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Inhibition of the *Trichoderma reesei* Cellulases by Cellobiose Is Strongly Dependent on the Nature of the Substrate

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Abstract: The inhibition effect of cellobiose on the initial stage of hydrolysis when cellobiohydrolase Cel 7A and endoglucanases Cel 7B, Cel 5A, and Cel 12A from *Trichoderma reesei* were acting on bacterial cellulose and amorphous cellulose that were [^{3}H]- labeled at the reducing end was quantified. The apparent competitive inhibition constant (K_i) for Cel 7A on [^{3}H]-bacterial cellulose was found to be 1.6 $\pm$ 0.5 m*M*, 100-fold higher than that for Cel 7A acting on low-molecular-weight model substrates. The hydrolysis of [^{3}H]-amorphous cellulose by endoglucanases was even less affected by cellobiose inhibition with apparent K_i values of 11 $\pm$ 3 m*M* and 34 $\pm$ 6 m*M* for Cel 7B and Cel 5A, respectively. Contrary to the case for the other enzymes studied, the release of radioactive label by Cel 12A was stimulated by cellobiose, possibly due to a more pronounced transglycosylating activity. Theoretical analysis of the inhibition of Cel 7A by cellobiose predicted an inhibition analogous to that of mixed type with two limiting cases, competitive inhibition if the prevalent enzyme-substrate complex without inhibitor is productive and conventional mixed type when the prevalent enzyme-substrate complex is nonproductive. © 2004 Wiley Periodicals, Inc.

Keywords: cellulase; cellulose; cellobiose; inhibition; hydrolysis; transglycosylation

INTRODUCTION

Cellulose is the major component of plant cell walls and the most abundant organic compound in the biosphere. In nature, the degradation of cellulose is carried out mainly by fungi and bacteria. These microorganisms produce a set of extracellular enzymes, the cellulolytic system. The well-studied cellulolytic enzyme system produced by the filamentous fungus *Trichoderma reesei*, consists of at least two cellobiohydrolases, Cel 7A (formerly CBH I; Henrissat et al., 1998) and Cel 6A (CBH II); five endoglucanases, Cel 7B (EG I), Cel 5A (EG II), Cel 12A (EG III), Cel 61A (EG IV), and Cel 45A (EG V); and two β-glucosidases (Bhikhabhai et al., 1984; Chen et al., 1992; Saloheimo et al., 1988). Typically, cellobiohydrolases have a tunnel-like catalytic site with many substrate-binding subsites (reviewed in Teeri, 1997) and are able to hydrolyze crystalline cellulose processively, releasing mainly cellobiose. The three-dimensional structure of Cel 7A reveals a 50 Å-long tunnel with at least seven substrate-binding subsites and two product subsites (Divne et al., 1994;1998). Cellobiose is found to bind almost exclusively to the +1 and +2 subsites. In contrast, endoglucanases have a more open substrate-binding groove (Teeri, 1997), exhibit comparatively poor activity toward crystalline substrates and presumably act mainly on the amorphous or disordered regions of cellulose. In contrast to cellobiohydrolases, endoglucanases produce significant amounts of glucose and higher cellooligosaccharides in addition to cellobiose (Karlsson et al., 2002; Medve et al., 1998).

A typical feature in the time course of cellulose hydrolysis is the pronounced decrease in rate observed already at a very low degree of conversion. Strong product inhibition by cellobiose has been reported among the rate-retarding factors (Gusakov and Sinitsyn, 1992; Holtzapple et al., 1990). Quantitative knowledge about product inhibition is important in the design of suitable reactors and schemes for the production of fermentable sugars from cellulosic materials. Cellobiose inhibition of Cel 7A acting on low-molecular-weight chromogenic substrates like para-nitrophenylcellobioside has been well studied and reveals strong competitive inhibition, with an inhibition constant around 20 μ*M* (Claeyssens et al., 1989; van Tilbeurgh and Claeyssens 1985; Vonhoff et al., 1999). Such a strong product inhibition would be a drawback from a biological point of view, limiting the levels of soluble sugars that can be produced. However, it has been shown that the hydrolysis of natural, cellulosic, substrates is more resistant to inhibition and product inhibition is not responsible for the gradual decrease in the hydrolysis rate at the early stage of the

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process (Väljamäe et al., 1998; Zhang et al., 1999). To date, there has been very little quantitative information about product inhibition during the initial stage of cellulose hydrolysis. Ryu and Lee (1986) studied a crude cellulase preparation from *T. reesei* and found a competitive inhibition constant of 3.75 mg/mL (11 m*M*) for cellobiose, while no corresponding data is available for purified enzymes. Analysis of progress curves cannot be used for determination of the inhibition constants for cellulose hydrolysis, since there are other factors, such as changes in cellulose structure that also influence the rate (Nidetzky and Steiner 1993; Väljamäe et al., 1998; Zhang et al., 1999). Determination of inhibition constants must instead be based on initial rate measurements, which are complicated by the high background level of product initially present in inhibition studies. Several attempts have been made to overcome this by monitoring the initial rates of dye release from dyed cellulose derivatives (Gusakov et al., 1985; Holtzapple et al., 1984; 1990). However, this approach fails for cellobiohydrolases where the tunnel-shaped active sites cannot accommodate bulky dye groups. The activity of endoglucanases might be influenced by the dye groups as well. Furthermore, there is very little information about the initial rate based k_{cat} values for cellulases acting on cellulosic substrates. The relatively low activity of cellulolytic enzymes already at the initial stage together with the rapid decrease in reaction rates, makes the estimation of true initial rates a difficult task.

