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# Do Cellulose Binding Domains Increase Substrate Accessibility?

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## Abstract

This article provides an overview of various theories proposed during the past five decades to describe the enzymatic hydrolysis of cellulose highlighting the major shifts that these theories have undergone. It also describes the effect of the cellulose-binding domain (CBD) of an exoglucanase/xylanase from bacterium *Cellulomonas fimi* on the enzymatic hydrolysis of Avicel. Pretreatment of Avicel with $CBD_{Cex}$ at 4 and 37°C as well as simultaneous addition of $CBD_{Cex}$ to the hydrolytic enzyme (Celluclast, Novo, Nordisk) reduced the initial rate of hydrolysis owing to irreversible binding of CBD proteins to the substrate's binding sites. Nonetheless, near complete hydrolysis was achieved even in the presence of $CBD_{Cex}$. Protease treatment of both pure and $CBD_{Cex}$-treated Avicel reduced the substrates' hydrolyzability, perhaps owing to proteolysis of the hydrolyzing enzyme (Celluclast) by the residual Proteinase K remaining in the substrate. Better protocols for complete removal of CBD proteins from the substrate need to be developed to investigate the effect of CBD adsorption on cellulose digestibility.

**Index Entries:** Cellulose; enzymatic hydrolysis; cellulases; cellulose-binding domain; *Cellulomonas fimi*.

## Introduction

Cellulose hydrolysis has been studied for many decades, while recently new and specific applications have been refueling this interest. The production of low-cost, high-quality animal feed from wood or agricultural residues has been one of the first applications of cellulose digestion using

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simple treatments. Today, however, enzymatic digestion of cellulose has applications in the textile industry; detergent manufacturing; and the production of energy, chemicals, and fuels from lignocellulosics. The wide range of applications for cellulose modification by biologically derived enzymes (cellulases) is a great example of how biotechnology can either replace or improve the traditional industrial processes while offering additional benefits, e.g., improving product quality and reducing environmental impacts.

The agricultural sector has always been interested in studying the mechanism of hydrolysis of cellulose. An understanding of the digestion of cellulosic materials (e.g., forage) in the digestive tract of feedlot cattle and other farm animals has enabled agricultural scientists to produce high-quality, low-cost feed from agricultural products and residues (e.g., feed crops, plant residues, or even wood). Cellulases can be added to grain-based fodder to reduce the feed viscosity and water uptake in animal digestive systems, thereby enhancing the amount of nutrients obtained from the feed *(1)*.

In the textile industry, hydrolysis of cellulose by fungal and bacterial enzymes has created new market opportunities and many economic benefits. Biopolishing of denim and other natural fabrics using cellulases has replaced, for the most part, the stonewashing operation for producing fabrics with softer texture and aged appearance, both in demand by the fashion market. Cellulases are also added to laundry detergents to remove "pills" formed on fabric surfaces during washing, hence improving the appearance of garments *(1)*. More technical studies have shown that changing the ratio of different components in an enzyme mixture, i.e., cellobiohydrolases to endoglucanases, can produce different results. Endoglucanase-rich mixtures are more effective in biopolishing (aging of denims) while complete enzyme mixtures enhance the depilling property in detergents without causing excessive abrasion *(2)*. Commercial cellulases have also been shown to enhance the whiteness, brightness, and color characteristics of cotton fabrics *(3)*.

The pulp and paper industry also takes advantage of the potential of cellulases in improving paper and fiber quality, reducing papermaking energy requirements, and removing ink from recycled paper. Cellulases have been shown to preferentially attack and hydrolyze shorter wood fibers, i.e., fines, produced during the refining operation. Refining, a mechanical action necessary for improving the physical properties of primary or secondary fibers, can generate small particles (fines) that can reduce the pulp's water drainage rate during papermaking operations *(4)*. By preferential hydrolysis of fines, enzymatic treatment can improve the pulp's drainage property and reduce the energy requirements of papermaking operations. A combination of cellulase treatment and refining has also been shown to provide a means for using coarse wood fibers (e.g., Douglas fir) to produce finer paper products *(5)*.

