Processivity of Cellobiohydrolases Is Limited by the Substrate

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Processive cellobiohydrolases (CBHs) are the key components of fungal cellulase systems. Despite the wealth of structural data confirming the processive mode of action, little quantitative information on the processivity of CBHs is available. Here, we developed a method for measuring cellulase processivity. Sensitive fluorescence detection of enzyme-generated insoluble reducing groups on cellulose after labeling with diaminopyridine enabled quantification of the number of erated insoluble reducing groups on cellulose after labeling processivity. Sensitive fluorescence detection of enzyme-generated reducible groups on cellulose and endo-mode initiations. Both CBHs TrCel7A from Trichoderma reesei and PcCel7D from Phanerochaete chrysosporium employed reducing-end exo- and endo-mode initiation in parallel. Processivity values measured for TrCel7A and PcCel7D on cellulose hydrolysis were more than an order of magnitude larger than the values of intrinsic processivity that were found from the ratio of catalytic constant ($k_{cat}$) and dissociation rate constant ($k_{off}$). We propose that the length of the obstacle-free path available for a processive run on cellulose chain limits the processivity of CBHs on cellulose. TrCel7A and PcCel7D differed in their $k_{off}$ values, whereas the $k_{cat}$ values were similar. Furthermore, the $k_{off}$ values for endoglucanases (EGs) were much higher than the $k_{off}$ values for CBHs, whereas the $k_{cat}$ values for EGs and CBHs were within the same order of magnitude. These results suggest that the value of $k_{cat}$ may be the primary target for the selection of cellulases.

As a major constituent of plant cell walls, cellulose is the most abundant biopolymer on Earth, and it has great potential as a renewable energy source (1–3). Linear cellulose chains are composed of glucose units linked together through β-1,4-glycosidic bonds. The degree of polymerization (DP) in native celluloses ranges from thousands to tens of thousands of glucose units. Cellulose chains interact with each other through hydrogen bonding and van der Waals interactions, forming insoluble crystalline microfibrils and higher order structures. In plant cell walls cellulose microfibrils are in turn associated with other polymers like hemicelluloses and lignin. In nature, cellulose is degraded by microorganisms, mainly fungi and bacteria, that secrete a set of cellulolytic enzymes. Cellulases have been historically divided into exoglucanases or cellobiohydrolases (CBHs) and endoglucanases (EGs). EGs cleave at random positions along the cellulose chain, whereas CBHs progressively release cellobiose units from the ends of cellulose chains. The most extensively studied cellulolytic system is that of the soft-rot fungus Trichoderma reesei (Tr) (4). The major component of the Tr cellulolytic system is a glyco-side hydrolase family 7 (Ref. 5, see the CAZy database) CBH, TrCel7A (formerly CBH I). Like most cellulases, TrCel7A consists of a catalytic domain connected through a linker peptide with the carbohydrate binding module (6). TrCel7A is a reducing end-acting processive CBH (7, 8). Its active site resides in a 50 Å-long tunnel containing 10 glucose unit binding sites (9, 10). The TrCel7A counterpart in another well characterized cellulolytic organism, the white-rot fungus Phanerochaete chrysosporium (Pc) (11), is PcCel7D (formerly CBH 58) (12). PcCel7D is also a reducing end-acting CBH (13, 14). A less abundant CBH produced by Tr is the non-reducing end-acting TrCel6A (formerly CBH II) (15). In addition to two CBHs, Tr also secretes a number of EGs. The most abundant EGs are TrCel7B and TrCel5A. The less abundant EG, TrCel12A, is smaller and does not contain carbohydrate binding module (16).

Efficient degradation of cellulose requires synergistic cooperation of CBHs and EGs. The major components of most of the cellulolytic systems are processive CBHs that are responsible for the degradation of crystalline cellulose (17). EGs are expressed in smaller amounts, and they serve to assist CBHs by generating new chain ends on cellulose. However, it has been recognized for some time now that the differentiation of cellulases into EGs and CBHs is an oversimplification; cellulases have evolved to a continuum of overlapping modes of action ranging from totally random EGs through processive EGs to strictly exo-acting highly processive CBHs (17). The exact roles of individual enzymes with different degrees of processivity and endo-activity in cellulose degradation are not known.