In this study we used [^{3}H] reducing-end labeled bacterial cellulose ([^{3}H]-BC) and amorphous cellulose ([^{3}H]-amorphous cellulose) as substrates for initial rate-based quantitative analysis of inhibition by cellobiose of cellobiohydrolase Cel 7A and endoglucanases Cel 7B, Cel 5A, and Cel 12A from *Trichoderma reesei*. We also give minimum estimates for the turnover numbers of those cellulases acting on cellulosic substrates. A theoretical analysis is also provided for the inhibition of Cel 7A by cellobiose.

MATERIALS AND METHODS

Reagents and Enzymes

Sodium boro [^{3}H] hydride with a specific activity of 48 Ci/mmol was from Amersham International. Scintillation cocktail QUICKSAFE A^{TM} was from ScintVaruhuset AB, Uppsala, Sweden. 2,2′-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) cellobiose, glucose oxidase and peroxidase were purchased from Sigma (St. Louis, Missouri, USA), β-glucosidase was from Fluka (Buchs, Switzerland).

Enzymes Cel 7A, Cel 7B, Cel 6A, Cel 5A, and Cel 12A were purified from culture filtrate of *T. reesei* strain QM 9414 as described in (Bhikhabhai et al., 1984; Håkansson et al., 1978; Saloheimo et al., 1988). The purity of the enzymes was confirmed by SDS/PAGE.

Enzyme Concentrations

Enzyme concentrations were determined from absorbance measurements at 280 nm using the molar absorption coefficients 78,800; 67,000; 92,000; 78,000 and 38,200 M^{-1} cm^{-1} for Cel 7A, Cel 7B, Cel 6A, Cel 5A, and Cel 12A, respectively.

Cellulose Concentration

Cellulose concentration was determined by the anthrone/sulphuric acid method using cellobiose as standard and absorbance measurements at 585 nm (Hörmann and Gollwitzer, 1962). Cellulose samples were solubilized completely by a mixture of *T. reesei* cellulases prior to analysis of total sugar.

Preparation of Bacterial Cellulose

Bacterial cellulose (BC) and bacterial microcrystalline cellulose were prepared from commercially available *Acetobacterium xylinum* cellulose (CHAOKOH® coconut gel in syrup from Thep. Padung Porn Coconut Co. Ltd., Bangkok, Thailand) as described in Väljamäe et al. (1999).

Preparation of Amorphous Cellulose

Amorphous cellulose (for review, see Dawsey and McCormick, 1990) was prepared from bacterial microcrystalline cellulose by the following procedure: Freeze-dried cellulose was dissolved in solution of 10% (w/v) LiCl in dry *N,N*-dimethylacetamide to give a cellulose concentration of 5 mg/mL. After dissolution, the sample was diluted with *N,N*-dimethylacetamide to a LiCl concentration of 1%. An equal volume of 98% ethanol was then added dropwise to the cellulose solution under vigorous stirring. Finally, one third volume of water was added dropwise and the suspension was left under stirring overnight. After regeneration, the cellulose was washed thoroughly with water and reaction buffer.

Labeling of the Reducing Ends on Cellulose With [^{3}H]

Labeling of the reducing ends on cellulose with [^{3}H] (Nutt et al., 1998) was carried out as follows: 1 mL of 2.0*M* sodium borohydride solution in 0.1*M* NaOH and 0.25 mL (25 MBq) of sodium boro[^{3}H]hydride in 0.1*M* NaOH was added to 350 mg of cellulose in 50 mL of 0.1*M* NaOH. The mixture was boiled for 1 h then a new amount of reagents was added and boiling was continued for another hour. The reduced cellulose was washed on a glass filter with water and stored in glycerol. Labeled cellulose was washed thoroughly with water and reaction buffer before use. In the case of amorphous cellulose, the step of regeneration from the solution of *N,N*-dimethylacetamide and LiCl was repeated after labeling. Labeled cellulose samples were solubilized completely by a mixture of *T. reesei* cellulases prior to analysis of specific activity. The specific activity of labeled cellulose

was 29400 CPM/mg and 33400 CPM/mg for [^{3}H]-BC and [^{3}H]-amorphous cellulose, respectively.

Enzymatic Hydrolysis of Cellulose

Enzymatic hydrolysis was carried out in 1.5 mL Eppendorff tubes by incubating cellulose suspensions (1–5 mg/mL) in 0.05*M* sodium-acetate (NaAc) buffer, pH 5.0, with enzyme at 25 °C in a total volume of 550 μL without agitation. In the inhibition studies the solutions were provided with the desired concentration of cellobiose (1–100 m*M*). The reaction was initiated by addition of enzyme and stopped after 10 s by addition of 1.0*M* NaOH to a final pH of 12.5 or by addition of 1.0*M* methylamine to a final pH of 11.5.

The cellulose residue was pelleted by centrifugation (16,000*g*, 5 min) and the radioactivity (and/or cellobiose) in the supernatant was quantified. Radioactivity was quantified using a Wallac WinSpectral^{TM} 1414 liquid scintillation counter. No significant variation in the counting efficiency among the different samples was observed. Zero data points were made by addition of the cellulase shortly after the alkali and were otherwise treated similarly. All data points were analyzed at least in triplicate and the data are represented as averages.

The cellobiose concentration (as total sugar) was determined by the following procedure: 600 μL supernatant aliquots were neutralized by addition of 41 μL of 1.56*M* acetic acid to obtain pH 5.0 followed by addition of 2.0 m*M* ABTS and a mixture of β-glucosidase, glucose oxidase, and peroxidase and incubated in a water bath at 25 °C overnight. The final concentrations of the reagents in the assay were 0.2 m*M* for ABTS, 0.5 U/mL for β-glucosidase, 2.0 U/mL for glucose oxidase, and 0.5 U/mL for peroxidase. Units of enzyme activity were as stated by the manufacturer. The oxidation of ABTS was followed by the increase in absorbance at 420 nm. Calibration curves were made using cellobiose as standard, including the addition of methylamine and acetic acid to mimic the sample treatment. The assay sensitivity allowed measurements from 0.5 μ*M* cellobiose in the hydrolysis mixture. Since β-glucosidase also degrades higher cello-oligosaccharides to glucose the above assay gives us the concentration of total sugar when cellulase action on pure cellulose is studied.

