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Isotherms for Adsorption of Cellobiohydrolase I and II from *Trichoderma reesei* on Microcrystalline Cellulose

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ABSTRACT

Adsorption to microcrystalline cellulose (Avicel) of pure cellobiohydrolase I and II (CBH I and CBH II) from *Trichoderma reesei* has been studied. Adsorption isotherms of the enzymes were measured at 4°C using CBH I and CBH II alone and in reconstituted equimolar mixtures. Several models (Langmuir, Freundlich, Temkin, Jovanovic) were tested to describe the experimental adsorption isotherms. The isotherms did not follow the basic (one site) Langmuir equation that has often been used to describe adsorption isotherms of cellulases; correlation coefficients (R^2) were only 0.926 and 0.947, for CBH I and II, respectively. The experimental isotherms were best described by a model of Langmuir type with two adsorption sites and by a combined Langmuir-Freundlich model (analogous to the Hill equation); using these models the correlation coefficients were in most cases higher than 0.995. Apparent binding parameters derived from the two sites Langmuir model indicated stronger binding of CBH II compared to CBH I; the distribution coefficients were 20.7 and 3.7 L/g for the two enzymes, respectively. The binding capacity, on the other hand, was higher for CBH I, 1.0 μmol (67 mg) per gram Avicel, compared to 0.57 μmol/g (30 mg/g) for CBH II. The isotherms when analyzed with the combined Langmuir-Freundlich model indicated presence of unequal binding sites on cellulose and/or negative cooperativity in the binding of the enzyme molecules.

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Index Entries: *Trichoderma reesei*; cellulase; cellobiohydrolase; adsorption isotherm; cellulose hydrolysis.

INTRODUCTION

Enzymatic hydrolysis of cellulose has attracted great interest in the last 25 y as a process that may contribute to the solution of energy and environmental problems. The cellulase system of the filamentous fungus *Trichoderma reesei* has been found to be one of the most effective for hydrolysis of cellulosic materials. *Trichoderma reesei* produces at least five genetically different cellulases: two cellobiohydrolases (CBH I and II; EC 3.2.1.91) and four endoglucanases (EG I, II, III, and V; EC 3.2.1.4). The enzymes appear in multiple isoforms in culture filtrates of *T. reesei* and act in synergism during hydrolysis of cellulose *(1)*.

Cellulose hydrolysis catalyzed by *Trichoderma* enzymes has been widely studied from both biochemical and technical points of view. It was found that the adsorption of the cellulases on the solid substrate is a prerequisite initial step in the hydrolysis. The importance of adsorption is reflected in the molecular structure of these enzymes. The *Trichoderma* cellulases have a similar structural organization with a catalytic domain (core) and a cellulose binding domain (CBD) connected by a flexible linker region *(1)*. Experiments with intact cellulases and their separated domains showed that the role of the cellulose binding domain is to enhance the binding and hydrolytic capacity of the catalytic domain in hydrolysis of cellulose *(2–5)*.

Increased knowledge on adsorption of cellulases is important in the development of cellulose hydrolysis processes with optimal use of the enzymes. Adsorption to the residue after hydrolysis leads to loss of the enzymes *(6)*. This is a serious problem since the cost of enzyme is a considerable fraction of the total hydrolysis cost *(7)*. On the other hand, adsorption to the fresh cellulose substrate can be utilized as a method for enzyme recycling. This has been studied, e.g., during hydrolysis of newspaper *(8)* and steam pretreated willow *(6)*. In one process, alternative hydrolysis was performed in an aqueous two-phase system. Also in this case, the adsorption of the cellulases was found to be important for recycling of the enzymes *(9,10)*.

In the future, when pure cellulase components produced by genetically engineered organisms will be available, a detailed knowledge of the adsorption characteristics of the single enzymes will be important in order to optimize the enzyme utilization in hydrolysis processes. Until recently, relatively few data have been published on the adsorption of pure cellulases *(2–4,11–13)*; in most adsorption studies the complete cellulase system of *Trichoderma* was used, i.e., culture filtrates or commercial enzyme preparations.

Important questions are how the enzymes interact during adsorption, and if they have common or specific binding sites. Only a limited number

of studies have been addressed to these questions *(12,14,15)*, probably because of the difficulties in quantifying cellulases in the presence of each other. In a previous work it was found that by studying a limited number of pure cellulases and using FPLC for the quantitative analysis of the enzymes at least partly, these questions could be answered *(16)*.

In the present paper the authors analysed the isotherms for CBH I and CBH II adsorption on microcrystalline cellulose. The isotherms were measured at 4°C in order to keep hydrolysis as low as possible. Avicel was used as a model substrate because of its frequent use in studies of cellulase–cellulose interaction. The adsorption isotherms of cellulases to various substrates have often been modelled with the Langmuir equation by assuming one *(2,6,11,17–21)* or two *(3,13)* binding sites. The Freundlich isotherm has also been used to model the adsorption of cellulases *(12)*. These and several other adsorption models were tested in order to find the best description of the experimental isotherms of pure CBH I and CBH II, which are the most abundant cellulases in *Trichoderma* culture filtrates *(1)*. Apparent binding parameters, i.e., binding capacities and distribution coefficients, were evaluated. Presence of unequal binding sites or negative cooperativity was analyzed by the combined Langmuir-Freundlich model. Our aim with studies of cellulase adsorption is to achieve more knowledge on optimal use of the enzymes in hydrolysis processes.