Enzymatic hydrolysis of cellulose is also a key step in the production of fuels and chemicals from lignocellulosic materials such as wood, grass,

and waste paper. In a biomass-to-ethanol bioconversion process, chemical and mechanical treatments remove the majority of hemicelluloses from the feedstock and produce a highly digestible, cellulose-rich substrate that needs to be further hydrolyzed to obtain monomeric glucose for fermentation to ethanol.

Understanding and improving the interactions between cellulose and cellulase enzymes can help reduce the use of costly enzymes and improve the economy of the overall process. A more economic process will reduce the price of ethanol fuel, hence enabling it to compete with gasoline as a transportation fuel for road vehicles. Many social, economic, and environmental benefits can result from using renewable resources for energy production *(6)*.

Hydrolysis of cellulose by cellulase enzyme continues to be a research priority for scientists from a variety of disciplines owing to its diverse applications and its multifaceted nature (e.g., the enzyme components and properties, substrate characteristics, reactor configurations and process design).

In this article, we provide an overview of the theories that have been proposed to describe the enzymatic hydrolysis of cellulose and highlight the major shifts that these theories have undergone during the past five decades. We also report our observations of the interactions between an isolated cellulose-binding domain (CBD) from *Cellulomonas fimi* exoglucanase/xylanase, a hypothetically hydrolysis-inducing agent, and a model cellulosic substrate, Avicel.

## *Enzymatic Hydrolysis of Cellulose*

Depolymerization of cellulose into its monomeric glucose units by cellulase enzymes is a complex phenomenon mediated by inter- and intramolecular synergism among the main components of the cellulase system—endoglucanases, cellobiohydrolases, and β-glucosidase. Both the enzyme mixture and the solid substrate have attributes that can strongly influence the dynamics of the reaction *(7)*. In addition, the reaction heterogeneity arising from the interaction between a solid substrate and the liquid enzyme further adds to the complexity of an enzymatic hydrolysis system. Adsorption of enzyme onto the solid surface, penetration into the crystalline structure, occurrence of the chemical reaction, desorption/readsorption or movement of proteins on the cellulose structure, and, finally, diffusion of the hydrolysis products (glucose, cellobiose, or short oligomer chains) out of the porous structure seems to be the logical order of events during hydrolysis.

Simultaneous or consecutive occurrence of these events on various reaction sites on the substrate creates an intense nonlinear dynamic within the first few hours of the reaction. At any moment, different fractions of the enzyme are engaged in different actions. It has been shown that almost all the enzyme activities, except β-glucosidase, are adsorbed onto the substrate very rapidly. Swelling of the substrate accommodates the penetra-

tion of enzyme proteins into the structure. Through concerted action of endo- and exo-acting enzymes, cellulose chains are cleaved and cellobiose units are released into the solution. Once in the liquid phase, cellobiose is further cleaved by the action of β-glucosidase to produce soluble glucose monomers.

During the first few hours of the reaction, the rate of glucose production is linear with time. The intensity of reaction, however, subsides after a few hours and the reaction gradually slows down, hence exhibiting the typical biphasic pattern observed in any cellulose hydrolysis profile, i.e., of glucose production (or percentage of hydrolysis) vs time. The cause of this gradual drop in the reaction rate is not fully understood, but it has been postulated that both enzyme- and substrate-related properties contribute to this effect *(7)*. Examples of substrate-related factors include the degree of crystallinity and polymerization of cellulose chains, extent of accessible surface area, substrate porosity, and presence of extraneous materials such as lignin and hemicellulose in cellulosic substrates. Enzyme-related factors include thermal or mechanically induced deactivation of enzymes due to mixing or exposure to high temperatures, sieving (separation) of enzyme components by the substrate and the loss of synergism following this separation, as well as product inhibition due to accumulation of cellobiose and glucose in the reaction medium. Conflicting observations have prevented scientists from identifying a single factor as the sole cause of gradual loss of efficiency during the hydrolysis of cellulose. This is further confounded by the fact that research has not been able to provide a detailed and all-encompassing mechanism for hydrolysis of cellulose by cellulolytic proteins.

