Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: adsorptions of two cellobiohydrolases, two endoglucanases and their core proteins on filter paper and their relation to hydrolysis

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Separate binding of several purified cellulolytic components of *Trichoderma reesei* on to filter paper was studied and concomitant hydrolysis rates evaluated. Enhancement of mass transfer from the bulk liquid to the solid substrate by agitation has two different effects on adsorption depending on the type of enzyme: (i) the fraction of cellobiohydrolase II (CBH II) and endoglucanase III (EG III) bound at equilibrium is increased, whereas (ii) the rate but not the extent of cellobiohydrolase I (CBH I) and endoglucanase I (EG I) adsorption is affected. The adsorption of CBH I core, a component lacking the cellulose-binding domain (CBD), is, however, not significantly influenced by mass transfer. The CBH I interdomain peptide (present in CBH I core b) does not participate in adsorption but enhances stability. The ad-

sorption of CBH I core proteins is a fully reversible process whereas that of the intact CBH I is not. Thus, the interaction of the CBD with filter paper apparently accounts for the mass-transfer-limited binding rate and also for the irreversible adsorption of intact CBH I. Adsorption isotherms at 50 °C indicate very similar relative association constants for the intact cellulases (0.24–0.30 l/g of cellulose), but drastically reduced values for CBH I core proteins (0.03 l/g of cellulose). The specific activities of adsorbed CBH I and of its core proteins are identical and a linear relationship between adsorption and rates of hydrolysis is found only for these enzymes. Thus, non-productive binding on to cellulose seems evident in the case of CBH II and EG III but not CBH I.

INTRODUCTION

Most fungal and many bacterial cellulases exhibit a bistructural-bifunctional organization: the active-site-containing core is connected via a flexible linker peptide to the cellulose-binding domain (CBD). The latter promotes adsorption of the cellulases to crystalline regions of the cellulosic substrate (Tomme et al., 1988; Kraulis et al., 1989; Gilkes et al., 1991, 1992). The linker peptide probably ensures an optimal interdomain distance between core and CBD (Teeri et al., 1992; Srisodsuk et al., 1993) and/or protects the enzyme against proteolytic attack (Langsford et al., 1987). However, the mechanisms involved in binding to cellulose and the exact role of the different domains for enzyme adsorption and substrate hydrolysis are not completely understood. The isolated fungal catalytic domain is capable of binding to cellulose, albeit with reduced affinity (Tomme et al., 1988) and a more 'active' role was attributed to both the fungal (Knowles et al., 1987) and bacterial CBD (Din et al., 1991; Kilburn et al., 1993), suggesting it may either facilitate single chain liberation from the cellulose crystals or cause non-hydrolytic fibre disruption.

Some important factors influencing the adsorption of individual cellulolytic components from *Trichoderma reesei* are the crystallinity of cellulose (Tomme et al., 1988) and other physical parameters such as temperature, ionic strength and pH (Kyriacou et al., 1988). This was ascribed to the predominance of hydrogen bonding in the adsorption process. Adsorption isotherms were reported, at least in a formal manner, to obey Langmuir-type equations (Beldman et al., 1987; Kyriacou et al., 1988). Some authors (Woodward et al., 1988; Ståhlberg et al., 1991) applied

Scatchard analysis to evaluate adsorption data and proposed two-site models based on the observation of non-linear (concave upward) plots. However, the validity of classical Scatchard analysis can be disputed as many different factors clearly influence cellulase adsorptions. Physicochemical heterogeneity of the substrate and lateral enzyme–enzyme interactions, especially in the case of high substrate coverage, could be important. Also the two-domain structure of the cellulases which may give rise to non-uniform interaction with the substrate will strongly influence adsorption (Gilkes et al., 1992).

Since adsorption of several cellulases is apparently irreversible (Beldman et al., 1987; Kyriacou et al., 1989), and probably accompanied by conformational changes in enzyme structures, the application of equilibrium models assuming an equilibrium between free and bound enzyme as determined by the adsorption isotherm is problematic. Previous studies on protein adsorption on to solid surfaces indicated that time-dependent conformational changes may be induced by surface binding and that adsorption of macromolecules on to solids is seldom an equilibrium process (Andrade and Hladky, 1986). It is concluded that adsorption is irreversible when no net desorption is observed upon removal or dilution of the free protein in solution. Irreversible protein binding thus may result in a hysteresis between adsorption and desorption isotherms (Jennissen and Botzet, 1979). Although enzyme binding to cellulose is considered a prerequisite step before substrate degradation, much less attention has been paid to correlating the adsorption of the individual cellulases and the hydrolysis rates (Beldman et al., 1987).