MATERIALS AND METHODS

Materials

Microcrystalline cellulose, Avicel (M 2331) was purchased from Merck (Darmstadt, Germany). Its water content was 5% at normal storage in room temperature. Other chemicals were of analytical grade.

Enzyme Purification

CBH I and CBH II of *T. reesei* QM 9414 were purified by ion-exchange chromatography as described elsewhere *(22)*. With isoelectric focusing (PhastGel IEF 3–9, Pharmacia Biotech, Sollentuna, Sweden) at high sample loading and silver staining CBH I gave a single band; CBH II showed a single major band and a few weak contaminating bands. The amount of contaminants was less than 1% and according to their pI values they were not any of the major cellulases. Before the adsorption experiments the purified CBH I and CBH II fractions were concentrated by ultrafiltration (10 mL Omegacell, cut-off 10 kDa, Filtron Technology, MA) and the buffer was changed to 50 m*M* sodium acetate pH 4.8. Enzyme concentrations were determined from the UV absorbance at 280 nm using the following extinction coefficients ($M^{-1} \times cm^{-1}$), CBH I, 78,800; CBH II, 92,000 *(4)*. Equimolar mixtures of CBH I and CBH II were prepared from solutions of the pure components. When the amount of enzymes are given in mg the following

molecular weights were used for the calculation: CBH I, 64,000 g/mol; CBH II, 53,000 g/mol *(4)*.

Adsorption Studies

Adsorption of CBH I and CBH II were studied using the enzymes alone and in mixtures with 1:1 molar ratio. Experiments were performed in 1.8-mL plastic tubes with screw cap (Nunc, Roskilde, Denmark) at pH 4.8 in 50 m*M* sodium acetate buffer using Avicel as substrate. The enzyme/substrate ratio was varied in the range of 0.08–5.12 μmol/g. The plastic tubes containing known amount (3.8–20 mg) of Avicel and the enzyme solutions with known concentration (0.4–51.2 μ*M*) of CBH I or/and CBH II were prethermostated in a cold room at 4°C. The experiments were started by pipetting a certain amount (0.3–1.5 mL) of enzyme solution on the Avicel. To ensure proper mixing the tubes were continuously inverted by rotation at 40 rpm during the incubation (90 min). At the end of the incubation, Avicel with the bound enzymes was separated from the hydrolysate containing the free enzymes and sugar products by quickly filtering the samples through a small syringe filter (Millex-GV4, low protein binding membrane, pore size 0.22-μm, diameter 4-mm, Millipore, Bedford, MA). The filtrate, without any further sample pretreatment, was analyzed by HPLC to determine hydrolysis products (cellobiose and glucose) and from that the residual concentration of Avicel and by FPLC to determine the free concentration of CBH I or/and CBH II present. Knowing the free enzyme and the residual substrate concentration the amount of bound enzyme was calculated from the initial enzyme concentration and was expressed as enzyme bound per gram residual substrate.

Quantitative Determination of CBH I and CBH II by FPLC

Free CBH I and CBH II in the supernatant from the adsorption experiments were quantitatively determined by means of anion-exchange chromatography on a Mono Q column in an automatized FPLC system (Pharmacia) *(16)*. Sample (10–300 μL) was diluted up to 6 mL with start buffer (20 m*M* triethanolamine-HCl, pH 7.6). The diluted sample was applied on the anion-exchange column then eluted by a 12 mL gradient (end buffer: 0.5*M* NaCl in 20 m*M* triethanolamine-HCl pH 7.6) at 1 mL/min flow rate. Ultraviolet absorbance of the eluent was followed at 280 nm. Calibration curves were used to determine the amount of enzyme(s) in the injected sample.

HPLC Analysis of the Hydrolysis Products

Hydrolysis products of Avicel (cellobiose and glucose) were analyzed on a Pharmacia HPLC system equipped with an Erma ERC-7515A refractive index detector (Erma, Tokyo, Japan) using an Aminex HPX-

87H column (300 × 7.8 mm, Bio-Rad Laboratories, Richmond, CA) as described elsewhere *(16)*. From the data residual concentration of Avicel was calculated.

Curve Fitting

Nonlinear curve-fitting to the experimental data was performed with the DeltaGraph Professional program (Delta Point, Monterey, CA).

ADSORPTION ISOTHERM MODELS

The models in this section describe how the adsorbed amount of a substance (enzyme) depends on the concentration of the substance in solution. The equations contain two or more parameters that either have physical meaning or are considered as empirical ones. In the notations used below B is the amount of the bound enzyme [μmol/g substrate] and $[F]$ is the concentration of the free enzyme in solution [μmol/L].

The Langmuir model assumes adsorption to uniform binding sites and that the adsorption energy does not depend on the saturation, i.e., a monolayer is formed and there is no interaction between the adsorbing molecules *(23)*.