The first attempt at establishing a mechanistic model for enzymatic hydrolysis of cellulose was made by Elwyn Reese in 1950 *(10)*. His theory, known as the $C_1$-$C_x$ model, suggested that a mechanical action (by the $C_1$ factor) precedes the actual hydrolytic action (by the $C_x$ factor). Although groundbreaking in its own time, this theory did not describe the nature of $C_1$ and $C_x$ factors. Building on this model, numerous attempts were made to attribute the $C_1$ and $C_x$ activities to different components of an enzyme mixture. For example, Wood and McCrae *(11)* equated the $C_1$ and $C_x$ factors with exo- and endo-acting enzymes, respectively, suggesting that exoglucanases that have a higher affinity for the cellulose chains start the reaction and that the action of endoglucanases would follow. While maintaining the same nomenclature ($C_1$ = exo and $C_x$ = endo), in a later revision of their original proposition, Wood and McCrae *(12)* proposed that it is the endoglucanases ($C_x$) that initiate the attack on the cellulose chains as they are more random in their action and can create susceptible chain ends suitable for exoglucanases ($C_1$) to act on. This was a clear deviation from the original assumption of the $C_1$-$C_x$ model that $C_1$ acts first and $C_x$ follows. While it can be argued that the differences are solely in the interpretations and have no bearing on the actual theory of hydrolysis, we find it worth

Table 1
Highlights of Various Concepts Developed During the Past Five Decades to Describe Mechanism by which Crystalline Cellulose Is Hydrolyzed

| Year | Reference | Proposed mechanism |
|---|---|---|
| 1950 | *10* | The $C_1$-$C_x$ model: A nonhydrolytic component ($C_1$) splits the structure of native cellulose and creates short linear chains and a hydrolytic component ($C_x$) depolymerizes the short chains into glucose. |
| 1972 | *11* | $C_1$ and $C_x$ factors are, respectively, the exo- and endo-acting enzymes; that is, the exoglucanases initiate the attack. |
| 1979 | *12* | Endoglucanases ($C_x$) act more randomly and therefore are more likely to initiate the attack ($C_x$ followed by $C_1$—the reverse of original Reese's model). |
| 1985 | *13* | The amorphogenesis action that swells, segments, and/or destratifies the substrate precedes any hydrolytic action by either exo or endo enzymes. Amorphogenesis may be mediated by some nonhydrolytic factors (*see* Table 2) or by $H_2O_2$ produced by other enzymes. |
| 1986 | *21* | $C_1$ is not a distinct component but a specific property of different enzymes, i.e., binding ability. The extent of catalysis is directly related to how tightly the enzyme is bound to the substrate. |
| 1989 | *23* | Synergism among CBHs and endoglucanases with varying stereospecificities cause the disaggregation of crystalline cellulose, which is then followed by the catalytic action. |
| 1990 | *22* | Mechanochemical action: Tightly bound enzymes attack the disturbed regions and disperse the crystalline cellulose by penetrating into the structure and opening new sites for the action of weakly absorbed enzymes. |
| 1991 | *20* | $C_1$ activity resides not in a system distinct from $C_x$ but in a discrete domain of each enzyme. Cellulose binding modules initially defibrillate the substrate and render it more susceptible to the action of the catalytic core. |

highlighting as it appears to be an interesting turning point in the evolution of cellulose hydrolysis theory, as summarized in Table 1.

In 1985, Coughlan *(13)* introduced the concept of amorphogenesis as a prerequisite for the hydrolytic action of the enzyme. His suggestion was that amorphogenesis (i.e., swelling, segmentation, or destratification of the substrate) renders the crystalline cellulose more accessible to the enzymes for hydrolytic dissolution. Thus, amorphogenesis was the equivalent of $C_1$-induced mechanical dispersion proposed by Reese *(10)*.