This report deals with cellulose binding studies of the four

Abbreviations used: CBH, cellobiohydrolase, 1,4- β -D-glucan cellobiohydrolase (EC 3.2.1.91); CBD, cellulose-binding domain; EG, endoglucanase, 1,4- β -D-glucan glucanohydrolase (EC 3.2.1.4.); CNP, 2'-chloro-4'-nitrophenyl; FLUOS, 5- (and 6-)carboxyfluorescein succinimidyl ester.

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major cellulases secreted by *Trichoderma reesei*, namely cellobiohydrolase (CBH) I, CBH II, endoglucanase (EG) I and EG III, and of catalytic core proteins of CBH I (Tomme et al., 1988) and EG III (Ståhlberg et al., 1988). The relationships between the adsorption of each individual cellulase and the rate of substrate hydrolysis have also been investigated.

EXPERIMENTAL

Materials

Whatman filter paper No. 1 (Whatman, Maidstone, U.K.) with a relative crystallinity index of 40% (Henrissat et al., 1985) was used as substrate. 2'-Chloro-4'-nitrophenyl (CNP) β -glycosides were prepared as described (Van Tilbeurgh et al., 1988). β -Glucosidase (Novozym 188; Novo, Bagsvaerd, Denmark) was used after partial purification by Biogel P6 (Bio-Rad, Richmond, CA, U.S.A.) gel filtration. Its activity was determined with 4'-nitrophenyl β -D-glucoside (Sigma, Deisenhofen, Germany) (Ghose, 1987). Fluorescamine (Sigma, Deisenhofen, Germany), 5- (and 6-)carboxyfluorescein succinimidyl ester (FLUOS; Lambda, Graz, Austria) were commercial supplies. Beckman Ready Gel (Fullerton, CA, U.S.A.) was used for scintillation counting. All other chemicals were reagent grade.

Enzyme purification

Intact CBH I, a truncated form (CBH Ib), CBH II, EG I, EG III and EG III core were purified from the culture filtrate of *T. reesei* by reported methods (Tomme et al., 1988; Ståhlberg et al., 1988; Macarron et al., 1993). Molecular mass (58 kDa) and carbohydrate content (2.3%, w/w) determinations proved that CBH Ib lacks the CBD but contains about 60% of the glycosylated linker peptide (Hayn et al., 1992). CBH I, CBH Ib, CBH II and EG I were further purified using an affinity column of *p*-aminobenzyl 1-thio- β -cellobioside (Van Tilbeurgh et al., 1984). CBH I core was prepared by limited proteolysis as described previously (Tomme et al., 1988). The purity of the individual enzymes was proven by analytical electrophoresis (SDS/PAGE and isoelectric focusing/PAGE) and by activity measurements on small, chromogenic substrates at 50 °C as described (Nidetzky et al., 1994). The concentration of the individual cellulases was determined using their molar absorption coefficients ($M^{-1} \cdot cm^{-1}$) at 280 nm: CBH I (67200), CBH Ib (63400), CBH I core (62500), CBH II (79100), EG I (54800), EG III (77000; Saloheimo et al., 1988), and EG III core (63600; Ståhlberg et al., 1988).

Labelling of CBH II

Fluorescamine labelling of CBH II was carried out in 0.1 M sodium bicarbonate using a 20 molar excess of fluorescamine dissolved in acetone. Unbound label was removed by repeated dialysis in Centricon tubes (10 kDa cut-off; Waters-Millipore, Eschborn, Germany). Labelling with FLUOS was performed as described by Koller (1991) using equimolar amounts of reagent and protein. Radioactive labelling with ^{125}I was performed according to the method of Sinn et al. (1988) using *N*-bromosuccinimide as the oxidizing agent. The catalytic activity of CBH II was determined against filter paper (20 g/l) and full retention of activity was found in the case of fluorescamine- and ^{125}I -labelled CBH II. FLUOS-labelled CBH II was catalytically inactive.

Enzyme stability

Stability of the individual cellulases (in the absence of substrate) was determined by activity measurement before and after incubation in 0.05 M sodium acetate buffer (pH 5.6) using the conditions specified. Similarly, the stability of the fluorescence- or radioactivity-labelled CBH II was determined.