$$B = n[F]/(K_d + [F]) \tag{1}$$

where n [μmol/g] is the number of binding sites per gram substrate and K_d [μmol/L] is the dissociation constant of the adsorbent-adsorbate complex. The affinity of the adsorbate to the adsorbent can be characterized, beside the K_d value, with the distribution coefficient that is equal to the ratio of the bound and the free substance ($B/[F]$) at infinitely low concentration ($[F] \approx 0$). The distribution coefficient is the initial slope of the Langmuir isotherm and can be calculated as

$$\alpha = n / K_d \tag{2}$$

where α has the dimension L/g *(2,11,19,24)*. The model can be extended assuming adsorption for more than one kind of binding site. The authors tested the Langmuir model for two binding sites:

$$B = n_1[F]/(K_{d1} + [F]) + n_2[F]/(K_{d2} + [F]) \tag{3}$$

n_1 and n_2 [μmol/g] are the number of adsorption sites per gram substrate; K_{d1} and K_{d2} [μmol/L] are dissociation constants for the two sites *(3,13)*. For the two site model the distribution coefficient is the sum of the distribution coefficients calculated for the two sites:

$$\alpha = n_1 / K_{d1} + n_2 / K_{d2} \tag{4}$$

The Freundlich model can be derived from the Langmuir equation by assuming that the adsorption energy for the surface sites decrease with the

logarithm of the saturation *(25)*, but it is often used as an empirical isotherm for adsorption to heterogeneous surfaces *(23,25)*.

$$B = K[F]^{1/m} \tag{5}$$

K is the Freundlich equilibrium constant and m is the power term of the Freundlich isotherm ($m > 1$).

The combined Langmuir Freundlich model is a combination of the Langmuir and Freundlich models and has been derived to describe adsorption to a heterogeneous surface *(26)*.

$$B = n[F]^{1/m} / (K + [F]^{1/m}) \tag{6}$$

where n [μmol/g] is the number of binding sites per gram substrate; m and K are empirical parameters. This model is analogous to the well-known Hill equation *(27)*.

The Temkin model describes the adsorption in the case of a linear decrease in adsorption energy with increasing saturation *(25)*.

$$B = k\, ln([F]) + b \tag{7}$$

The parameter k is equal to $RT/q_0\alpha$ and b is equal to $k\, ln(a_0)$, where R is the gas constant, T the absolute temperature, q_0 the heat of adsorption at zero saturation, α and a_0 are constants.

The Jovanovic model was originally derived for adsorption of gases *(28)*, but has also been used to describe adsorption of peptides and proteins on ion-exchange adsorbents *(29,30)*.

$$B = n[1 - \exp(-[F]/K_d)] \tag{8}$$

The authors have also used the extension of the Jovanovic model for two adsorption sites:

$$B = n_1(1 - \exp[-[F]/K_{d1}]) + n_2(1 - \exp[-[F]/K_{d2}]) \tag{9}$$

The parameters in the Jovanovic model have the same physical meaning as in the Langmuir model: n, n_1, and n_2 [μmol/g] are number of binding sites per gram substrate; K, K_{d1}, and K_{d2} [μmol/L] are dissociation constants.

RESULTS AND DISCUSSION

In an earlier study *(16)* the authors published data on adsorption isotherms at 4°C of CBH I and CBH II on Avicel. Adsorption was studied using the enzymes alone and in reconstituted equimolar mixtures. By comparing the isotherms the following could be observed: At most concentrations CBH I adsorbed to considerably higher extent than CBH II, both when they were studied alone and together; but at low enzyme/substrate ratio (E/S < 0.2 μmol/g) the difference between them was very small. At high concentrations, for both cellobiohydrolases, a reduction in adsorption was observed in presence of the other enzyme; adsorption of CBH II was