It must be noted here that the alkaline pH used for termination of the cellulase reaction can influence the assay. A time-dependent reduction in the assay signal was observed when the cellobiose was incubated in the presence of Cel 7A at pH values above 12 before the assay. This effect was, however, negligible at lower pH values and pH 11.5 was found to be optimal for rapid termination of the enzymatic hydrolysis.

THEORETICAL ASPECTS

The substrate concentration ($S_{0.5}$, mg/mL) corresponding to half the limiting release rate of label (P_{lim}, CPM $\cdot$ mL$^{-1}\cdot$s^{-1}) were calculated by nonlinear regression of radioactivity data according to the equation:

$$p = \frac{P_{\lim} \cdot [S]}{[S] + S_{0.5}} \tag{1}$$

where [*S*] and *p* stand for the cellulose concentration (mg/mL) and the initial rate of the release of radioactivity (CPM $\cdot$ mL$^{-1}\cdot$s^{-1}), respectively. Equation (1) was also used for the estimation of kinetic parameters for the hydrolysis of nonlabeled celluloses. In this case *p* represents the initial rate of the release of soluble sugars (as cellobiose $\mu M \cdot s^{-1}$) and P_{lim} is corresponding limiting rate (as cellobiose $\mu M \cdot s^{-1}$).

Apparent competitive inhibition constants (K_i) were calculated using nonlinear regression of hydrolysis data obtained in the presence of different concentrations of supplied cellobiose [*I*] (m*M*) according to the equation for competitive inhibition:

$$p = \frac{P_{\lim} \cdot [S]}{[S] + S_{0.5} \cdot \left(1 + \frac{[I]}{K_i}\right)} + H \tag{2}$$

where [*S*] and *H* stand for the cellulose concentration (mg/mL) and the background radioactivity (CPM $\cdot$ mL$^{-1}\cdot$ s^{-1}), respectively. $S_{0.5}$ was fixed at a value obtained from Eq. (1).

Derivation of the steady-state rate equation for the mechanism depicted in Figure 5 was carried out using the procedure proposed by King and Altman (1956). The following concentration terms were used:

e_0	total enzyme
[*S*]	free substrate
[*I*]	free inhibitor
[E]	free enzyme
[EI]	enzyme-inhibitor complex
[*EIS*]	enzyme-inhibitor-substrate ternary complex
[*ES*]	productive enzyme-substrate complex
[*ES′*]	nonproductive enzyme-substrate complex

The definitions of the constants used in Eq. (3), are the following:

$$K_S = \frac{k_{-1}}{k_1},\ K_{EI} = \frac{k_{-2}}{k_2},\ K_{EIS} = \frac{k_{-4}}{k_4},\ K_{ES}\frac{k_{-6}+k_{cat}}{k_6}$$

Theoretical Analysis of the Inhibition of Cel 7A by Cellobiose

Due to the polymeric nature of the substrate and the multiple glucosyl-unit binding tunnel of Cel 7A the enzyme can combine with substrate in different ways, both productively, where a glucosidic bond is positioned for cleavage, and nonproductively, where the substrate has to move to be positioned correctly. The simplest reasonable mechanism for Cel 7A acting on polymeric substrate is depicted in Figure 5. Derivation of the steady-state rate expression according to the mechanism presented in Figure 5 using the method proposed by King and Altman

(1956) results in Eq. (3). The only simplifying assumption made was that the second-order rate constants for the binding of substrate were not affected by the inhibitor and vice versa, i.e, $k_1 = k_3$ and $k_2 = k_4$.

$$v = \frac{\frac{k_{cat} \cdot e_0 \cdot [S]}{1 + K_{ES}\left(1 + \frac{[I]}{K_{EIS}}\right)}}{[S] + \frac{K_S \cdot K_{ES}\left(1 + \frac{[I]}{K_{EI}}\right)}{1 + K_{ES}\left(1 + \frac{[I]}{K_{EIS}}\right)}} \quad (3)$$

Equation (3) is analogous, but not identical, to the expression for mixed-type inhibition where both limiting rate and apparent dissociation constant is affected by the inhibitor. Eq. (3) has two limiting cases:

1. If K_{ES} (for the definition of K_{ES} and other constants see above) is very small then the term $1 + K_{ES}$ $(1+ [I]/K_i)$ approaches 1 and Eq. (3) reduces to

$$v = \frac{k_{cat} \cdot e_0 \cdot [S]}{[S] + K_S \cdot K_{ES}\left(1 + \frac{[I]}{K_{EI}}\right)}$$

   i.e., the equation for competitive inhibition.

2. If K_{ES} is large, the term $1 + K_{ES}$ $(1+ [I]/K_i)$ is approximated by K_{ES} $(1+[I]/K_i)$ and Eq. (3) reduces to

$$v = \frac{\frac{k_{cat} \cdot e_0 \cdot [S]}{K_{ES}\left(1 + \frac{[I]}{K_{EIS}}\right)}}{[S] + \frac{K_S\left(1 + \frac{[I]}{K_{EI}}\right)}{\left(1 + \frac{[I]}{K_{EIS}}\right)}}$$

   i.e., the equation for conventional mixed-type inhibition.