Adsorption experiments

Individual cellulases were dissolved in 5 mM sodium acetate buffer (pH 5.6) to give a final volume of 100 μ l in each experiment. Filter paper discs (weight: 2.05 ± 0.05 mg), dug out with an office perforator, were added to the enzyme solutions pre-incubated at 50 °C and further incubated in the absence or presence of different intensities of agitation (maximally 1350 rev./min; Thermomixer 5436; Eppendorf, Germany).

Adsorption was followed according to a suitable time schedule (four samples taken within the first 15 min) up to 3 h. The reaction was stopped by centrifugation (10000 rev./min (8000 *g*), 1 min), the supernatant withdrawn and used for further determinations of either non-adsorbed enzyme concentrations or the amount of soluble hydrolysis products formed (see below).

Adsorption isotherms (50 °C) were determined in the absence of mass transfer limitation (agitation at 1350 rev./min) using a reaction time sufficiently long to reach the adsorption equilibrium (25–60 min). In all experiments the amount of adsorbed enzyme was determined from concentration measurements of the individual enzyme in solution before and after incubation with filter paper (activities measured with chromogenic substrates, or fluorescence/radioactivity measurements in the case of CBH II).

To ensure complete conversion of all soluble hydrolysis products into D-glucose (and prevent product inhibition by cellobiose of some of the enzymes), 0.2 i.u. of β -glucosidase/ml (if not already present) was added to each of the centrifuged samples, which were then further incubated for 30 min at 50 °C, and hydrolysis rates expressed as D-glucose released per min.

Desorption experiments

CBH I or CBH I core (3.55 μ M each) were incubated with filter paper discs (20 g/l) as described above. After 30 min at 1350 rev./min 70 μ l of the reaction mixture was withdrawn and the same volume of fresh buffer added. Enzyme desorption, first without agitation, was followed by measuring the time-dependent change in enzyme concentration in the supernatant (activity measurements with chromogenic lactoside as described below). After 60 min, stirring was started (1350 rev./min) and desorption followed for another 60 min.

Analytical methods

The activities of the individual cellulases were measured at 50 °C using chromogenic CNP-glycosides as recently reported (Nidetzky et al., 1994). CBH I and EG I with the lactoside, and EG III with the cellotrioside. D-Glucose formed in hydrolysis experiments using filter paper was determined by the glucose oxidase/peroxidase (GOD/POD) assay (Boehringer, Mannheim, Germany) as described elsewhere (Nidetzky et al., 1994). The concentration of labelled CBH II (in stock solutions) was determined by the dye-binding assay (Pierce, Rockford, IL, U.S.A.) using non-labelled CBH II as a standard. Fluorescence measurements of filtered samples (0.2 μ m pore diam.; Millipore) were performed with a Shimadzu fluorimeter Md RF-5001 PC (Shimadzu, Duisburg, Germany) using a constant slit width of 5 nm and the following wavelengths (excitation/emission): fluorescamine-labelled CBH II (350 nm/455 nm), FLUOS-

Figure 1 Kinetics of cellulase adsorption on to 20 g/l filter paper at 50 °C without agitation (closed symbols) and at 1350 rev./min (open symbols)

(a) 1.25 μ M CBH I ($\square$, $\blacksquare$) and 1.25 μ M CBH I core ($\triangle$, $\blacktriangle$). (b) 0.15 μ M EG I ($\square$, $\blacksquare$). (c) 0.74 μ M EG III ($\triangle$, $\blacktriangle$) and 0.74 μ M EG III core ($\square$, $\blacksquare$). (d) 1.25 μ M 125 I-CBH II ($\triangle$, $\blacktriangle$) and 1.25 μ M FLUOS-labelled CBH II ($\square$, $\blacksquare$).

labelled CBH II (394 nm/518 nm). Radioactivity was measured by liquid-scintillation counting in a Beckman scintillation counter Model LS 3800.

Computational methods

Parameters in the mathematical models were estimated by non-linear least-squares regression using the BMDP statistical software package (BMDP Statistical Software, Inc., Los Angeles, CA, U.S.A.).