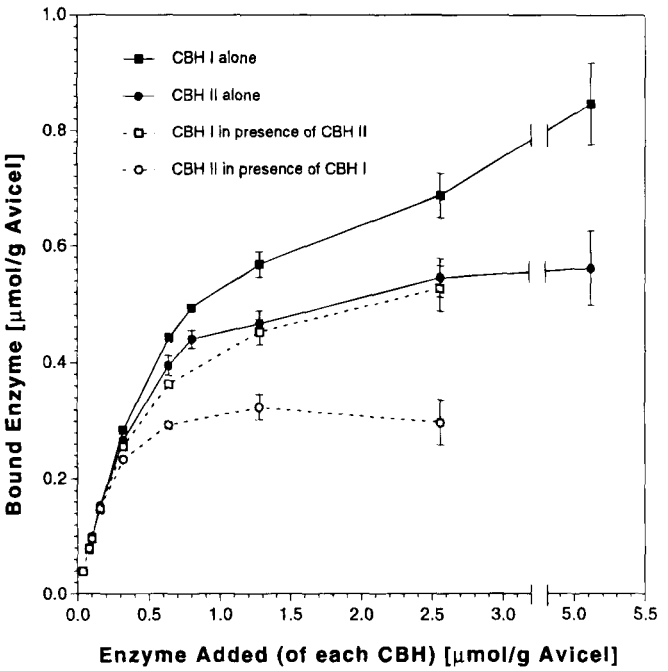
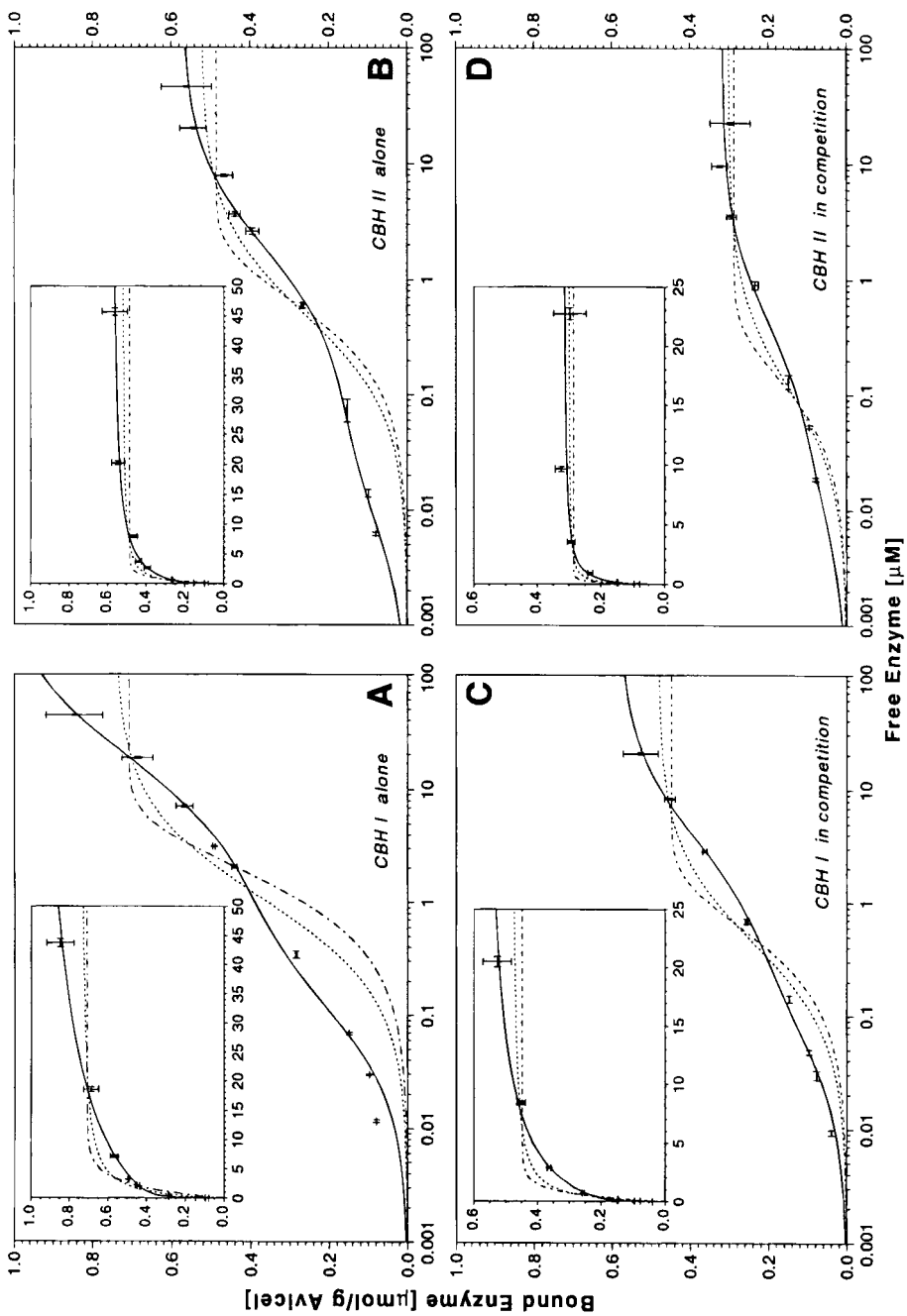


Fig. 1. Adsorption isotherms of CBH I and CBH II after 90 min incubation at 4°C. The enzymes were studied alone and in mixtures with 1:1 molar ratio. The enzyme/substrate ratio was varied in the range of 0.08–5.12 µmol/g by varying the volume and concentration of the enzyme solutions and the amount of Avicel used in the experiment (0.3–1.5 mL 0.4–51.2 µ*M* CBH I or/and CBH II was added to 3.8–20 mg Avicel). The enzyme concentration on the *x*-axis denotes the total amount (bound + free) added of each cellobiohydrolase per gram Avicel. Error bars show the standard deviation of the data calculated from repeated experiments and analyses. (■) CBH I alone; (●) CBH II alone; (□) CBH I in presence of CBH II; (○) CBH II in presence of CBH I.

more strongly influenced by the presence of CBH I than vice versa. At low saturation (E/S < 0.2 µmol/g), on the other hand, the effect of the other enzyme was negligible. It was interpreted that the decrease in adsorption when the two cellobiohydrolases were studied together was a result of competition for binding sites on cellulose. By calculating the total enzyme adsorption and comparing it with the isotherms for the single enzymes it could also be concluded that, beside competition for common binding sites, adsorption to sites that are specific for CBH I and CBH II, respectively, contribute to the total adsorption.

In the present work the adsorption isotherm data for CBH I and CBH II is analyzed. Nonlinear curve fittings to the experimental isotherms (shown in Fig. 1) were performed using several adsorption models. The aim was to find the best description of the experimental isotherms of pure CBH I and CBH II and, if possible, to get conclusions from the models about the adsorption process itself. Figures 2 and 3 show the results of curve fitting. In the main charts a semilogarithmic plot is used, whereas in the inserts the


A
CBH I alone
B
CBH II alone
C
CBH I in competition
D
CBH II in competition
Bound Enzyme [µmol/g Avicel]
Free Enzyme [µM]

results are shown using linear scale. It is important to point out that using conventional linear plots most of the models seem to represent the experimental data relatively good whereas semilogarithmic plots much better reveal how the fitted curves deviate from the experimental data. This is especially important in the low concentration region that is compressed when using linear scale. The correlation coefficients for seven models tested on the four experimental isotherms are collected in Table 1. Tables 2 and 3 contain apparent binding parameters deduced from the fitted curves.