Amorphogenesis has been attributed to the $H_2O_2$ produced by some enzymes *(14)*, some iron-containing proteins in fungal filtrates *(15)*, or even to CBH I produced by *Trichoderma reesei (16)*. Table 2 summarizes some of

Table 2
Evidence for Existence of Nonhydrolytic Component in Cellulase Mixtures[a]

| Reference | Nonhydrolytic factor | Substrate | Method of monitoring | Observation |
|---|---|---|---|---|
| *17* | MGF from *T. reesei* (~5 kDa) | Filter paper and corn leaf | SEM<br>Glucose:phenol–sulfuric acid | MGF increased microfibril content of filter paper (SEM). Incubation of filter paper with 4 mL of MGF solution (23 µg/mL) for 24 h released 5.9 mg of soluble carbohydrates but did not enhance filter paper's enzymatic digestibility. |
| *20* | $CBD_{CenA}$ (~11 kDa) | Ramie fiber (~72% crystalline) | SEM to examine fibers<br>Carbohydrates: orcinol–sulfuric acid<br>UV absorbance at 600 nm to measure particles | Treatment with $CBD_{CenA}$ alone disrupted fiber structure and roughened the surface (SEM), and released some small particles ($A_{600nm}$) but no sugar. |
| *18* | CBH $II_{cp}$ from *T. reesei* (~36 kDa) | Avicel and cotton linters | TEM<br>Reducing sugar by DNS | Incubation of 2.5 mg of cotton fibers with 194 µg of CBH $II_{cp}$ at 23°C for 18 h dispersed the fibers (TEM). Pretreatment with CBH $II_{cp}$ enhanced Avicel's enzymatic digestibility but did not produce any reducing sugars from Avicel or cotton. |
| *19* | FFP from *T. reesei* (11 to 12 kDa) | Filter paper (Whatman no. 1) | PCM<br>UV absorbance at 600 nm<br>Sugars by phenol–sulfuric acid | Incubation of 20 mg of filter paper with 0.3 mL of FFP (50–100 µg) at 10°C for 24 h caused partial disruption of filter paper (PCM) and produced free fibrils ($A_{600nm}$) but no sugars. |

[a]MGF, microfibril-generating factor; $CBD_{CenA}$, CBD from *C. fimi* endoglucanase; CBH $II_{cp}$, catalytic domain of CBH II; FFP, fibril-forming protein; SEM, scanning electron microscopy; TEM, transmission electron microscopy; DNS, dinitrosalicylic acid; PCM, phase contrast microscopy.

the more recent studies that have tried to elucidate the nature of a nonhydrolytic factor responsible for mechanical dispersion of a substrate prior to catalytic reaction. Several groups *(17–19)* have been able to isolate a component or a portion of a component from *T. reesei* filtrate that had the ability to cause dispersion in the substrate. The microfibril-generating factor (MGF; 5 kDa) isolated by Krull et al. *(17)* increased the microfibril content of filter paper as observed by scanning electron microscopy. It also released soluble carbohydrates from filter paper but did not improve the digestibility of filter paper after a 24-h pretreatment at 50°C.

In a similar study, Banka et al. *(19)* isolated a larger nonhydrolytic component, referred to as fibril-forming protein (FFP; 11 to 12 kDa), from *T. reesei* that was able to disrupt filter paper as detected by phase contrast microscopy and produce free fibrils. Thus, the MGF and FFP seemed to play the role of $C_1$ factor in Reese's model *(10)*. Another interesting observation by Woodward et al. *(18)* showed that the catalytic domain (36 kDa) of CBH II from *T. reesei* (CBH $II_{CP}$) had no catalytic activity toward Avicel and cotton linters; nevertheless, transmission electron microscopy revealed that the cotton fibers were dispersed upon treatment with this protein. This nonhydrolytic effect makes the CBH $II_{CP}$ a good candidate for the $C_1$ activity and also implies that the hydrolytic activity of this particular catalytic domain is contingent on the presence of its related binding domain. Din et al. *(20)* observed that the catalytic domain of *C. fimi* endoglucanase could hydrolyze cotton to a limited extent, even in the absence of its binding domain. This is perhaps owing to the differences in the origin of the two enzymes and how their domains were isolated (e.g., intensity of papain digestion).