Processivity is an important characteristic of polymer active enzymes (18). However, the lack of methods for measurement of cellulase processivity has been a major obstacle in elucidation of the costs and benefits of processivity in cellulose degradation (19, 20). Most of the information on the processivity of glycoside hydrolases has been derived from the structures of the enzymes and is qualitative. Enzymes that contain more
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Monomer unit binding sites and have more closed active site topology are thought to be more processive (21). Information on the processivity of CBHs has also been derived from microscopy studies of residual polymers after treatment with enzyme (22, 23). Direct observation of processive movement of TrCel7A on cellulose has also been reported (8). Quantitative measurements of the processivity of glycoside hydrolases have been complicated by the heterogeneous and ill-defined nature of the substrates (20). For cellulases and chitinases, attempts have been made to quantify the processivity using the ratio of released soluble sugars and reducing end-groups generated to the insoluble substrate (24–30). This approach is strictly applicable only for enzymes that employ an endo-processive mode of action. The pattern of the released soluble products (cellbiose to glucose ratio) has also been used as an indication of the processivity of CBHs (12, 29, 31). Numerical estimates of processivity published for cellulases and chitinases range from 5 to 25 (12, 24–31). Whether the relatively low processivity values reflect the properties of the enzyme or the substrate or the limitations in methodology used to measure the processivity is not known.

In this study we developed a method for quantitative determination of the processivity of cellulases. Processivity of CBHs was found to be dependent on the nature of the substrate. Processivity values measured for CBHs on cellulose were much lower than the processivity values calculated from the ratio of catalytic constant ($k_{cat}$) and dissociation rate constant ($k_{on}$).

**EXPERIMENTAL PROCEDURES**

**Materials**—Anthranilic acid (AA), glucose, cellbiose, sodium borohydride, and 2,6-diaminopyridine (DAP) (Aldrich D2,440-4) were purchased from Sigma. Sodium cyanoborohydride and 2,2′-biquinoline-4,4′-dicarboxylic acid disodium salt hydrate (BCA) were from Fluka. n-[U-14C] glucose with specific activity of 262 mCi mmol$^{-1}$ was from Hartmann Analytic Gmbh. DAP was further purified using vacuum sublimation. All other chemicals were used as purchased.

**Enzymes**—TrCel7A, TrCel5A, and TrCel12A were purified from the culture filtrate of Tr QM 9414 as described previously (32–34). Pccel7D was purified from the culture filtrate of the P. cellulolyticus K3 as previously reported (35). Enzyme concentration was determined from absorbance at 280 nm using molar extinction coefficients of 84,400 (14), 74,900 (14), 78,000, and 38,200 M$^{-1}$ cm$^{-1}$ for TrCel7A, Pccel7D, TrCel12A, and TrCel12A, respectively.

**Celluloses**—Bacterial cellulose (BC) was prepared by laboratory fermentation of the Acetobacter xylinum strain ATCC 53582 (14). Amorphous cellulose (AC) (prepared from Whatman no. 1 filter paper) and reduced cellulose were prepared as described previously (14). For preparation of DAP-cellulose, DAP labeling was performed in 50 ml total volume. Cellulose concentration was 3.5 g liter$^{-1}$ and other conditions were as described in the determination of insoluble reducing groups (IRGs) with DAP labeling.

**Determination of Soluble Reducing Groups (SRGs)**—SRGs were determined with a modified bicinchoninic acid (BCA) method (14, 36). Supernatant (0.5 ml) from cellulose hydrolysate was mixed with 0.5 ml of BCA reagent and heated at 75 °C for 30 min. Concentration of SRGs was found from the absorbance at 560 nm, and cellobiose was used for calibration. For zero data points, supernatants after centrifugation of cellulase suspensions without cellulases were used. At low cellulase concentrations used in this study, the concentration of free cellulase was negligible, and cellulases were omitted from the zero data points for SRGs.

**Determination of IRGs on Cellulose**—IRGs were determined using fluorescence labeling with DAP (for CBHs) or AA (for EGs). After hydrolysis with cellulase, the residual cellulose was washed with 0.2 M NaOH. All cellulose washings were performed through centrifugation (1 min, 10,000 $\times$ g) and re-suspension steps. Cellulose was further washed with proteinase K buffer (2% SDS in 50 mM Tris-HCl, pH 9.5). Proteinase K and CaCl$_2$ were added to the final concentrations of 40 μg ml$^{-1}$ and 1 mM, respectively, and incubated at 60 °C for 30 min. After proteinase K treatment the cellulose was washed with water, and the volume of suspension was adjusted to 0.1 ml. For DAP labeling, 0.4 ml DAP reagent was added to 0.1 ml of the above-described cellulose suspension in water. DAP reagent was prepared freshly by dissolving DAP and sodium cyanoborohydride in buffered methanol (4% sodium acetate trihydrate and 2% boric acid in methanol) (37). Concentrations of cellulose, DAP, and sodium cyanoborohydride were 2 g liter$^{-1}$, 75 mM, and 0.5 M, respectively. After 3 h at 80 °C the DAP-labeled cellulose was thoroughly washed with water and 50 mM sodium acetate, pH 5.0.