In the absence of inhibitor, Eq. (3) is in the form of Michaelis-Menten equation with the following parameters:

$$V_{\lim} = \frac{k_{cat} \cdot e_0}{1 + K_{ES}} \text{ and } K_M = \frac{K_{ES} \cdot K_S}{1 + K_{ES}}$$

RESULTS

Estimation of Kinetic Parameters for Cellulases on Cellulose

The initial rate of the release of soluble sugar from amorphous cellulose by *T.reesei* cellobiohydrolases Cel 7A and Cel 6A and endoglucanases Cel 7B, Cel 5A, and Cel 12A as a function of substrate concentration is shown in Figure 1. Initial rates were based on the measurements of the released soluble sugars (calibrated with cellobiose) after 5 s or 10 s of hydrolysis. These time points were in the linear region of the time course. The k_{cat} and $S_{0.5}$ values

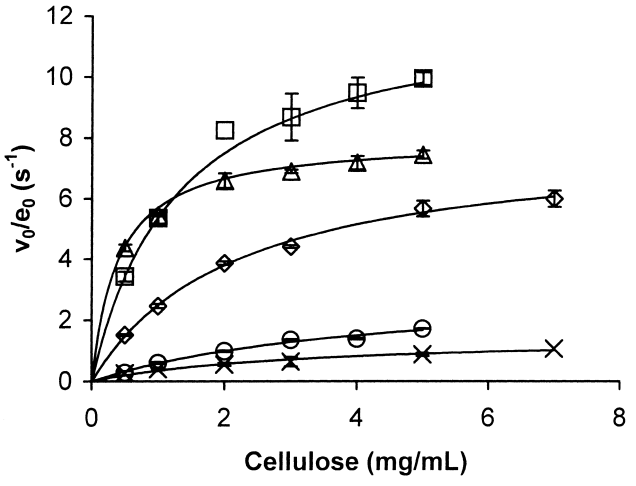

Figure 1. Initial rate based activities (v_0 / e_0) in hydrolysis of amorphous cellulose. Amorphous cellulose (drBMCC) was incubated with enzyme in 0.05*M* sodium acetate buffer, pH 5.0, at 25°C. Initial rates were based on cellobiose formation after 5–10 s hydrolysis. (◇) 0.5 μ*M* Cel 6 A, (×) 1.0 μ*M* Cel 7A, (□) 1.0 μ*M* Cel 7B, (△) 1.0 μ*M* Cel 5A and (○) 1.0 μ*M* Cel 12A. The solid lines are based on Eq. (1).

calculated from the hydrolysis data by nonlinear regression according to Eq. (1) are listed in Table I. The estimated apparent $S_{0.5}$ values were dependent on the enzyme concentration, with higher $S_{0.5}$ values observed at higher enzyme loadings (Table I). The results obtained at lower enzyme loads are regarded as more reliable since the estimation of k_{cat} is more error prone at higher $S_{0.5}$ values. The estimated k_{cat} for Cel 7A was highest ($2.5 \pm 0.5\ s^{-1}$) on bacterial microcrystalline celluloses. For endoglucanases the highest reliable k_{cat} ($12.4 \pm 0.2\ s^{-1}$) was found in the case of Cel 7B on amorphous cellulose (Table I). Although the $S_{0.5}$ value for Cel 6A on amorphous cellulose was dependent on the total concentration of enzyme, the k_{cat} of $8\ s^{-1}$ observed was virtually unaffected.

Hydrolysis of [^{3}H]-Cellulose at Different Substrate Concentrations

The normalized rate (p/P_{lim}) of the release of radioactivity after 10 s of hydrolysis as a function of substrate concentration is depicted in Figure 2. The substrates used were reducing-end-labeled bacterial cellulose ([^{3}H]-BC) in the case of Cel 7A and reducing-end-labeled amorphous cellulose ([^{3}H]-amorphous cellulose) in the case of endoglucanases. The substrate concentrations ($S_{0.5}$) causing half limiting release rate of label (P_{lim}) were calculated from the hydrolysis data by nonlinear regression according to Eq. (1) and are listed in Table I. A necessary condition for the use of radioactively reduced celluloses in the study of product inhibition is that the release of label must be strictly related to the general activity of the enzyme. The $S_{0.5}$ value of 2.2 ± 1.0 mg/mL for Cel 7A acting on [^{3}H]-BC is consistent with the $S_{0.5}$ value of 2.1 ± 0.5 mg/mL found using nonreduced bacterial cellulose and measurements of released cellobiose

Table I. Estimated apparent parameters k_{cat}, $S_{0.5}$, and K_i values for the hydrolysis of [^{3}H]-labeled and nonlabeled celluloses by *Trichoderma reesei* cellulases. The substrate concentration ensuring half limiting release rate of label or soluble sugars ($S_{0.5}$ and k_{cat} values based on the release of soluble sugars were calculated according to Eq. (1). The apparent competitive inhibition constants (K_i) were calculated according to Eq. (2).

Substrate	Enzyme	Enzyme concentration (μM)	$S_{0.5}$[a] mg/mL	k_{cat}[a] s^{-1}	$S_{0.5}$[b] mg/mL	K_i[b] mM
AC	Cel7A	1.0	3.2 ± 1.2	1.5 ± 0.3	n.d.	n.d.
AC	Cel6A	0.5	2.2 ± 0.2	8.0 ± 0.3	n.d.	n.d.
		0.25	1.6 ± 0.3	7.8 ± 0.6	n.d.	n.d.
AC	Cel7B	1.0	4.3	20	5.8 ± 1.2	11 ± 3
		0.25	1.3 ± 0.2	12.4 ± 0.2	n.d.	n.d.
AC	Cel5A	1.0	0.4 ± 0.3	8.0 ± 0.1	5.6 ± 0.7	34 ± 6
	Cel12A	1.0	4.2 ± 1.8	3.1 ± 0.7	4.1 ± 0.3	Activation
		2.0	4.1	3.6	n.d.	n.d.
BC	Cel7A	0.5	2.1 ± 0.5	1.7	n.d.	n.d.
		1.0	n.d.	n.d.	2.2 ± 1.0	1.6 ± 0.5
BMCC	Cel7A	1.0	2.9 ± 1.2	2.5 ± 0.5	n.d.	n.d.