While the search for an individual component that can fit the role of Reese's $C_1$ factor continued, an interesting study by Klyosov et al. *(21)* shifted the concept of $C_1$ factor as a "component" to a "property." Klyosov suggested that "$C_1$ factor is not an individual substance or enzyme with a particular specificity but rather a property of already known enzymes, namely the capacity of cellulases for binding onto the surface of insoluble cellulose." He further elaborated his theory *(22)* and proposed that the tightly bound enzymes initiate the attack at the disturbed regions of the crystalline cellulose and disperse the structure through a mechanochemical action, creating more-accessible areas of attack for the weakly bound, more-mobile enzymes that will carry out the catalytic reaction. At the amorphous regions, however, the quality of binding is not an issue, and the quantity of enzyme available will determine how fast and how much of the substrate will be hydrolyzed.

Klyosov's *(22)* theory also had interesting implications in terms of synergism. It is known that during the hydrolysis of cellulose multiple components of an enzyme mixture act in harmony to produce a synergistic effect that is greater than the sum of individual effects. The complementary role of different components in an enzyme mixture has been attributed to their different stereospecificities *(23)*; however, Klyosov *(22)* proposes that

the synergism occurs because of the existence of multiple enzyme components with various adsorption capacities. The presence of a tightly bound endoglucanase ensures effective dispersion of the substrate and entails an efficient hydrolysis, even if other components (e.g., CBHs) are only weakly bound. Thus, the tightness of binding is equated with $C_1$ factor as the perquisite for an efficient catalytic degradation.

The pioneering work of Din et al. *(20)* in 1991 with the cellulose-binding domain (CBD) of endoglucanase A (CenA) from the bacterium *C. fimi* brought yet another shift to the way the nonhydrolytic component was perceived. This work suggested that the $C_1$ activity resides not in a system distinct from $C_x$ but in a discrete domain of each enzyme. It was shown that the isolated $CBD_{CenA}$ causes a roughening of the surface of cellulosic fibers (cotton and ramie) and releases small particles into the solution that can be detected spectrophotometrically at 600 nm. It was also proposed that this protein could penetrate into structure and create free chain ends; however, it could not be verified whether the protein is indeed capable of destabilizing the hydrogen bonds within the crystal. This nonhydrolytic effect was observed only with the binding domain, and not the isolated catalytic core of the same enzyme.

Recently, a 50-kDa protein called swollenin was reported in *T. reesei (24)*. Incubation of cotton fibers with a crude swollenin preparation appears to result in an opening and swelling of fiber structure without hydrolysis. The protein comprises an N-terminal CBD joined by a linker to a region with sequence similarity to expansins (molecules implicated in cell wall extension in higher plants).

The primary role of carbohydrate-binding domains is to increase the "local" concentration of enzyme, thereby enhancing the hydrolysis reaction rate. However, it has been postulated, and in some cases shown, that the binding domains of cellulases perform functions other than physical or chemical binding *(20,25)*. These additional functions also seem important for initiating and maintaining an efficient hydrolysis. For example, it has been proposed that CBDs of exoglucanases can act as a plough, delaminating cellulose layers and releasing free chain ends. The enzymes' catalytic domains will then act on these free ends and depolymerize the cellulose structure *(25)*.