DAP-cellulose was completely solubilized by the mixture of Tr cellulases (Celluclast 1.5L) before the measurement of fluorescence. After suitable dilution with 0.5 M sodium acetate, pH 4.0, the fluorescence was recorded using a Hitachi F-4500 fluorimeter using excitation and emission wavelengths of 338 and 400 nm, respectively. The relative fluorescence intensity of 813.5 ± 615 μM$^{-1}$ determined for DAP-glucose was used for calibration (see Fig. S1).

For AA labeling was performed in buffered methanol at 80 °C for 1 h. Final concentrations of cellulose, AA, and sodium cyanoborohydride were 2 g liter$^{-1}$, 50 mM, and 0.5 M, respectively. Excitation and emission wavelengths were set to 330 and 425 nm respectively. Relative fluorescence intensity of 730 ± 50 μM$^{-1}$ determined for AA-glucose was used for calibration (14). The rest of the procedure was the same as in the case of DAP labeling.

**Determination of Total Reducing Groups (RG$_{tot}$) for Measurements of k$_{cat}$**—Reduced cellulose (1.0–5.0 g liter$^{-1}$) was incubated with 0.1 μM cellulase in 50 mM sodium acetate, pH 5.0, at 30 °C for 10 s. Hydrolysis was stopped by the addition of an equal volume of BCA reagent. After heating at 75 °C for 30 min, cellulose was separated by centrifugation, and absorbance of supernatant at 560 nm was recorded. Zero data points were treated identically, but cellulases were added after BCA reagent.

**Hydrolysis of DAP-cellulose**—DAP-cellulose (1.0 g liter$^{-1}$) was incubated with CBB (5.0–50 mM) in 50 mM sodium acetate, pH 5.0, at 30 °C. At defined times, the cellulose was separated by centrifugation, and the supernatant was used for determination of SRGs and DAP-sugars.
Hydrolysis of Reduced Cellulose—Reduced cellulose (1.0–5.0 g liter⁻¹) was incubated with cellulase (5.0–50 nIU) in 50 mM sodium acetate, pH 5.0, at 30 °C. At defined times, the cellulose was separated by centrifugation, and the supernatant was used for determination of SRGs. After washing of residual cellulose (see determination of IRGs on cellulose), the number of IRGs was determined by labeling with DAP or AA. To make zero data-points, cellulose was separated by centrifugation before the addition of cellulase, and supernatant was used for the zero data points in measurements of SRGs. In the case of zero data points for IRGs, 0.2 M NaOH was first added to the cellulose pellet followed by the addition of cellulase, and the rest of the treatment was identical to other data points.

RESULTS AND DISCUSSION

Measurement of Cellulase Processivity; Method Development—Processivity is an important kinetic property of polymer active enzymes. True or intrinsic processivity (P_app) is determined by the dissociation probability (P_d) of the enzyme from the polymer. It has also been demonstrated that P_app corresponds to the average number of catalytic acts performed before the dissociation of the enzyme from the polymer (38). P_app can realize only on an ideal polymer where P_d is independent of the position of the enzyme on polymer. Real polymers often contain structural heterogeneities or steric obstacles that may influence P_d. Thus, it is appropriate to define apparent processivity (P_app) as an average number of catalytic acts (N_catal) performed per initiation of a processive run (N_init) on a real polymer.

\[ P_{app} = \frac{N_{catal}}{N_{init}} \] (Eq. 1)

The experimental challenge for the quantification of P_app is the determination of the N_init. This is the reason for the virtual absence of quantitative data on the processivity of cellulases. For CBHs acting exclusively from the reducing end of a cellulose chain, a substrate with label on the reducing end can be used to reveal the reducing-endexo initiations (N_init)_R-exo (7). If the release of SRGs is followed in parallel with the released end-label (EL) (Fig. 1C), then P_app can be found from Equation 2.

\[ P_{app} = \frac{[SRG] + [EL]}{[EL]} = \frac{N_{catal}}{(N_{init})_{R-exo}} \] (Eq. 2)

Because the first catalytic act will release the EL that cannot be detected as an SRG, the term [EL] has to be included to account for N_catal. If CBH can use an initiation mode other than reducing-end exo in parallel, the P_app will be overestimated, as only reducing-end initiations are detected.