Note: Abbreviations: AC; amorphous cellulose, n.d.; not determined.
[a]Parameter values are based on the release of soluble sugars.
[b]Parameter values are based on the release of labeled primary end groups.

(Fig. 2, Table I). At the same time, parallel measurements of the released cellobiose and released label at a fixed [^{3}H]-BC concentration resulted in a linear relationship between these quantities at the initial stage of hydrolysis (data not shown). It must be noted here that for another *T. reesei* cellobiohydrolase, Cel 6A, a linear time course for the release of label was not achieved and the inhibition data cannot be presented, probably because Cel 6A acts preferentially from the nonreducing end (Boisset et al., 2000; Nutt et al., 1998). The $S_{0.5}$ values for endoglucanases Cel 7B and Cel 12A on [^{3}H]-amorphous cellulose were also in acceptable correlation with the values found using the nonreduced substrates (Table I). However, the $S_{0.5}$ value for endoglucanase Cel 5A acting on [^{3}H]-amorphous cellulose was an order of magnitude higher than the value found on nonreduced substrate (Table I) and, thus, the activity measurements based on the following release of reduced primary end groups might not be representative of the general activity of that enzyme.

The relative instability of the [^{3}H]-labeled celluloses, especially under alkaline conditions, was a drawback in the inhibition studies and sets the limits for the sensitivity of the assay. Time-dependent and leveling-off release of radioactivity was observed under alkaline conditions (Fig. 3). "When cellulose has been treated once with alkali until the release of radioactivity was leveling off and thoroughly washed, a second cycle of alkalification causes a much lower, but still noticeable new burst of radioactivity release

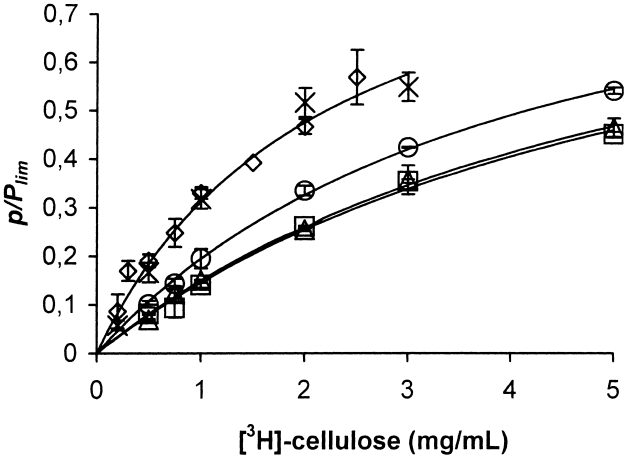

Figure 2. Normalized activity (p/P_{lim}) of hydrolysis of [^{3}H]-labeled celluloses with cellulases. [^{3}H]-labeled cellulose was incubated with enzyme in 0.05M NaAc buffer, pH 5.0, at 25°C for 10 s. In the case of Cel 7A (×) the enzyme concentration was 0.5 μM and the substrate was [^{3}H]-BC. Concentrations of Cel 7B (□), Cel 5A (Δ), and Cel 12A (○) were 1.0 μM and the substrate was [^{3}H]-amorphous cellulose. The solid lines are based on Eq. (1) on the normalized scale (p/P_{lim}). (◇) refer to the data of 1.0 μM Cel 7A on nonreduced BC and measurements of the released total sugar.

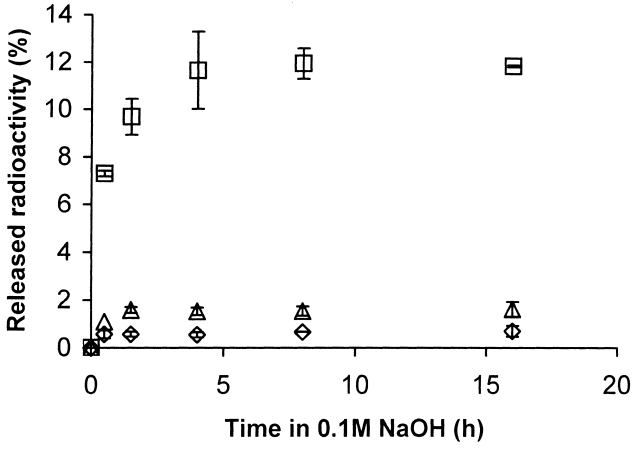

Figure 3. Alkali-induced release of radioactivity from tritium labeled celluloses. Tritium labelled cellulose samples were incubated in 0.1M NaOH at 25°C. [^{3}H]-BC (Δ), [^{3}H]-amorphous cellulose (□), second treatment of alkali pretreated [^{3}H]-amorphous cellulose(◇).