The study by Din et al. *(20)* also showed that "small particles" from cotton were released into the medium as a result of treatment with $CBD_{CenA}$. These particles are assumed to be small cellulose fragments that are noncovalently bound to the fiber surface and sloughed off during CBD binding. The isolated catalytic domain of this enzyme, on the other hand, was shown to have a polishing effect on the fiber surface. It has been suggested that this effect is caused by the hydrolysis of glycosidic bonds at fibers' mechanically damaged regions, which, in turn, removes the short fibers attached to these areas and smooths the substrate's surface.

Jervis et al. *(26)* did not observe such disruptive effects in treatment of highly crystalline cellulose from *Valonia ventricosa* with $CBD_{CenA}$. However,

they showed that the majority (70%) of bound CBDs are mobile on the crystalline surface, hence undermining the "binding-site exclusion" theory suggested by McGhee and von Hippel *(27)*. Thus, this study showed that binding of a CBD molecule does not permanently exclude the corresponding binding sites from the pool of substrate-binding sites. Instead, the mobility of CBD makes those sites available for new binding upon its displacement. In this study, surface diffusion, however, was not found to be the rate-limiting factor during hydrolysis of cellulose, although it might have important implications in terms of processivity of cellulase action on cellulose.

Creagh et al. *(28)* studied the structural changes of bacterial microcrystalline cellulose (BMCC) after treatment with increasing amounts of $CBD_{Cex}$ using confocal microscopy. The degree of dispersion of the BMCC fibrils was found to be dependent on the concentration of bound $CBD_{Cex}$.

The work presented herein is our first step in testing the hypothesis that the dispersing effect of CBDs can increase the interfibrillar space, thereby increasing the available surface area and enhancing the rate and extent of hydrolysis of a cellulosic substrate.

## Materials and Methods

### $CBD_{Cex}$ Protein

The CBD of *C. fimi* exoglucanase/xylanase (Cex) was produced at the University of British Columbia Biotechnology Laboratory according to protocols described previously *(29)*. The protein solution was concentrated to 1–1.5 mg/mL by ultrafiltration using a YM1 (1 kDa) membrane (Millipore, Bedford, MA). Avicel was used as the solid substrate in all experiments.

### Adsorption Assays

For quantitative analysis, $CBD_{Cex}$ (1–30 μ*M*) was mixed end-over-end with 5 mg of Avicel (1% slurry) in a final volume of 1 mL of buffer (50 m*M* potassium phosphate, pH 7.0) at 4 or 37°C. After 90 min, at 4 or 37°C, cellulose was removed by centrifugation (13,000*g*, at respective temperature), and the free protein left in the supernatant was measured (280 nm) and used to calculate the amount of $CBD_{Cex}$ bound to Avicel. All experiments were performed in duplicate. The results were fitted to a Langmuirian equation in which the affinity binding constants ($K_a$) and the saturation capacity ($N_o$) were obtained.

### Determination of Protein Concentration

The concentration of $CBD_{Cex}$ was determined by ultraviolet (UV) absorbance at 280 nm, in which the molar extinction coefficient of 0.027625 $\mu M^{-1}cm^{-1}$ was used.

### Proteolysis by Proteinase K

Saturating amounts of $CBD_{Cex}$ protein (20 μ*M*) were mixed (4°C) end-over-end with 5 mg of Avicel in a final reaction volume of 1 mL of buffer

(50 m*M* potassium phosphate, pH 7.0). After 90 min, cellulose was spun down by centrifugation (13,000*g*, 4°C), and the concentration of free protein was measured using a fluorimeter (Perkin-Elmer LS50 Luminescence Spectrometer), which, in turn, was used to measure the amount of $CBD_{Cex}$ bound to Avicel. This entailed constructing a standard curve of the fluorescence emission at 537 nm vs $CBD_{Cex}$ concentration. The cellulose pellet was rinsed with Tris buffer five times with centrifugation (13,000*g*, 4°C) following each rinse. The pellet was then treated with Proteinase K (50 µg/mL, 37°C, 16 h) to remove bound protein. After overnight incubation, the pellet was centrifuged (13,000*g*, 4°C, 15 min), and the free $CBD_{Cex}$ was measured using the fluorimeter. No fluorescence was detected from Proteinase K alone. The cellulose pellet was further rinsed with potassium phosphate buffer (pH 7.0) five times and centrifuged (13,000*g*, 4°C, 5 min) after each rinse. The pellet was then treated with guanidinium hydrochloride (6 *M*, room temperature, 5 h) to remove any bound protein remaining after the protease treatment; this method was used to estimate the amount of $CBD_{Cex}$ removed after Proteinase K treatment. The pellet was centrifuged (13,000*g*, 4°C, 15 min), and the free protein was measured using the fluorimeter.