RESULTS AND DISCUSSION

Enzyme stability

Intact CBH I, CBH II and EG I were fully stable for 3 h at 50 °C and 1350 rev./min. To diluted solutions of EG III (or its core) (at concentrations < 1 μ M) 0.1 g/l BSA (itself not adsorbing on to filter paper) was added to avoid inactivation both at 4 and 50 °C. CBH I core (< 2 μ M) was rapidly inactivated under the same conditions whereas CBH Ib was perfectly stable, the glycosylated linker peptide in CBH Ib (Hayn et al., 1992) probably having a stabilizing effect. Approx. 10% of the radioactive or

fluorescamine label of the derivatized CBH II was lost when incubated under the same conditions (3 h; 1350 rev./min). This did not affect CBH II quantification during its fast adsorption on to filter paper.

Kinetics of adsorption and hydrolysis

As specified in the Experimental section adsorption of all cellulases was performed in the presence of β -glucosidase. It was proved that this did not influence the binding behaviour of any of the other enzymes.

The time courses of CBH I and CBH I core adsorption are shown in Figure 1(a). In agreement with the results of Tomme et al. (1988), the absence of the CBD in the core results in a drastic (40%) decrease of the binding capacity. Binding kinetics and the amounts of bound enzyme were identical for both truncated forms of the enzyme: CBH I core and CBH Ib (results not shown).

Agitation enhances considerably the mass transfer through the boundary film at the surface of cellulose and consequently the time required to reach the apparent adsorption equilibrium was strongly reduced (Figure 1a). The film at the liquid–solid interface

Table 1 Specific activities on filter paper (20 g/l) at 50 °C.

Enzyme	Initial enzyme concn. (μM)	Specific activity (μM D-glucose/ μM adsorbed cellulase per h)	
		Agitation (1350 rev./min)	No agitation
CBH I	1.25	700	670
CBH I core	1.25	674	660
CBH II	0.30	1083	1584
EG I	0.15	3887	2655
EG III	0.74	1339	1302
EG III core	0.74	1555	1315

Table 2 Binding constants of *T. reesei* cellulases for filter paper

The reaction conditions were: 50 °C, 1350 rev./min, 20 g of filter paper/l; reaction time: 30 min (CBH I, CBH Ib, CBH I core), 40 min (EG I) and 60 min (EG III, EG III core). r = correlation coefficient.

Enzyme	$10 \times B_{\text{max}}$ ($\mu\text{M/g}$)	$10^{-6} \times K_a$ (l/mol)	$10 \times K_t$ (l/g of cellulose)	r
CBH I	1.70 ± 0.08	1.41 ± 0.24	2.40 ± 0.42	0.963
CBH Ib	1.49 ± 0.13	0.22 ± 0.04	0.33 ± 0.07	0.987
CBH I core	1.48 ± 0.22	0.21 ± 0.08	0.31 ± 0.13	0.927
CBH II (FLUOS)	2.58 ± 0.13	0.95 ± 0.12	2.46 ± 0.33	0.967
CBH II (^{125}I)	2.60 ± 0.40	1.15 ± 0.29	2.99 ± 0.88	0.977
EG III	3.08 ± 0.24	0.91 ± 0.13	2.80 ± 0.46	0.989
EG III core	0.80 ± 0.08	2.44 ± 0.48	1.95 ± 0.43	0.975
EG I*	1.66 ± 0.55	0.56 ± 0.26	0.93 ± 0.53	0.918

* High S.D.s resulted because substrate saturation could not be reached.

may be up to 0.1 cm thick in systems without stirring (Andrade and Hlady, 1986) and thus boundary film resistance may effectively decrease the rates of enzyme adsorption. In contrast, for CBH I core (Figure 1a) and CBH Ib (not shown) no mass-transfer resistance was evident and thus binding of these two enzymes seems to be in a 'reaction-limited' regime (Fogler, 1992). Without agitation, adsorption of CBH I was biphasic (Figure 1a). A fast initial phase, similar to the rate of adsorption of the truncated enzymes, was followed by a slower one, absent in the case of CBH I core and CBH Ib. Presoaking of the filter paper discs in buffer for 60 min did not alter the kinetics of intact CBH I adsorption (results not shown) and therefore this fast initial rate (Figure 1a) may not be explained simply by an unspecific uptake of liquid by the dry substrate. Biphasic adsorption kinetics of CBH I could then be due to reaction-limited adsorption of the core part and mass-transfer-dominated binding by the CBD (Figure 1a). With diffusion no longer limiting (agitation at 1350 rev./min) adsorption of intact CBH I occurred in a single phase (Figure 1a). The specific hydrolytic activities of CBH I and its core proteins were almost identical both under stirring and static conditions (Table 1).