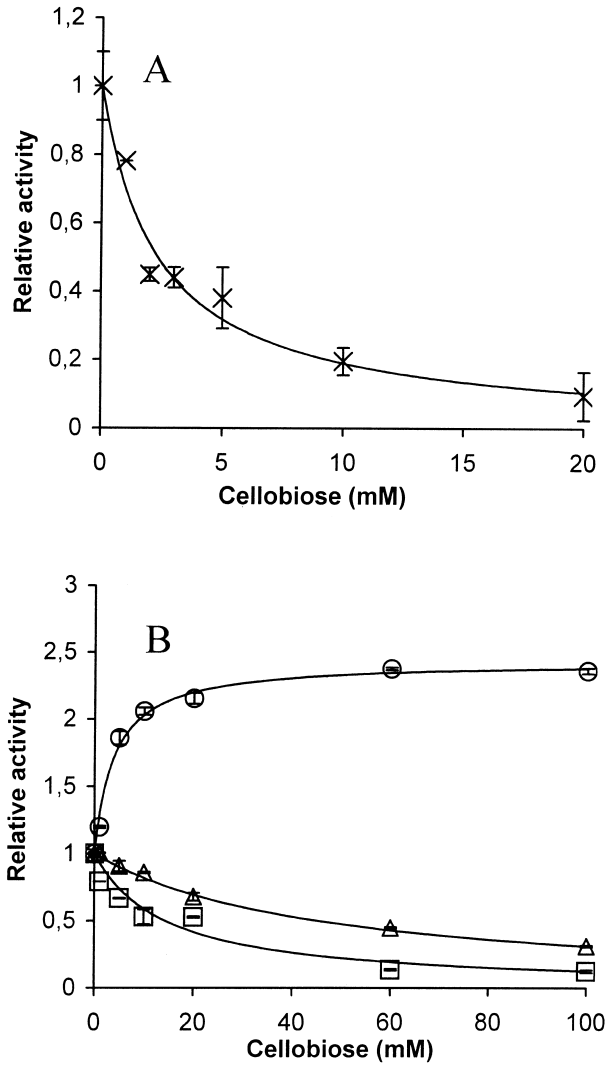

Figure 4. Inhibition of the initial rates of hydrolysis of [^{3}H]-labeled celluloses by cellobiose. [^{3}H]-labeled cellulose was hydrolyzed with 1 μ*M* enzyme in 0.05*M* NaAc buffer, pH 5.0, at 25°C for 10 s in the presence of externally supplied cellobiose (0–100 m*M*). (A) Cel 7A (×) [^{3}H]-BC 1 mg/mL. (B) Cel 7B (□), Cel 5A (△) and Cel 12A (○) on [^{3}H]-amorphous cellulose 2 mg/mL. The solid lines are based on Eq. (2) on the normalized scale (p_I/p_I=0).

(Fig. 3)." These results indicate that alkali-induced release of radioactivity from the [^{3}H]-reduced cellulose is at least partly of reversible. Thus, all samples used for our data (including zero data points) were left under alkaline conditions for 2 h between the termination of the enzyme reaction by alkali and counting of the scintillation to ensure that the nonenzymatic release of radioactivity was equal in all data points.

Hydrolysis of [^{3}H]-Cellulose in the Presence of Cellobiose

Since high $S_{0.5}$ values observed for the initial rates of cellulose hydrolysis are difficult to measure precisely (the physical properties of the cellulose suspensions set limits on the highest usable concentration) the conventional approach involving the measurements of apparent parameters (P_{lim}, $S_{0.5}$) at different inhibitor concentrations is not applicable. This demanded an alternative approach to assess the strength of inhibition. The conventional expression for competitive inhibition [Eq. (2)] relates the initial rate of product release (p) to the concentration of substrate [S] and inhibitor [I]. If the series of initial rates of hydrolysis is measured at different cellobiose concentrations at a fixed substrate concentration the value of apparent K_i can be estimated by nonlinear regression analysis. To do that, the $S_{0.5}$ value obtained without added cellobiose needs to be fixed. The relative initial activity (p_I/p_I=0) of Cel 7A on [^{3}H]-BC in the presence of cellobiose at different concentrations is shown in Figure 4. Table 1 lists the estimates of apparent competitive K_i using the fixed $S_{0.5}$ values obtained on the labeled substrates listed in the same table. The apparent competitive K_i thus obtained for the cellobiose inhibition of Cel 7A can be estimated around 1.5 m*M*.

Since crystalline cellulose is a poor substrate for many endoglucanases, amorphous cellulose was used to assess their inhibition by cellobiose. The $S_{0.5}$ values for all endoglucanases on [^{3}H]-amorphous cellulose were in the range of 5 mg/mL (Fig. 2, Table I). Based on these numbers and the data represented in Figure 4B, apparent competitive K_i values of 11 ± 3 m*M* and 34 ± 6 m*M* were calculated for Cel 7B and Cel 5A, respectively.

Hydrolysis of [^{3}H]-amorphous cellulose by Cel 12A revealed, most interestingly, that cellobiose had an apparent activating effect on the release of label.

DISCUSSION

In this study we assessed the inhibition of cellulases by cellobiose using reducing-end [^{3}H]-labeled celluloses as a substrate. Such substrates have been used earlier to study the mode of action of cellulases and it has been shown that the reduction of free aldehyde groups in cellulose does not affect the hydrolysis by cellulases (Nutt et al., 1998). We found a linear relationship between the release of radioactivity and that of cellobiose when labelled cellulose was degraded by cellobio-hydrolase Cel 7A and endoglucanases Cel 7B and Cel 12A. This makes the release of radioactivity a good measure of the general activity of these enzymes and provides a tool for discrimination between the externally added product and that resulting from hydrolytic activity, thus enabling measurements of initial rates in the presence of high levels of externally supplied cellobiose. Although, due to the continuous alterations in cellulose structure and complex kinetics (Väljamäe et al., 1998; 2003), there might not exist a true steady state in cellulose hydrolysis we took the initial linear stage of the time curve as an indicator for apparent steady state. The major drawback of [^{3}H]-reduced celluloses as substrates for cellulases was a certain instability of the labeling under alkaline conditions. Although we do not know the exact mechanism, the release of radioactivity under alkaline

conditions is probably caused by alkaline degradation of cellulose (Knill and Kennedy, 2003). Since the alkalification is the best method for rapid (for 5–10 s scale kinetics) termination of cellulase reaction the sensitivity of the [^{3}H]-cellulose-based assay is limited by the high background radioactivity. This makes the assessment of the exact type of inhibition impossible. The value of apparent competitive inhibition constant found for Cel 7A on [^{3}H]-BC was at around 1.5 m*M*, roughly 100-fold higher than that reported for Cel 7A acting on low-molecular-weight substrates (Claeyssens et al., 1989; van Tilbeurgh and Claeyssens 1985; Vonhoff et al., 1999). It must be noted here that exact knowledge about the type of inhibition was not crucial to reveal the strength of inhibition under our experimental conditions using substrate concentrations around 1/2 $S_{0.5}$. Analysis of hydrolysis data according to the conventional equation for pure noncompetitive inhibition resulted in only slightly higher apparent K_i values which however, did not differ from the competitive inhibition constants more than the standard error.