### *Hydrolysis of $CBD_{Cex}$-Treated Avicel*

Avicel slurry (1%) was prepared in sodium acetate buffer, pH 4.8, to which $CBD_{Cex}$ was added (final concentration of 2 or 20 µ*M*) in a reaction volume of 10 mL of buffer (0.05 *M* sodium acetate, pH 4.8). After overnight incubation at either 4 or 37°C, cellulose was removed by centrifugation (14,000*g*, room temperature, 15 min), and the pellet was rinsed with buffer (sodium acetate, pH 4.8) and transferred volumetrically into 10-mL flasks. Celluclast (40 filter paper units [FPU]/g cellulose) was added to the mixture, which was then supplemented with Novozyme 188 (80 cellobiase units [CBU]/g cellulose). The final volume was brought to 10 mL by the addition of 0.05 *M* sodium acetate buffer, pH 4.8. The samples were transferred to 50-mL Erlenmeyer flasks and incubated in a 45°C shaker bath. Samples (300 µL) were taken at 1, 5, 24, 50, 75, and 100 h and boiled for 5 min prior to storage at –20°C. Controls included samples with no $CBD_{Cex}$ pretreatment (0.05 *M* sodium acetate buffer, pH 4.8 only) followed by Celluclast, and samples that received $CBD_{Cex}$ treatment but no Celluclast (0.05 *M* sodium buffer, pH 4.8 only). Also, there were samples that did not receive any protein treatment but consisted only of Avicel and buffer (0.05 *M* sodium acetate buffer, pH 4.8). All samples were run in duplicate. Enzymes used in this study, i.e., Celluclast (complete cellulase system, 98.06 FPU/mL) and Novo 188 (β-glucosidase, 462.6 IU/mL) were provided by Novo Nordisk (Denmark).

### *Sugar Analysis*

Glucose concentration was measured by high-performance liquid chromatography (HPLC). The HPLC system (Dionex DX-300; Dionex, CA) was equipped with an ion-exchange PA1 (Dionex) column, a pulsed

