^+ (M+22, *m/z* 364.3) contained ^{18}O (21.0 ± 0.6%).

The presence of ^{18}O in both CTri and CB indicated that CBH II cleaved ^{18}O-CP at two different glycosidic bonds. The region of the spectrum corresponding to unreacted ^{18}O-CP (not shown) contained 52.8 ± 1.2% ^{18}O, a loss of 20% of the initial label. This loss is not time-dependent, as a shorter incubation gave a larger decrease. The total amount of ^{18}O in ^{18}O-CB and ^{18}O-CTri (56.4%) was similar to the amount in unreacted ^{18}O-CP (52.8%). Assuming that each cellopentaose is hydrolyzed to one cellotriose and one cellobiose (or one cellotetraose and one glucose), this observation showed that the loss of ^{18}O was proportional for all the COligos within a sample. This loss most likely occurred during the sample workup, after hydrolysis. For all the experiments in this paper, the amount of ^{18}O in unreacted ^{18}O-CP was in the range 47.5−53.5%.

Table 2: Proportion of COligos Released from the Reducing Ends of ^{18}O-CP and ^{14}C-CP by Exocellulases[a]

substrate	enzyme	% CTri	% CB	SD[b]	% CTet	% glucose	SD[b]
^{18}O-CP	E3	61	39	0.0 (2)			
	CBH II	63	37	0.8 (2)			
	E4	11	89	0.4 (2)	20[c]	80[c]	*d*
	CBH I	28	72	3.1 (2)			
^{14}C-CP	CBH I	33.1	66.9	2.3 (4)			
	E6	25.3	74.7	0.3 (2)	0.5	99.5	0.2 (4)

[a] CP can be hydrolyzed to either CTri and CB, or CTet and Glucose. The values shown here treat the products from each type of cleavage separately. [b] Standard deviation applies to both COligo products. The values in parentheses represent the number of independent determinations. [c] The value is approximate. For explanation, see Results. [d] Single determination made it impossible to calculate the standard deviation.

The % ^{18}O values calculated from the IS-MS spectra were used to determine the preferred mode of action of each enzyme on ^{18}O-CP. Using the assumption stated above, the fraction of each product (such as CB) that is released from the reducing end of ^{18}O-CP was calculated by dividing the % ^{18}O of that product by the sum of the label in both products (CB and CTri). This comparison is valid for % ^{18}O values from a single sample, for (as discussed above) any loss of label is proportional to all products, and cancels out. The values are expressed as percentages, and are shown in Table 2. They show the existence of two classes of exocellulase, differing in their preferred mode of action on cellopentaose. E3 and CBH II preferentially released cellobiose from the nonreducing end, as 61% and 63% (respectively) of the ^{18}O was found in cellotriose. In contrast, cellobiose was released from the reducing end by CBH I (72%) and E4 (89%). When E4 cleaved CP to CTet and glucose, the glucose was also preferentially released from the reducing end (80% of the time). However, this value is approximate since IS-MS is unable to detect glucose under the conditions used. As a result, this value was based only on the amount of ^{18}O present in CTet (20% of that present in the unreacted starting material).

For E3, E4, and CBH II, the sum of the % ^{18}O found in CB and CTri was close to that found in unreacted ^{18}O-CP. However, the products from CBH I contained 31% less ^{18}O than the unreacted material. To determine if the ^{18}O was being lost from the products through some unknown interaction with CBH I, a mixture of ^{18}O-CB and ^{18}O-CTri (each 1.9 mM) was incubated with CBH I under the standard conditions. Analysis of the resulting mass spectra showed no loss of ^{18}O from either product, when compared to controls containing either no enzyme or CBH I denatured with 5% trichloroacetic acid.