Inhibition of Cel 7A by cellobiose with an apparent inhibition constant of 1.5 m*M* is far more reasonable in terms of efficient cellulose degradation by fungus than the figure of 20 μ*M* found in the studies with low-molecular-weight substrates. Cel 7A is the most abundant cellulase produced by *T. reesei* and is the key component of the cellulolytic system of the fungus. Since Cel 7A is designed to produce cellobiose as a food source it is unlikely that the activity of the enzyme is constrained to the extent expected for a product K_i in the range of 20 μ*M* *Trichoderma reesei* produces two β-glucosidases that serve to hydrolyze cellobiose to glucose thus relieving the product inhibition of Cel 7A. The K_M and k_{cat} values for cellobiose are 2.1 m*M* and 51.5 s^{-1} for β-glucosidase I and 11.1 m*M* and 18.6 s^{-1} for β-glucosidase II (Chen et al., 1992). Thus, they appear to be designed for a steady-state concentration of cellobiose approaching the 1 m*M* range.

The differences in the strength of inhibition by cellobiose found between the hydrolysis of low-molecular-weight substrates and cellulose, respectively, is most likely related to the particular experimental system. Conventional enzyme kinetics, derived for enzyme reactions in homogeneous solutions, is not directly applicable to insoluble cellulose hydrolysis but is still often used as an empirical approximation. The cellulose–cellulase system displays dual saturation character, i.e., both enzyme and substrate can be saturated with each other (reviewed in Lynd et al., 2002). This is different from the Michaelis-Menten model, which features saturation with substrate but not with enzyme. Saturation of substrate with enzyme results in an apparent K_M value that is increased at higher enzyme concentrations. This is consistent with our data with Cel 6A and Cel7B on amorphous cellulose (Table I), our unpublished results with Cel 7A on bacterial microcrystalline cellulose, and also with others' results (Bernardez et al., 1994;Wald et al., 1984). However, a cellulose concentration that is much higher than the total concentration of the enzyme will result in hydrolysis kinetics consistent with the conventional Michaelis-Menten model with K_M independent on enzyme concentration (Lynd et al., 2002). In that case, the cellulose concentration should be described in terms of chain ends available for an exocellulase, such as Cel 7A. The amount of chain ends in our BC preparations is in the range of a few micromoles per gram BC (Väljamäe et al., 1999) meaning that under our experimental conditions in the present study, and also in common laboratory practice and probably under natural conditions, the concentration of the enzyme and that of the true substrate is within the same order of magnitude. Thus, the true inhibition constant can not be found using the apparent K_M values ($S_{0.5}$ in the present study).

The apparent inhibition of endoglucanases by cellobiose was one order of magnitude weaker than for cellobiohydrolase Cel 7A, in accordance with earlier observations for low-molecular-weight substrates (van Tilbeurgh and Claeyssens 1985; van Tilbeurgh et al., 1988), and is probably related to the more open structure of the substrate-binding region in endoglucanases. The data also suggest that product inhibition of the endoglucanases will not create a serious ''bottleneck'' in synergistic cellulose degradation. However, since the soluble sugar and primary end release based apparent $S_{0.5}$ values for Cel 5A were different, the resultant inhibition constant must be treated with caution.

Hydrolysis of [^{3}H]-amorphous cellulose by Cel 12A revealed, most interestingly, that cellobiose had an apparent activating effect on the release of label (Fig. 4B). All cellulases are, in principle, capable of transglycosylation. All of the enzymes studied here have a retaining hydrolysis action (Henrissat and Davies, 1997) involving the covalent enzyme-glycosyl intermediate, which normally is split by water. At a sufficiently high cellobiose concentration it has a high probability to combine with this intermediate so that its free 4′-hydroxyl group can possibly compete with water as nucleophile, partly due to its optimal positioning. Such a situation will indeed lead to the more rapid release of the enzyme from the intermediate into solution in the presence of cellobiose and allow it to act again at a new site. This will be revealed as an apparent activation since we are following the release of primary reduced end groups only. Obviously, there is no activation on the level of net cellobiose production. The probability for the transglycosylation reaction may also be enhanced by a high ratio between the primary product release rate and the covalent intermediate hydrolysis rate. A similar activating effect of cellobiose on Cel 7A possibly occurs at cellobiose concentrations above 30 m*M*, as reflected by a slight increase in the release of label which is inconsistent with Eq. (2) (data not shown). The transglycosylating activity of Cel 12A proposed here may be related to the recent observation that this enzyme can cause plant cell wall expansion, an activity that may involve transglycosylation (Yuan et al., 2001).

As a separate part of this study we derived a rate equation for Cel 7A acting on polymeric substrate at the presence of inhibitor. A comprehensive theoretical analysis of cellulase action on polymeric substrate, including product inhibition,