Using reduced cellulose as a substrate and detection of CBH generated IRGs on residual cellulose enables measuring of the sum of endo-mode initiations, (N_init)_endo and (N_init)_R-exo (Fig. 1D). Reduced cellulose contains the sugar alditol in its former reducing end and will not go through the reductive amination with amines (Fig. 1A). Upon action by a reducing-end exo-enzyme, the sugar alditol is released, and IRG is generated. The latter can be visualized by labeling with fluorescent amines like DAP or AA (Fig. 1A). Internal cuts resulting from endo-mode actions will also generate new reducing ends on cellulose and are visualized (Fig. 1D). However, if a cellulose chain is completely degraded, IRG will not be produced, and P_app will be overestimated. Therefore, substrates with high DP must be used to minimize this possibility. P_app on reduced cellulose is given by Equation 3.

\[ P_{app} = \frac{[IRG] + [SRG]}{[IRG]} = \frac{[IRG]}{(N_{init})_{R-exo} + (N_{init})_{endo}} \] (Eq. 3)

[IRG]_total is the total concentration of reducing groups generated during the hydrolysis of reduced cellulose. As the first catalytic act in the processive run does not produce a detectable SRG but produces detectable IRG, the term [IRG] has to be included to account for N_catal. Comparison of the number of initiations measured using DAP-cellulose and reduced cellulose as a substrate (Fig. 1, C and D) enables us to find the probability of endo-mode initiations (P_endo).

\[ P_{endo} = \frac{[IRG] - [EL]}{[IRG]} = \frac{(N_{init})_{endo}}{(N_{init})_{R-exo} + (N_{init})_{endo}} \] (Eq. 4)

Concentrations of IRGs and released EL for insertion into Equation 4 must be measured at the same value of [SRG]. However, both approaches (Fig. 1, C and D) fail to reveal the exo-mode initiations from the non-reducing end, and its presence leads to the overestimation of P_app. Unfortunately, the non-reducing end lacks specific chemistry, and initiations from this end cannot be visualized.

Sensitive Detection of Reducing End-groups on Cellulose Using Fluorescence Labeling—To reveal the number of initiations, the hydrolysis must be conducted under single-hit conditions, meaning that upon dissociation the possibility for the enzyme to attack the chain end that has been already attacked would be negligible. To avoid the possible limitation of P_app by the length of the cellulose chain, substrates with high DP must be used. High DP celluloses have low numbers of chain ends, meaning that sensitive detection of IRGs is necessary to follow the hydrolysis under single-hit conditions. Among absorbance-based methods, the highest sensitivity and greatest reliability can be achieved with a modified BCA method (14, 36). However, the sensitivity of the BCA method was not sufficient for the purposes outlined in this study. Fluorescence measurements provide about 2 orders of magnitude greater sensitivity than absorbance measurements. Recently we optimized the labeling of cellulose with AA (14). However, about 10 times greater sensitivity can be achieved by the use of DAP. Here we optimized the DAP labeling of IRGs on cellulose. Labeling efficiency was found to be independent of cellulose concentration. Comparative determination of CBH generated IRGs by AA and DAP labeling indicated that DAP labeling was quantitative. See Fig. S1 for determination of fluorescence parameters of DAP-sugar conjugate and optimization of DAP labeling of cellulose. In addition to the detection of CBH generated IRGs on cellulose, DAP labeling was also used for...
Cellobiohydrolases Can Use Endo-mode Initiation—With the aim to quantify the processivity of TrCel7A and PcCel7D, we first assessed the number of initiations in the hydrolysis of reducing-end DAP-labeled bacterial cellulose (DAP-BC). Released DAP-sugar conjugates, hereafter referred to as end-label, were detected by fluorescence. Using this approach we can reveal the number of initiations from the reducing-end \((N_{\text{init}})_{R-\text{exo}}\) (Equation 2, Fig. 1C). However, the resulting \(P_{\text{app}}\) values increased with increasing enzyme to substrate ratio, indicating the presence of another mode of initiation that has lower affinity and that was used in parallel with \((N_{\text{init}})_{R-\text{exo}}\).

To see the possible contribution of endo-mode initiations \((N_{\text{init}})_{\text{Endo}}\), comparative experiments were performed on reduced BC (rBC). After treatment of rBC with CBH, the CBH generated IRGs on residual cellulose were measured using DAP labeling. This approach provides us with the sum of \((N_{\text{init}})_{R-\text{exo}}\) and \((N_{\text{init}})_{\text{Endo}}\) (Equation 3, Fig. 1D). The general activity of CBHs on rBC was similar to that on DAP-BC (Fig. 2A), but the number of initiations measured on rBC was significantly higher than that measured on DAP-BC (Fig. 2B). The concentration of released EL from the DAP-BC hydrolysis and the concentration of IRGs generated to rBC allowed calculation of the probability for endo-initiation \(P_{\text{Endo}}\) (Equation 4).