Evaluation of the Models

Basic Models

The basic Langmuir equation, Eq. (1), assumes adsorption to a uniform surface and no interaction between the adsorbing molecules. Nonuniform surface or cooperativity (positive or negative) in adsorption may result in deviation from the Langmuir isotherm. Although both structural heterogeneity of the adsorbent and interaction between the adsorbed molecules are presumably important factors in cellulase adsorption, the Langmuir isotherm has frequently been used for describing the adsorption of cellulases on cellulose *(2,6,11,17–21)*. The reason for this could be that the model is simple, widely used and the interpretation of its parameters is straightforward.

A deviation from the Langmuir model can also occur because of a statistical exclusion of adjacent binding sites. A bound ligand can exclude binding to neighboring binding sites. This has been demonstrated for ligand binding to linear biopolymers, e.g., DNA *(31)*. The statistical exclusion of binding sites has also been discussed in a study on adsorption of the bacterial cellulase CenA *(24)*. A simulation model that can treat adsorption to overlapping sites on the cellulose surface has been developed *(32)*.

When the Langmuir model was used for the data, the correlation coefficients were rather low (Table 1); the fitted curves could not follow the experimental results (Fig. 2). The apparent binding parameters deduced from the model are shown in Table 2.

The Freundlich equation, Eq. (5), is often considered to be an empirical equation. However, it is possible to make a theoretical derivation of the model for adsorption to a nonuniform surface by assuming a decrease in

Fig. 2. (opposite page) Curve fitting to the experimental isotherms of CBH I and CBH II (data from Fig. 1) by nonlinear regression analysis using three adsorption models: (solid line) two-site Langmuir model (Eq. [3]); (dotted line) basic (one-site) Langmuir model (Eq. [1]); (dot-dashed line) Jovanovic model (Eq. [8]). Parameters of the fitted curves for the Langmuir models are given in Table 2. Correlation coefficients are shown in Table 1. The experimental results are represented by error bars calculated from repeated experiments and analyses. In the inserts the data are shown conventionally on a linear scale. In the main figures a semilogarithmic scale is used in order to show the low values of the free concentrations better. **(A)** CBH I alone, **(B)** CBH II alone, **(C)** CBH I in presence of equimolar amount of CBH II, **(D)** CBH II in presence of equimolar amount of CBH I.

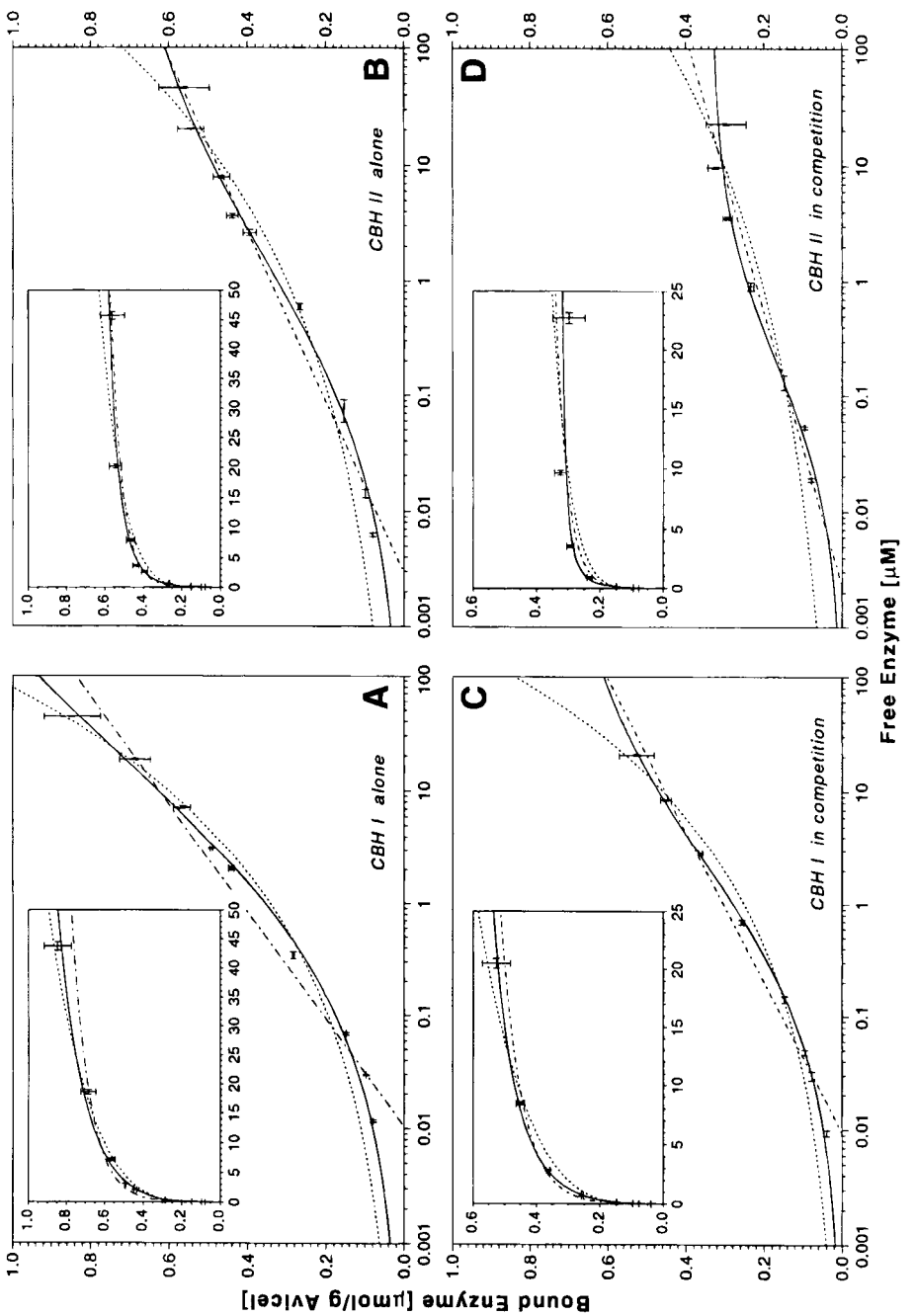

A
CBH I alone
B
CBH II alone
C
CBH I in competition
D
CBH II in competition
Bound Enzyme [µmol/g Avicel]
Free Enzyme [µM]