was given by Holtzapple et al. (1984; 1990). At that time, the three-dimensional structures of cellulases were not available and the existence of nonproductive complex before the productive one was not confirmed. Now we know that the two-domain architecture of cellulases based on catalytic domain and cellulose-binding domain allows the nonproductive binding and concentration of the enzymes on the cellulose surface (reviewed in Linder and Teeri, 1997). It is also reported that the major diffusion mode of Cel 7A is two-dimensional, lateral diffusion (Väljamäe et al., 2001). Furthermore, based on the long tunnel-shaped active site structure of Cel 7A we now know that nonproductive complexes can occur also in the case when the cellulose chain is already captured by active site. The simplest reasonable mechanism for Cel 7A acting on polymeric substrate is depicted in Figure 5. The steady-state rate equation for the scheme in Figure 5 is analogous to the equation for mixed-type inhibition, since both limiting rate and apparent dissociation constant is affected by the inhibitor [see Eq. (3)]. In the derivation of Eq. (3) we assumed that the second-order rate constants for the binding of substrate were not affected by the inhibitor, and vice versa. The above assumption might hold, since second-order rate constants are related to the diffusion from solution. Note that in the scheme in Figure 5 the catalytic cleavage results, beside the product cellobiose, in the formation of a nonproductive enzyme-substrate complex rather than a free enzyme so the net rate constant for the conversion of *ES* to *ES′* is equal to $k_{-6} + k_{cat}$, instead of k_{-6} only. Thus, according to our definition of $K_{ES} = (k_{-6} + k_{cat})/k_6$ the K_{ES} is a dimensionless parameter that represents the ratio of nonproductive enzyme substrate complex to the productive one so that $K_{ES}=[ES']/[ES]$. Equation (3) has two limiting cases depending on whether $[ES]$ or $[ES']$ is the prevalent complex:

1. If K_{ES} is much smaller than unity then the prevalent complex without inhibitor is the productive enzyme-substrate complex *ES* and we have a competitive inhibition. The affinity of the enzyme for the substrate (K_M) is a product of both K_S and K_{ES} so that $K_M=K_S \cdot K_{ES}$ and the limiting rate (V_{lim}) is equal to $k_{cat} \cdot e_0$.

2. If K_{ES} is much larger than unity then the prevalent complex without inhibitor is the nonproductive enzyme-substrate complex ES′ and we have a conventional mixed-type inhibition. In this case $V_{lim} = k_{cat} \cdot e_0 / K_{ES}$ and $K_M = K_S$. Pure noncompetitive inhibition is a special case when $K_{EI} = K_{EIS}$, i.e., the binding of the substrate is not affected by the inhibitor, and vice versa.

Essentially the same results were obtained by Holtzapple et al., (1990) with competitive and noncompetitive inhibition as limiting cases while competitive inhibition was expected for the cellulases having a strong tendency for productive complex formation.

The lower limit estimate, (based on the assumption that at saturating cellulose concentration all of the enzyme is productively bound) of k_{cat} on nonreduced celluloses was also found. For Cel 7A the highest value of k_{cat} (2.5 ± 0.5 s^{-1}) was observed on bacterial microcrystalline cellulose (Table I). The k_{cat} value around 8 s^{-1} that was found for Cel 6A on amorphous cellulose, is in fair accordance with the reported k_{cat} value of 12–14 s^{-1} for Cel 6A acting on cellohexaose (Harjunpää et al., 1996). This k_{cat} value was also, contrary to the half-saturating substrate concentration ($S_{0.5}$), independent of enzyme concentration. The k_{cat} values for endoglucanases on amorphous cellulose were 12.4 ± 0.2 s^{-1}, 8.0 ± 0.1 s^{-1} and around 3.5 s^{-1} for Cel 7B, Cel 5A, and Cel 12A, respectively (Table I). Those figures can be compared to 118 s^{-1}, 65 s^{-1}, and 14 s^{-1} on cellopentaose for Cel 7B, Cel 5A, and Cel 12A, respectively, found by Karlsson et al. (2002). Lower k_{cat} values observed with amorphous cellulose as substrate are probably caused by the random nature of the endoglucanase attack. Every hydrolytic cleavage of cellopentaose results in the formation of soluble sugar and is therefore detectable by assaying soluble sugars. Cleavage of cellulose chains, on the other hand, results in the formation of soluble sugar only in the case when the cleavage happens to be close to the chain end since higher cello-oligosaccharides (starting from celloheptaose) are insoluble.

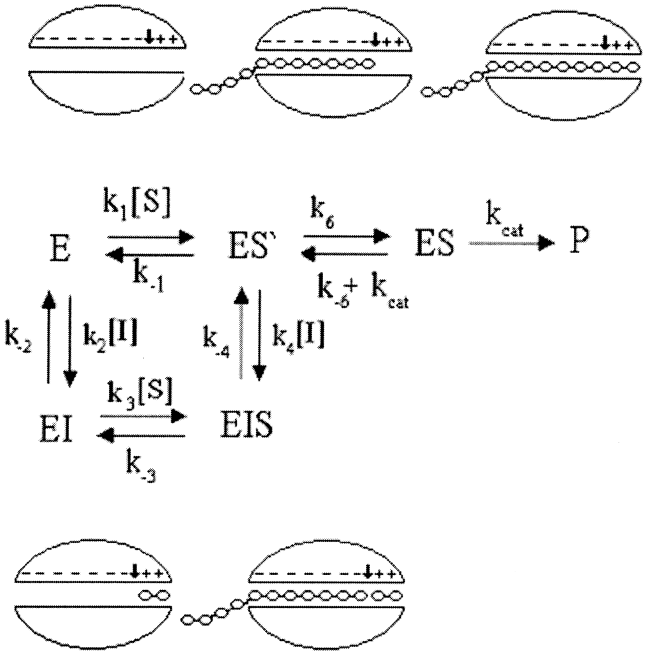

Figure 5. Schematic representation of Cel7 A inhibition by cellobiose. Five species with the following concentration terms: [E] free enzyme; [ES'] nonproductive enzyme-substrate complex; [ES] productive enzyme-substrate complex; [EI] enzyme-inhibitor complex, and [EIS] enzyme-inhibitor-substrate ternary complex are depicted in the cartoons. [S] and [I] denotes the concentration of free substrate and free inhibitor respectively.

NOMENCLATURE

BC	bacterial cellulose
[^{3}H]-BC	[^{3}H] reducing end labeled bacterial cellulose

[^{3}H]-amorphous cellulose	[^{3}H] reducing-end labeled amorphous cellulose
NaAc	sodium acetate
K_i	inhibition constant

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