**FIGURE 1. Principle of the measurement of cellulase processivity.** A, labeling of reducing groups on cellulose via reductive amination with fluorescent DAP is shown. Elimination of water between the aldehyde group of the reducing end of the cellulose chain and the amino group of DAP results in the formation of a Schiff base intermediate (not shown), which is reduced by NaCNBH3 to form a stable aminoditol derivative of cellulose (DAP-cellulose). The stronger reducing agent NaBH4 can be used to reduce aldehyde groups of cellulose to corresponding alditols, which do not give reductive amination with DAP. B, the active site tunnel of TrCel7A and PcCel7D contains 10 glucose unit binding sites (not numbered) and the cleavage site (indicated with arrowheads) is between binding sites \(-2\) and \(+2\). Association \((k_{\text{on}})\) results in the formation of the enzyme-substrate complex that can dissociate \((k_{\text{off}})\) or go through catalytic events \((k_{\text{cat}})\) to form cellobiose and an enzyme-substrate complex localizing one cellobiose unit further on the cellulose chain. Intrinsic processivity \(P_{\text{Intr}}\) can be found from the values of \(k_{\text{on}}\) and \(k_{\text{off}}\). In this study the \(k_{\text{off}}\) values were found on reduced cellulose (Equation 6) using the assumption that \(k_{\text{off}}\) is limiting for enzyme recruitment and \(k_{\text{off}} \gg k_{\text{cat}}\). C, release of DAP-sugar end-label (EL) in hydrolysis of DAP-cellulose reveals the number of initiations from the reducing end of cellulose \((N_{\text{init}})_{R-\text{exo}}\). D, hydrolysis of reduced cellulose and detection of enzyme-generated IRGs on residual cellulose reveals the sum of endo-initiations \((N_{\text{init}})_{\text{Endo}}\) and \((N_{\text{init}})_{R-\text{exo}}\). Apparent processivity \(P_{\text{app}}\) is given by the ratio of enzyme-released SRGs and IRGs (Equation 3). Comparison of the number of initiations measured using DAP-cellulose and reduced cellulose as substrate allows calculation of the probability for endo-initiation \(P_{\text{Endo}}\) (Equation 4).
Tr
earlier studies possible contamination of CBH preparations

The concentration of cellulose was 1.0 g l⁻¹, and enzyme was 20 nm

different time points (see supplemental Table S1).

formation of IRGs in hydrolysis of rAC is shown.

TABLE 1

Probabilities of endo-mode initiation for

TABLE 1

Probabilities of endo-mode initiation for TrCel7A and PcCel7D on bacterial cellulose and amorphous cellulose

Probability of endo-mode initiation (P_endo) was found from comparative hydrolysis of DAP-cellulose and reduced cellulose (Fig. 1, C and D, Equation 4). The concentration of cellulose was 1.0 g l⁻¹, and averages are taken from 5–7 different time points (see supplemental Table S1).

| Enzyme  | Bacterial cellulose | Amorphous cellulose |
|---------|---------------------|---------------------|
|         | TrCel7A             | PcCel7D             |
|         | TrCel7A             | PcCel7D             |
|         | PcCel7D             | TrCel7A             |
|         | PcCel7D             | TrCel7A             |

A more open active site structure of Cel7A is surrounded by four loops with the higher affinity than the reducing-end exo initiation. On AC, the P_endo values were determined also for EGs TrCel5A and TrCel12A. Both enzymes displayed P_endo values above 0.97 (supplemental Fig. S2). To rule out the possibility that observed endo-mode initiations were caused by the modifications at the reducing ends of DAP-BC and rBC, the use of endo-mode initiation by CBHs was also confirmed on native unmodified BC (supplemental Fig. S3).

Reports on possible endo-activity of family 7 CBHs have been contradictory (39). Kinetic studies with tetrasaccharide carrying bulky substituents on both of its ends suggested a possible endo-character of TrCel7A (40). Endo-activity of TrCel7A on cellulose has also been reported before (41) but in earlier studies possible contamination of CBH preparations with EGs was often suspected (39). Numerous control experiments showed that contamination of CBH preparations by EG activity cannot be the reason for the high P_endo values observed here (for the results and more detailed discussion, see supplemental material and supplemental Figs. S3 and S4).