Table 1
Correlation Coefficients Obtained from Nonlinear Regression Analyses of CBH I and CBH II Adsorption Isotherms by Using Different Models

	Correlation coefficients (R^2)			
	Alone		In competition	
Model	CBH I	CBH II	CBH I	CBH II
Basic models:				
Langmuir (Eq. [1])[a,c]	0.926	0.947	0.955	0.954
Freundlich (Eq. [5])[b]	0.987	0.961	0.981	0.887
Temkin (Eq. [7])[b]	0.971	0.980	0.983	0.950
Jovanovic (Eq. [8])[a]	0.887	0.904	0.916	0.905
Extended models:				
Combined Langmuir-Freundlich (Eq. [6])[b,d]	0.996	0.995	0.999	0.983
Two-site Langmuir (Eq. [3])[a,c]	0.995	0.996	0.998	0.987
Two-site Jovanovic (Eq. [9])	0.987	0.983	0.993	0.984

[a]Fitted curves shown in Fig. 2.
[b]Fitted curves shown in Fig. 3.
[c]Fitted parameters shown in Table 2.
[d]Fitted parameters shown in Table 3.
The enzymes were studied alone and in mixtures with 1:1 molar ratio at 4°C and 90 min incubation.

adsorption energy with the logarithm of the saturation *(25)*. The Freundlich model could be used to describe the adsorption of bovine serum albumin to an ion-exchange resin (QAE-dextran) at the isoelectric point of the protein; at pH values different from the pI the adsorption of BSA followed the Langmuir equation instead *(33)*. For the adsorption of phosphorylase *b* to agarose substituted with alkyl residues the Freundlich isotherm gave a better description than the Langmuir *(34)*. In both aforementioned cases in which the Freundlich model was successful, the main forces involved in the protein adsorption were other than electrostatic forces. This indicates that the Freundlich isotherm is more adequate to describe protein adsorption when hydrogen bonding, hydrophobic, or van der Waals interactions are dominating. For protein adsorption to uncharged carbohydrate polymers, like starch or cellulose, the major inter-

Fig. 3. (opposite page) Curve fitting to the experimental isotherms of CBH I and CBH II (data from Fig. 1) by nonlinear regression analysis using three adsorption models: (solid line) combined Langmuir-Freundlich model (Eq. [6]); (dotted line) Freundlich model (Eq. [5]); (dot-dashed line) Temkin model (Eq. [7]). Parameters of the fitted curves for the combined Langmuir-Freundlich model are given in Table 3. Correlation coefficients are shown in Table 1. See also the caption for Fig. 2.

Table 2
Apparent Binding Parameters from Langmuir Models for Adsorption to Avicel of *T. reesei* Cellobio-hydrolases when Studied Alone and in Equimolar Mixtures

	Apparent binding parameters						
	Binding capacities				Distribution coefficients		
	[μmol/g]			[mg/g]	[L/g]		
Isotherm for	High n_1	Low n_2	Total n^a	Total n	High α_1	Low α_2	Sum α^b
CBH I alone							
One-site model			0.74	48			0.69
Two-site model	0.40	0.64	1.04	67	3.6	0.033	3.7
CBH II alone							
One-site model			0.52	28			1.0
Two-site model	0.16	0.41	0.57	30	20.5	0.22	20.7
CBH I in presence of CBH II							
One-site model			0.48	31			1.1
Two-site model	0.21	0.37	0.58	37	3.6	0.095	3.7
CBH II in presence of CBH I							
One-site model			0.30	16			2.5
Two-site model	0.095	0.22	0.32	17	12.3	0.47	12.8

[a] $n = n_1 + n_2$ for the two site model.

[b] $\alpha = \alpha_1 + \alpha_2$ for the two site model.

The values were obtained from the adsorption isotherms shown in Fig. 1 by nonlinear regression analysis using the basic (one-site) and the two-site Langmuir models (Eq. [1–4]). The fitted curves are shown in Fig. 2. The correlation coefficients are given in Table 1. The total binding capacity is given both on molar and weight basis for easier comparision with data in the literature. Abbreviations: High=high affinity binding site, Low=low affinity binding site.

actions are of this kind. The Freundlich isotherm has also been used to describe adsorption of α-amylase to starch granules *(35)* and of pure CBH I and CBH II to cellulose *(12)*. For cellulase adsorption to cellulose, recent findings indicate that hydrophobic interactions are important. One side of the cellulose binding domain of CBH I has three exposed tyrosines *(36)* that have been shown to be directly involved in the binding to cellulose *(37)*. The tyrosines can interact by hydrophobic "stacking" interaction with glucose molecules. In accordance with this discussion, it was found that the adsorption of CBH I and CBH II was better described by the Freundlich than the Langmuir model in most cases (Fig. 2 and 3, Table 1).