Biphasic adsorption kinetics similar to those of CBH I were observed with EG I under static conditions (Figure 1b). Whereas in the second, slower phase, which is obviously controlled by enzyme diffusion, the effect of agitation on the adsorption rate could be clearly demonstrated, the adsorption equilibrium was not influenced by mixing. Among all the other cellulases studied here EG I showed the highest specific activity on filter paper (Table 1) and, interestingly, this was even 1.4 times higher at 1350 rev./min.

The time courses of substrate binding by EG III and its core are shown in Figure 1c. When compared with intact EG III

approx. 30 and 10 % less core enzyme was adsorbed (respectively under mixing and static conditions). In contrast to the adsorption of CBH I and EG I the kinetics of EG III binding did not seem to be diffusion-controlled. Agitation, on the other hand, was found to increase the amounts of equilibrium-bound EG III and EG III core by 35 % and 25 % respectively (Figure 1c). Without mixing, the specific activities of EG III and its core were identical (Table 1). At 1350 rev./min, however, the adsorbed core was approx. 20–30 % more active than the intact enzyme, indicating that binding was less productive for intact EG III under these conditions.

Time-dependencies of the adsorptions using either ^{125}I - or fluorescamine-labelled CBH II (both catalytically active) were identical (Figure 1d). Mixing at 1350 rev./min led to an increase of CBH II adsorption by 30–35 %. Catalytically inactive FLUOS-labelled CBH II was capable of binding to filter paper when the reaction system was well mixed (here at 1350 rev./min; Figure 1d) and approx. 30–35 % was adsorbed. These results indicate that with stirring different CBH II binding modes could exist, the active site not being involved and therefore probably non-productive. This was confirmed further by the data in Table 1 which shows that, under static conditions of incubation, the initial value of D-glucose formation per mol of CBH II adsorbed was approx. 1.5-fold higher than during the experiment with agitation.

Adsorption Isotherms

Mathematical and statistical analysis of the adsorption isotherm of each cellulase (50 °C) was performed using a simple 'Langmuir-type' model [see eqn. (1)].

$$B = (B_{\text{max}} \cdot K_a F) / (1 + K_a F) \quad (1)$$

Figure 2 Relationship between adsorbed enzyme and activity (p-glucose released/min) at 50 °C and 1350 rev./min

(a) CBH I (Δ), CBH Ib (■), and CBH I core (□). (b) EG III (□) and EG III core (■). (c) ¹²⁵I-CBH II (■) and EG I (□). The incubation times were respectively 30 min (a), 60 min (b) and 30 min (c).

where B is bound enzyme (μmol/g of cellulose), $B_{\max}$ is available binding sites (μmol/g of cellulose), F is free enzyme concentration (μM) and K_a the association constant (l/mol). According to

Figure 3 Desorption of CBH I and CBH I core (Initial concentration 3.55 μM) after adsorption on to 20 g/l filter paper at 50 °C

Gilkes et al. (1992) a relative association constant (K_r) can be defined as the product of available binding sites and association constant ($K_r = B_{\max} \cdot K_a$). The K_r value can be used to compare roughly the 'affinities' of related cellulase ligands for a given substrate, provided that the number of available substrate sites does not differ too much. The K_r value is a measure for the slope of the adsorption isotherm at its origin ($B = 0$), i.e. where no (lateral) interaction of the ligands takes place.

The adsorption constants defined by eqn. (1) were calculated by doubly weighted, non-linear least-squares regression analysis and are summarized in Table 2. Although the Langmuir-type adsorption model is a very simplified approach to describe cellulase binding (Gilkes et al., 1992), statistically significant data were obtained. Furthermore, the calculated adsorption constants provide a useful tool to compare the binding properties of the individual cellulases.

The adsorption constants of CBH Ib and CBH I core (Table 2) are identical, Thus for binding on to filter paper the glycosylated linker peptide in CBH I is probably not involved, contradicting the results of Chanzy et al. (1984) and Van Tilbeurgh et al. (1986). Our conclusion finds further support in the above results, showing that the kinetics of binding to cellulose was also identical for both CBH Ib and CBH I core (Figure 1a). A comparison of the binding constants calculated for intact CBH I and the core proteins demonstrates that lack of the CBD leads to a drastic decrease of the (relative) association constants but not of the number of available substrate sites. Thus during CBH I adsorption on to filter paper the role of the CBD in decreasing the K_a values appears to render the enzyme more economical.