Based on its structure, TrCel7A has been considered a highly processive strictly reducing-end exo-acting enzyme (17). The active site tunnel of TrCel7A is surrounded by four loops with the major one known as an “exo-loop” that roofs the tunnel (9, 10, 12). Although for family 6 CBHs the mobility of active site loops that allows occasional opening of the tunnel and endo-mode of initiation is well documented (42, 43), it has not been demonstrated for family 7 CBHs. Results presented here suggest this possibility also for family 7 CBHs. The structure of PcCel7D is very similar to that of TrCel7A but reveals a deletion of six residues in the corresponding exo-loop (12). A more open active site structure of PcCel7D can account for the higher P_endo values observed here. Observed higher P_endo values on AC compared with BC apparently reflect the higher amount of sites available for endo-action on AC (Table 1). Alteration between endo- and exo- modes of action depending on the crystallinity of the substrate have been suggested before for a cellulase as well as κ-carrageenase (44, 45). High P_endo values observed here for TrCel7A and PcCel7D together with earlier reports of mixed exo/endo character of cellulases (23) and chitinases (28) suggest that strict exo-acting enzymes may be rare if present at all among cellulases and chitinases.

Apparent Processivity of Celllobiohydrolases Depends on the Substrate—Apparent processivity (P_app) was measured on rBC and rAC using the DAP labeling of cellulase generated IRGs on the residual cellulose (Fig. 1D). Data were plotted in coordinates of [RG_Tot] versus [IRG]. If single hit conditions

FIGURE 2. Comparative hydrolysis of DAP-cellulose and reduced cellulose reveals the use of endo-mode initiation by celllobiohydrolases TrCel7A and PcCel7D. Experiments were performed in 50 mM sodium acetate, pH 5.0, at 30 °C. Cellulose concentration was 1.0 g liter⁻¹, and enzyme was 20 nm TrCel7A (□) and ▪ or PcCel7D (● and △). Open symbols (□ and ●) refer to DAP-cellulose, and filled symbols (▪ and △) refer to reduced cellulose. Error bars are from three independent measurements. A, release of SRGs in hydrolysis of DAP-BC and rBC is shown. B, release of EL in hydrolysis of DAP-BC and formation of IRGs in hydrolysis of rBC is shown. C, release of SRGs in hydrolysis of DAP-AC and reduced rAC is shown. D, release of EL in hydrolysis of DAP-AC and formation of IRGs in hydrolysis of rAC is shown.
are met, the data plotted in these coordinates should follow the straight line with the slope equal to \( \frac{P_{\text{app}}}{P_{\text{off}}} \) (Fig. 3, Equation 3). On rBC, the \( P_{\text{app}} \) values of 61 ± 14 and 52 ± 5 were found for \( \text{TrCel7A} \) and \( \text{PcCel7D} \), respectively. \( P_{\text{app}} \) values measured for \( \text{TrCel7A} \) and \( \text{PcCel7D} \) on rAC were also similar to each other but lower than those measured on rBC (Fig. 3 and Table 2). On rAC, \( P_{\text{app}} \) values were also measured for EGs \( \text{TrCel5A} \) and \( \text{TrCel12A} \) (Fig. 3B). \( P_{\text{app}} \) values of 4.7 ± 0.2 and 2.7 ± 0.2 found for \( \text{TrCel5A} \) and \( \text{TrCel12A} \), respectively, were significantly lower than corresponding figures for CBHs (Table 2).

The most distributive EG was \( \text{TrCel12A} \). \( P_{\text{app}} \) found for \( \text{TrCel5A} \) is similar to the processivity of 4.3 estimated for another family 5 cellulase, EG Cel5H, from the bacterium \textit{Saccharophagus degradans} (30).

Although reports on the values of processivity of glycoside hydrolases are rare, approximate figures can be found in the literature. Detection of enzyme generated IRGs on unmodified cellulose by the use of absorbance-based methods have revealed processivity values for CBHs and processive EGs between 5 and 25 (24–27, 29). Although low, these figures are expected to be overestimated for two reasons; (i) if single-hit conditions are not satisfied, repeated attacks on an IRG, once attacked by the enzyme, will not be detected, and (ii) detection of IRGs on unmodified cellulose can reveal only the number of endo-initiations. Assessment of the processivity of chitinases from \textit{Serratia marcescens} on partly deacetylated soluble chitin derivative under single-hit conditions but using the assumption that all initiations are endo-mode revealed processivity values of 9.1 and 3.4 for ChiA and ChiB, respectively (28). ChiA and ChiB are effective in degradation of crystalline chitin, and the structure of both enzymes is in accordance with the processive mode of action (46, 47). For CBHs, the ratio of released cellobiose to glucose has also been used as a measure of the processivity, but the validity of this approach depends on the preferences for the initial binding mode between CBH and cellulose (31). The latter approach also results in processivity values between 5 and 25 (12, 29, 31).