Exocellulase Hydrolysis of ^{14}C-CP. To see if this unexplained ^{18}O loss altered the apparent mode of action of CBH I, we incubated CBH I with CP labeled in a different manner. CBH I (and also E6) was incubated with ^{14}C-CP, and the preferred modes of action of these two enzymes on ^{14}C-CP

were calculated in the same manner as with ^{18}O-CP. The results (Table 2) show that 66.9% of the cellobiose released from ^{14}C-CP by CBH I was from the reducing end, compared to 72% from ^{18}O-CP. E6 cleaved CP in the same manner as did CBH I, releasing 74.7% of the CB (and 99.5% of the glucose) from the reducing end. E6 also preferred to hydrolyze CP to CB and CTri, producing those products (instead of CTet and glucose) 66.1% (SD = 0.8) of the time.

DISCUSSION

The results of the experiments reported in this paper consistently show that exocellulases fall into two distinct classes. In one class (containing E3 and CBH II), the enzymes preferentially attack the nonreducing end of the substrate, while in the other class the enzymes (E4, E6, and CBH I) preferentially attack the reducing end of the substrate. These two classes correlate with the reactivity of the enzymes on CPol. The experiments with CTetol were inconclusive, not only because of slow hydrolysis by the enzymes, but also because of the nature of the substrate, since reducing end and nonreducing end cleavage of CTetol would both produce CB and CBol.

With ^{18}O-CP and ^{14}C-CP, all of the enzymes give a mixture of hydrolysis products (Table 2). We believe that the product mixture is a result of the physical restraints on substrate binding imposed by the structures of the β-1,4-glucose chain and the enzyme active site (Ruohonen et al., 1993). Cellulose is made of repeating units of cellobiose, with the α-faces of adjacent glucosyl units facing in the opposite direction. As a result, each cellulose strand has two nonequivalent faces that are related by rotating 180° around an axis running the length of the strand. The specificity of the glucosyl binding sites in cellulases alternates in the same manner, with adjacent sites designed to bind different faces of glucose. The constricted nature of exocellulase active site tunnels would be expected to limit severely the conformational flexibility of a substrate once it has entered, forcing it to present only one of its faces to the sugar binding region. Even if substrate always enters in the same direction (reducing or nonreducing end first), it will bind in different positions depending on the orientation it possesses when entering the active site. These different binding positions cause the substrate to be cleaved at different sites, separated by one glucose unit. The net result is a mixture of hydrolysis products, with an exocellulase that releases ^{18}O-CB from the reducing end of ^{18}O-CP also releasing some ^{18}O-CTri and ^{18}O-glucose.

The loss of ^{18}O in experiments with CBH I is consistent with a recently proposed "multiple attack" mechanism for that enzyme, based on detailed studies of CBH I hydrolysis of COligos up to cellooctaose (Nidetzky et al., 1994). This mechanism involves the processive cleavage of cellobiose from a COligo bound in the active site of CBH I, with no release of substrate between cleavages. For example, CBH I would cleave ^{18}O-CP at a low frequency to CTet and ^{18}O-glucose. The ^{18}O-glucose is undetectable by IS-MS, and is "lost." The CTet produced is subsequently cleaved to CB, which mixes with the CB produced from the cleavage of ^{18}O-CP to CB and CTri. In this fashion, the mole fraction of ^{18}O in the cellobiose mass spectrum decreases, altering the apparent mode of action of CBH I. This sequence of events is supported by the TLC data for CBH I (Figure 1) which show glucose, but not CTet, as a product of CP cleavage. The above process would be expected to occur with ^{14}C-CP as well, but, since this substrate is labeled at the other (nonreducing) end, this would produce the opposite result and alter the mode of action of CBH I in the other direction. The net effect is that the preferred mode of action of CBH I lies between the results determined with ^{18}O-CP and ^{14}C-CP, both of which show CBH I preferentially attacking the reducing end.

The data on exo/exo synergism fit well with what would be expected from the existence of two classes of exocellulases, as enzymes in the same class do not give synergism with each other while enzymes in different classes do give synergism. The only exception to this behavior is E4 which gives synergism with all of the other exocellulases. E4 differs from the other exocellulases in having a relatively high activity on CMC, probably because it has an open active site more accessible than the closed active sites of CBH I and CBH II. As a result, E4 may be attacking cellulose molecules that are not accessible to the other exocellulases, and this allows it to give synergism with them.