The Temkin model, Eq. (7), can also be derived theoretically for adsorption to a nonuniform surface, by assuming a linear decrease in

Table 3
Adsorption Parameters from Langmuir Freundlich Models for *T. reesei* Cellobiohydrolases when Studied Alone and in Equimolar Mixtures

	Adsorption parameters		
Isotherm for	n [μmol/g]	K [–]	1/m [–]
CBH I alone	1.5	3.1	0.37
CBH II alone	0.73	1.2	0.40
CBH I in presence of CBH II	0.72	1.6	0.47
CBH II in presence of CBH I	0.33	0.37	0.60

The values were obtained from the adsorption isotherms shown in Fig. 1 by nonlinear regression analysis using the combined Langmuir-Freundlich model (Eq. [6]). The fitted curves are shown in Fig. 3. The correlation coeffi-

adsorption energy with saturation *(25)*. According to our knowledge, this isotherm has not previously been used to describe adsorption of cellulases. The Temkin model gives a straight line in the semilogarithmic plots used (Fig. 3). The correlation coefficients were somewhat better compared to those obtained with the Langmuir model and were close to the coefficients obtained with the Freundlich model (Table 1). The better representation of the experimental data obtained with the Freundlich and Temkin isotherms, compared with the Langmuir, suggests that models developed for adsorption to nonuniform surfaces are more adequate to describe adsorption of cellobiohydrolases to Avicel.

The Jovanovic equation, Eq. (8), has been used to describe adsorption of proteins and peptides on ion-exchange adsorbents *(29,30)*. Regression analysis using this model resulted in fitted curves far from the experimental data (Fig. 2) and very low correlation coefficients (Table 1).

Two Site Models and the Combined Langmuir-Freundlich Model

Curve fittings to adsorption data were made using equations developed from the four basic model isotherms. The Langmuir two-site model, Eq. (3), was successfully used, as well as the combined Langmuir-Freundlich model, Eq. (6), and the Jovanovic model for two adsorption sites, Eq. (9). Fittings with the Temkin and Freundlich type of models with two sites did not improve the results compared to the respective one-site models or resulted in meaningless parameters. Results from calculations with the two-site Langmuir and the combined Langmuir-Freundlich models are shown in Figs. 2 and 3, respectively. These two models were found to give the best description of the adsorption of CBH I and CBH II both when the enzymes were studied alone and together. The fitted curves could follow the experimental data over the entire concentration range tested, and the correlation coefficients obtained were normally higher than or equal to 0.995 (Table 1). (For CBH II in presence of CBH I the R^2 values were somewhat lower.) Fittings with the two-site

Jovanovic model resulted in similar binding parameters (data not shown) compared to the two-site Langmuir model, but the R^2 values were lower.

Evaluation of the Apparent Binding Parameters

The two-site Langmuir and the combined Langmuir-Freundlich models were found to be the best ones to describe the adsorption of cellobiohydrolases; the basic Langmuir model, on the other hand, has often been used in the literature to characterize adsorption isotherms of cellulases. The binding parameters derived using these three models are collected in Tables 2 and 3.

The Langmuir Models

It has been noted that the apparent fit of the (one-site) Langmuir model to the experimental isotherms of cellulases does not imply that the underlying assumptions in the Langmuir adsorption theory are necessarily obeyed. The adsorption of several cellulases has been shown to be irreversible *(2,11,14)* so no physical meaning should be attributed to the K_d values calculated *(11)*. Nevertheless, the binding capacity and the distribution coefficient obtained from the (one-site) Langmuir model were suggested for characterizing the extent of cellulase adsorption and the degree of interaction, respectively *(2,11,19)*. This applies for the two-site Langmuir model as well. In this case, the characteristic parameters are the total binding capacity ($n_1 + n_2$ in Eq. [3]) and the distribution coefficient calculated as given by Eq. (4). The binding parameters obtained for the single sites should be treated as apparent ones.

The distribution coefficients (Table 2) were higher for CBH II than for CBH I indicating stronger binding of CBH II. The binding capacities, on the other hand, were higher for CBH I. At most concentrations used, these binding parameters will result in a higher amount of bound CBH I compared to CBH II, which agrees with the experimental results. The lower binding capacity observed for CBH II is in accordance with the observations made by electron microscopy that CBH II binds preferably to cellulose crystal ends and CBH I over the whole length of the crystal *(38,39)*, but conflicts with the data of Tomme et al. *(12)* who found the binding capacity for CBH II similar to that for CBH I, 64 and 70 mg/g, respectively.

The two-site Langmuir model has previously been used by Woodward et al. *(13)* and Ståhlberg et al. *(3)* to describe the adsorption of pure cellulases to Avicel. In the present study the binding parameters for CBH I (Table 2) are similar to those obtained by Ståhlberg et al. *(3)*, despite the differences in the experimental methodology used for the studies (e.g., they used direct photometric or fluorometric fluorometric detection of the free enzyme in solution) and the different temperatures (they performed the experiments at room temperature). Their results were (the present results in parenthesis) n_1 = 0.21 (0.40) μmol/g, n_2 = 0.85 (0.64) μmol/g, $n_1 + n_2$ = 1.06 (1.04) μmol/g, K_{d1} = 0.052 μ*M* and K_{d2} = 6.0 μ*M*, which give distribution coefficients of α_1 = 4.0

(3.6) L/g, α_2 = 0.14 (0.033) L/g, and $\alpha_1 + \alpha_2$ = 4.14 (3.7) L/g. The similar parameters obtained at different temperatures agree with earlier results that showed that by increasing the temperature from 4 to 40°C, the adsorbed fraction of cellobiohydrolases just slightly decreased *(16)*. The parameters from the study of Woodward et al. *(13)* cannot be compared to this data since they used very short adsorption time (2 min).