We have reported processivity values for \( \text{TrCel7A} \) around 90 and 40 cellobiose units on AA-labeled BC and AA-labeled bacterial microcrystalline cellulose, respectively (7). However, these figures may be overestimated because only \( N_{\text{init}}^{\text{b-exo}} \) was detected. In that study the lower processivity on AA-bacterial microcrystalline cellulose was explained by possible limitations of processivity by the DP of AA-bacterial microcrystalline cellulose (7). The average DPs of rBC and rAC used in this study (determined before the reduction of cellulose using the BCA method) (14) were 2300 and 1800 glucose units, respectively, meaning that measured \( P_{\text{app}} \) values cannot be limited by the DP of the substrate. The fact that \( P_{\text{app}} \) values for \( \text{TrCel7A} \) and \( \text{PcCel7D} \) were similar to each other but differed on different celluloses (Table 2) suggests that the \( P_{\text{app}} \) of CBHs is determined by the physicochemical properties of the substrate.

**Estimation of Intrinsic Processivity of Cellulases**—Finding that \( P_{\text{app}} \) of CBHs was dependent on the nature of the substrate prompted us to estimate the values of intrinsic processivity \( (P_{\text{intr}}) \) of cellulytic enzymes. \( P_{\text{intr}} \) depends on the values of two rate constants, the dissociation rate constant of the enzyme-substrate complex \( (k_{\text{off}}) \), and the catalytic constant \( (k_{\text{cat}}) \).
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represents the rate constant leading from one productive complex through catalytic events to another productive complex one product unit further on the polymer chain (Fig. 1B). For processive enzymes, \( k_{\text{cat}} \gg k_{\text{off}} \) and \( P_{\text{Intr}} \) approximates to

\[
P_{\text{Intr}} \approx \frac{k_{\text{cat}}}{k_{\text{off}}}
\]

(Eq. 5)

Thus, to estimate \( P_{\text{Intr}} \) we need estimates for \( k_{\text{cat}} \) and \( k_{\text{off}} \).

Measurement of the values of kinetic parameters for cellulases acting on cellulose has been complicated by non-linear kinetics that is caused by the heterogeneous nature of the insoluble substrate (48). Fig. 4 shows the formation of RG\(_{\text{Tot}}\) in time upon hydrolysis of rAC by TrCel7A, PcCel7D, or TrCel5A. Both CBHs displayed characteristic “burst” kinetics (7) with the first rapid formation of RG\(_{\text{Tot}}\) followed by the much slower but linear formation of RG\(_{\text{Tot}}\) in time (Fig. 4). In this linear stage the rate of enzyme recruitment is at a steady state (7). In our previous study we demonstrated that the association of CBH and cellulose (step 1 in Fig. 1B) was fast and proposed that the rate of recruitment of CBH was limited by the slow dissociation of CBH from the cellulose chain (49). Thus, using the assumption that \( k_{\text{off}} \) is rate-limiting for enzyme recruitment and \( k_{\text{cat}} \gg k_{\text{off}} \) the value of \( k_{\text{off}} \) can be found from the rate of initiations (\( \Delta N_{\text{init}}/\Delta t \)) in the linear stage that follows the initial transient burst caused by the first rapid binding.

\[
k_{\text{off}} = \frac{\Delta N_{\text{init}}}{\Delta t[E_{\text{S}}]} = \frac{\Delta [\text{IRG}]}{\Delta t[E_{\text{Tot}}]}
\]

(Eq. 6)

The term \([E_{\text{S}}]\) in Equation 6 stands for the concentration of enzyme-substrate complex, and \([E_{\text{Tot}}]\) is total enzyme concentration. Approximation of \([E_{\text{S}}] = [E_{\text{Tot}}]\) is justifi ed because of the low enzyme and high substrate concentrations used in this study (49).

\( k_{\text{off}} \) values were found from the linear region of the formation of IRGs in time (Fig. 2, B and D, supplemental Fig. S5, A and B) and are listed in Table 2. For both TrCel7A and PcCel7D, the \( k_{\text{off}} \) values on rBC were about 3 times lower than on rAC, indicating that interactions between the cellulose chain and the active site tunnel of CBHs are not sole determinants of \( k_{\text{off}} \). The \( k_{\text{off}} \) value of 0.029 ± 0.003 s\(^{-1}\) (30 °C) has been reported for dissociation of isolated carbohydrate binding module of TrCel7A from bacterial microcrystalline cellulose (50). This figure is about 30 times higher than the \( k_{\text{off}} \) found for dissociation of TrCel7A from rBC, suggesting that dissociation of TrCel7A is not limited by the dissociation of its carbohydrate binding module. On rAC the \( k_{\text{off}} \) values were also measured for the EGs TrCel5A and TrCel12A. \( k_{\text{off}} \) values for the EGs were about 2 orders of magnitude higher than those for CBHs (Table 2). This finding is consistent with a shorter and more open active site architecture of EGs (17).