The exocellulase classification demonstrated in this paper is consistent with the available cellulase structural data. The active site tunnel of CBH I (Divne et al., 1994) contains seven glucosyl binding sites (A−G), with cleavage occurring between sites B and C. The reducing end of a small ligand binds in site A, consistent with CBH I attacking cellulose from the reducing end. E4 is in the same cellulase family (E) as *Clostridium thermocellum* CelD, an endoglucanase whose structure is known (Juy et al., 1992). The CelD active site contains six glucosyl binding sites (A−F), with the reducing end at site F and hydrolysis between sites D and E. Assuming that the number of glucosyl binding sites and the position of cleavage are the same for all cellulases in a given family [as was demonstrated for the family B cellulases E2 and CBH II (Spezio et al., 1993)], the CelD structure indicates that E4 would prefer to attack the reducing end. Finally, both E3 and CBH II are members of cellulase family B (Zhang et al., 1995). The active site of CBH II (Rouvinen et al., 1990) contains at least four glucosyl binding sites (A−D), with cleavage between sites B and C. Ligands bind with the nonreducing end in site A, indicative of CBH II and E3 attacking cellulose from the nonreducing end.

With high molecular weight substrates, we propose that each exocellulase exclusively attacks one type of cellulose end. On acid-swollen cellulose, all of the enzymes except E4 release cellobiose as the major product, as would be expected for exocellulases. However, these enzymes also release a small amount of glucose on this substrate (data not shown). We believe that the glucose is produced as a result of exocellulases binding the two orientations of the cellulose chain at different positions in the active site, as previously described for cellopentaose. Further experiments are needed to determine the level of specificity an exocellulase has on insoluble cellulose.

Of the exocellulases studied here, those that catalyze cellulose hydrolysis by retention of configuration release cellobiose from the reducing end. CBH I is a retaining exocellulase, while CBH II, E3, and E4 are all in cellulase families that hydrolyze by inversion (Gebler et al., 1992). The gene for E6 is currently being sequenced (D. Irwin, personal communication). Preliminary results classify E6 as a member of cellulase family L, which contains an enzyme

(CbhB from *C. fimi*) that hydrolyzes by inversion (Shen et al., 1994). We propose that all retaining exocellulases will cleave from the reducing end. Hydrolysis by retention is a two-step process, passing through an intermediate where part of the substrate (the nonreducing end portion) is covalently bound to the enzyme (Sinnott, 1987). This means that when a retaining exocellulase attacks the cellulose reducing end, cellobiose is released free into solution while the rest of the cellulose chain is covalently bound to the enzyme. If a retaining exocellulase were to attack the nonreducing end, it would form a covalent bond to cellobiose and release the residual cellulose chain. The first process seems more likely, since the cellulose chain would remain bound in the active site. A similar hypothesis cannot be made for inverting exocellulases, and we have shown that both exocellulase classes contain inverting enzymes.

We speculate that exo/exo synergism is caused by two exocellulases from different classes exposing new hydrolysis sites for each other, with one enzyme attacking the reducing ends and the other attacking the nonreducing ends. Most of these "new" sites existed previously, but were inaccessible, since activity assays on filter paper indicated that over 90% of the new reducing ends produced by exocellulases are soluble (Irwin et al., 1993). The mechanism for this type of synergism is different than that for endo/exo synergism, where the action of the endocellulase is believed to create new sites for exocellulase hydrolysis. This model for exo/exo synergism also allows hydrolysis from all accessible cellulose chain ends (nonreducing and reducing), which was not allowed under the previous definition of exocellulases.

Overall, the results presented in this paper show the value of using several approaches to investigate cellulase function. With small substrates, we have developed a new method to introduce an isotopic label into COligos and demonstrated its utility in assaying the specificity of individual exocellulases. The ^{18}O label can be routinely detected and quantitated by ion-spray mass spectrometry, a new technique that is rapid and requires minimal sample preparation. With high molecular weight substrates, we have used filter paper assays to show the behavior of exocellulases in mixtures. These approaches with different types of substrates complement each other and help to illuminate the roles of exocellulases in cellulose hydrolysis.

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