Beldman et al. *(11)* used the one-site Langmuir model to characterize cellulase adsorption to Avicel. For CBH I (Exo III in their nomenclature) they reported the binding capacity and the distribution coefficient to be 63 mg/g and 0.44 L/g, respectively. The corresponding binding parameters achieved using the same model for our data (Table 2) agrees rather well, 48 mg/g and 0.69 L/g, respectively.

Kyriacou et al. *(19)* studied the effect of physical parameters (pH, temperature, and ionic strength) on the adsorption to Solka floc of partly purified cellulases and used the basic Langmuir model to evaluate the isotherms. They found distribution coefficients for the CBH I fraction in the range of 0.19–1.1 L/g, which agree well with the present results using the one-site model (0.69 L/g). On the other hand, the binding capacity they reported was substantially lower, less than 4 mg/g at 5°C, compared to the present study (48 mg/g), which is probably because of the difference in the properties of the substrate. Similar conclusion can be drawn from comparison of the data with that of Nidetzky et al. *(2)* who reported parameters for one-site Langmuir adsorption isotherms of pure cellulases on filter paper. The distribution coefficient they found for CBH I was 0.24 L/g and the binding capacity was 0.17 μmol/g, equivalent to 11 mg/g.

Comparing the results obtained with the one-site and the two-site Langmuir models, it is seen that the parameters derived from the one site model underestimates the binding capacity and especially the distribution coefficients for the enzymes (Table 2). The one-site model cannot follow both the initial and the saturating parts of the experimental isotherms. A one-site Langmuir curve fitted only to the low concentration region of the isotherm would strongly underestimate the binding capacity. A curve fitted only to the high concentration region of the isotherm would strongly underestimate the distribution coefficient. The curve fitted to the entire concentration range goes under the experimental values both at low and high free concentrations (and goes above them at medium concentrations) (Fig. 2). This leads to lower values obtained for both the binding capacity and especially the distribution coefficient (the initial slope of the curve) compared with the two-site model. Thus, it can be concluded that the one-site Langmuir model is not adequate to describe the adsorption isotherms and the parameters obtained cannot be used, not even as apparent ones, to characterize the adsorption.

The Combined Langmuir-Freundlich Model

A limitation of the Freundlich isotherm is that it does not reach a saturation when the concentration in solution is increased. To solve this inade-

quacy the Langmuir and Freundlich isotherms may be combined Eq. (5). The combined Langmuir-Freundlich equation has been used to describe adsorption of proteins and peptides on ion-exchange adsorbents *(26)*. Although this model is empirical, it could describe the adsorption of the cellobiohydrolases with very good correlation (Fig. 3, Table 1). The adsorption parameters derived using this model are shown in Table 3. Parameter n is the binding capacity, K and m have no physical meaning. Unfortunately, not even a distribution coefficient can be derived for this model, since the initial slope of this kind of function is infinite, thus, the parameters do not reflect the strength of the binding.

On the other hand, it should be noted that the combined Langmuir-Freundlich model is analogous to the well-known Hill equation that is used to describe cooperative binding of ligands to enzymes with multiple binding sites *(27)*. The power term of the Hill equation (corresponds to $1/m$ in Eq. [5]) shows the degree of cooperativity. In the present case, cellobiohydrolases act as ligand molecules binding to the cellulose microfibrils that are acting as large adsorbent molecules with multiple binding sites. The obtained power terms were smaller than one in each case (Table 3). This indicates either presence of unequal binding sites or negative cooperativity in binding, i.e., binding of one enzyme molecule on the cellulose surface obstructs the binding of the next one. It is likely that in the case of adsorption of cellulases on cellulose both factors contribute to the process.

CONCLUSIONS

The adsorption isotherms for CBH I and CBH II did not follow the basic (one site) Langmuir equation that has been widely used to describe cellulase adsorption; the apparent binding parameters derived using this model underestimated the binding capacities and specially the distribution coefficients for the enzymes. The Freundlich and Temkin isotherms gave somewhat better correlation coefficients than the basic Langmuir one, indicating that models developed for adsorption to nonuniform surfaces are more adequate to describe adsorption of cellobiohydrolases to Avicel; but not even these models are flexible enough to follow the experimental data over a wide concentration range. The Jovanovic isotherm is not applicable for cellulase adsorption at all. A model of Langmuir type with two adsorption sites and a combined Langmuir-Freundlich model could be used to describe the isotherms with very good correlation. These models should be preferred when the aim is to describe experimental isotherms of cellulases. The total binding capacity ($n_1 + n_2$) and the distribution coefficient ($\alpha_1 + \alpha_2$) derived from the two-site Langmuir isotherm are meaningful parameters to characterize the adsorption. The distribution coefficient for CBH II was higher than for CBH I indicating stronger bind-

ing of CBH II; the binding capacities, on the other hand, were higher for CBH I. The adsorption of cellulases on a complex substrate like cellulose is a complicated process that cannot be fully described by the used models. Therefore, the derived binding parameters should be considered as empirical ones. The results from the combined Langmuir-Freundlich model indicated presence of unequal binding sites on cellulose and/or negative cooperativity in the binding of the enzyme molecules.

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