However, this finding does not provide a general explanation for the role of the CBD in cellulose binding by other *T. reesei* cellulases. On filter paper almost four times more available sites ($B_{\max}$ value) were found for intact EG III than for its core. In contrast, the association constant K_a was 2.7 times higher for the core protein (Table 2).

The CBD of an intact *T. reesei* cellulase may therefore be capable of increasing (i) the number of binding sites available to an independently acting catalytic domain (as in the case of EG III) and (ii) the association constant (as in the case of CBH I). As for other carbohydrate-binding proteins (Quioco, 1986), hydrogen bonds probably account mainly for the interactions between the β -1,4-glycosidic chains and the CBD (Kyriacou et al., 1988) and this seems to be mediated mostly by tyrosine (Claeysens and Tomme, 1990; Teeri et al., 1992) and tryptophan residues (Reinikainen et al., 1992). The increases in B_{max} and K_a values for an intact cellulase compared with its core protein (Table 2) are probably caused by additional hydrogen bonds between the CBD and sites involved not only in hydrolysis but also elsewhere.

Within statistical significance identical adsorption constants were found for ^{125}I - and fluorescamine-labelled CBH II, indicating that different labels do not influence the adsorption properties of CBH II.

The calculated K_f values for CBH I, CBH II and EG III (Table 2) point to similar overall 'affinities' of the intact cellulases for filter paper, whereas both important binding constants, the number of available sites on filter paper and the association constant, showed considerable differences among the individual cellulases (Table 2). The K_f values in Table 2 are in agreement with data reported by Kyriacou et al. (1988) for Solka Floc cellulose but significantly lower than those found by Beldman et al. (1987) and Woodward et al. (1988) using Avicel.

Relationship between adsorption and hydrolytic activity

Identical and linear relationships between adsorbed enzyme and D-glucose formation were found in the case of CBH I, CBH Ib, and CBH I core (Figure 2a). These data corroborate the conclusion of Nidetzky et al. (1994) that during filter paper degradation the CBD does not endow the intact enzyme with any additional active properties.

For all other cellulases studied here a non-linear relationship between enzyme adsorbed and the respective hydrolytic activities was observed (Figures 2b and 2c). Two explanations could be valid: (1) the substrate (filter paper) is heterogeneous in terms of physicochemical properties, clearly influencing the hydrolysis rates (Nidetzky et al., 1993), and (2) non-productive binding is induced by the CBD as in the case of CBH II (Figure 1d). Also for EG III, especially at high substrate saturation ($> 1 \mu\text{M}$), deviation from linearity is more evident for the intact enzyme than for the core protein (Figure 2b).

Reversibility of adsorption

If adsorption of cellulases, especially of CBH I, is irreversible as reported (Beldman et al., 1987; Kyriacou et al., 1989), interpretation of binding constants in terms of an equilibrium model becomes meaningless. The time courses of CBH I and CBH I core desorption were measured and are shown in Figure 3. Initially, similar amounts of both enzymes were desorbed after fresh buffer had been added. In the case of CBH I core the extent of enzyme desorption corresponded exactly to the prediction of the 'equilibrium'-adsorption model given by eqn. (1) using the constants in Table 1. Therefore, and because no re-adsorption of CBH I core took place when further incubated at 1350 rev./min (Figure 3), the binding of CBH I core to filter paper is fully reversible both under stirring and static conditions.

On the other hand, the adsorption of intact CBH I to filter paper is irreversible (or only partly reversible) because: (i) desorbed CBH I was re-adsorbed on to cellulose when agitation

was started (Figure 3); and (ii) less enzyme was initially desorbed from the substrate, as could have been expected from the results in Table 1 assuming equilibrium between free and bound CBH I. The desorption of CBH I (Figure 3) can be ascribed tentatively to what was previously called 'core adsorption' of the intact enzyme [Figure 1(a) and text] and irreversibility in binding on to the substrate is obviously due to the interactions of the CBD and cellulose.

The data in Figure 3 indicate that non-uniform interactions of intact CBH I with cellulose may take place, possibly including pure 'core' and combined 'core and CBD' adsorption. Whether this applies for all other intact *T. reesei* cellulases remains unclear. In the case of CBH II and EG III, but not CBH I, evidence for hydrolytically non-productive adsorption (during agitation with filter paper) was found and this could be interpreted in terms of a binding mediated exclusively by the CBD without any interaction involving the catalytic domain.

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