Recently we proposed a mechanism for initial rapid rate retardation in CBH-catalyzed cellulose hydrolysis (49). According to this, the rate of formation of hydrolysis products after the first transient stage is governed by the value of \( k_{\text{off}} \) and the average length of the obstacle-free path of CBH on a cellulose chain. Thus, to measure the value of \( k_{\text{cat}} \) (step 2 in Fig. 1B), one must follow the progression of hydrolysis in the first transient stage before most of the CBH molecules have not yet passed through the average obstacle-free path. Therefore, \( k_{\text{cat}} \) values were found from the initial rates (based on activity measurements after 10 s of hydrolysis) of the formation of RG\(_{\text{Tot}}\) in hydrolysis of rBC and rAC at different concentrations (supplemental Fig. S5, C and D) and are listed in Table 2. With all cellulases, the \( k_{\text{cat}} \) values obtained here are in accordance with the corresponding figures found from the initial rates of the hydrolysis of amorphous and crystalline celluloses (51). Although the \( k_{\text{cat}} \) values for EGs were higher than corresponding figures for CBHs, the difference was much less prominent than in the case of \( k_{\text{off}} \) values (Table 2).

Provided with the estimates for \( k_{\text{cat}} \) and \( k_{\text{off}} \) we can now estimate the values of \( P_{\text{Intr}} \) using Equation 5. The highest \( P_{\text{Intr}} \) value (4000 ± 570) was found for TrCel7A on rBC, \( P_{\text{Intr}} \) values for PcCel7D were lower than those for TrCel7A on both rBC and rAC, and the difference was governed by differences in \( k_{\text{off}} \) values (Table 2). For both CBHs, \( P_{\text{Intr}} \) values were more than an order of magnitude higher than measured \( P_{\text{Intr}} \) values, confirming the limitation of the CBH processivity by the substrate. We propose that it is the average length of the obstacle-free path on the cellulose chain that sets limits for processive movement of CBHs. For EGs, the differences between \( P_{\text{Intr}} \) and \( P_{\text{Intr}} \) values were less obvious (Table 2). Because of their lower intrinsic processivity, EGs are expected to be less prone to hindrance by obstacles.

CBHs have evolved for the degradation of lignocellulosic substrates. The average length of the obstacle-free path available for processive runs (49) in lignocelluloses is expected to be much shorter than \( P_{\text{Intr}} \) values estimated for CBHs (Table 2). This suggests that the value of \( P_{\text{Intr}} \) itself cannot be the primary target for the selection of CBHs. CBHs TrCel7A and PcCel7D differed in \( k_{\text{off}} \) values, whereas their \( k_{\text{cat}} \) values were similar (Table 2). Furthermore, large differences between the \( P_{\text{Intr}} \) values for EGs and CBHs were also caused by widely different \( k_{\text{off}} \) values, whereas \( k_{\text{cat}} \) values for CBHs and EGs were
within the same order of magnitude (Table 2). These results suggest that the value of $k_{\text{off}}$ may be the primary target for the selection of cellulases. $k_{\text{off}}$ values in the range of $10^{-3}$ s$^{-1}$ (Table 2) correspond to the half-lives of CBH cellulose complexes, around 10 min. One may speculate that halted CBHs are in the “standby” position to catch the momentum to move forward if an obstacle dissociates (49), but the biological rationale of these stable non-productive complexes remains to be solved. On the other hand, low $k_{\text{off}}$ apparently limits the performance of CBHs on soluble and amorphous polymers where the accessibility of glycosidic bonds is high. On these substrates enzymes with high $k_{\text{off}}$ values and, therefore, high rates of enzyme recruitment, like EGs, are at an advantage (Fig. 4). The disadvantage of processivity on soluble and amorphous polymers was first clearly demonstrated for chitinases ChiB (19) and ChiA (52) from S. marcescens but has also been suggested for cellulases (12) and endosialidase (53). However, biomass pretreatment technologies that result in the production of amorphous cellulose are not yet cost effective for large scale industrial applications, meaning that the degradation of crystalline cellulose remains a key issue in commercialization of second generation biofuels. It should be noted that not all cellulytic systems rely on processive CBHs (30, 54, 55). Therefore, the exact mechanism of different strategies that have evolved for cellulose degradation and the role of the processivity of enzymes remain intriguing questions.

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