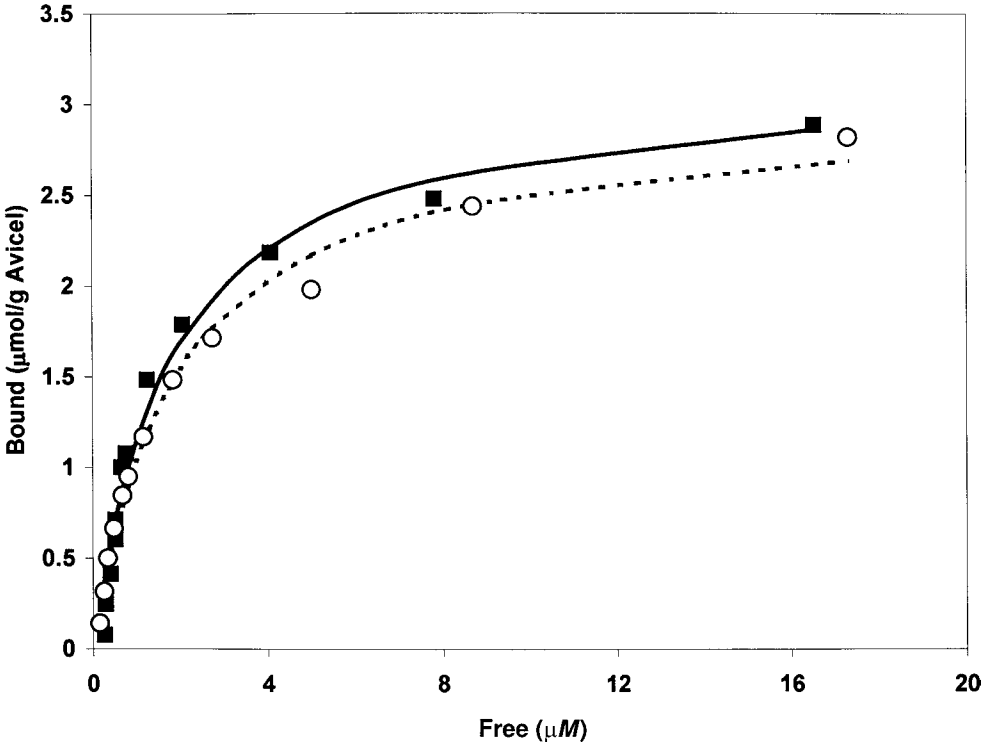



Fig. 1. Adsorption isotherms of $CBD_{Cex}$ on Avicel. Lines represent Langmuirian fit to the experimental data. Five milligrams of Avicel (1% slurry) was incubated with 500 μL of $CBD_{Cex}$ solution (1–30 μ*M*) at 4 (■) or 37°C (○) overnight. Free protein concentration was measured by UV absorption at 280 nm (ε = 0.027625 L/[μ*M* · cm]), and bound protein concentration was calculated by subtracting the free from total protein added.

amperometric detector with a gold electrode, and a Spectra AS3500 autoinjector (Spectra-Physics, CA). Prior to injection, samples were filtered through 0.45-μm HV filters (Millipore, Bedford, MA) and a volume of 20 μL was loaded. The column was equilibrated with 250 m*M* NaOH and eluted with deionized water at a flow rate of 0.8 mL/min.

### *Defibrillation Experiments*

Twenty-milligram pieces of filter paper (Whatman no. 1) were treated with 300 μL of $CBD_{Cex}$ solution (20 μ*M*) at room temperature. Samples were taken at 24 and 48 h and were analyzed for glucose content, small particle release (adsorption at 600 nm), and filter paper weight loss after being freeze-dried.

## Results and Discussion

We generated two isotherms for adsorption of $CBD_{Cex}$ on Avicel at 4 and 37°C. These isotherms (Fig. 1) revealed that temperature did not have

Table 3
Kinetic Parameters of Langmuir Equation
for Binding of $CBD_{Cex}$ to Avicel at 4 and 37°C[a]

| Substrate | $K_a$ ($M^{-1}$) | $N_o$ (mol/g Avicel) |
|---|---|---|
| Avicel | | |
| 4°C | $0.4 \times 10^6$ | $3.3 \times 10^{-6}$ |
| 37°C | $0.5 \times 10^6$ | $3.0 \times 10^{-6}$ |
| Solka floc (4°C) | $0.5 \times 10^6$ | $2.9 \times 10^{-6}$ |

[a]The experimental data were fitted to a Langmuir-type adsorption model: $[B] = [N_o]K_a[F]/1 + K_a[F]$, in which $[B]$ and $[F]$ are the concentrations of bound (mol/g) and free (mol/L) protein in the supernatant, respectively; $N_o$ is the initial concentration of binding sites (mol/g); and $K_a$ is the affinity constant (L/mol). At either 4 or 37°C, 5 mg of Avicel or Solka Floc (1% slurry) was mixed with 1–30 µ*M* $CBD_{Cex}$ (for 90 min) in 50 m*M* potassium phosphate (pH 7.0) to a final volume of 1 mL. The concentration of free protein $[F]$ was measured spectrophotometrically ($A_{280\ nm}$), and the amount of bound protein was determined by subtracting $[F]$ from the protein initially added.

an appreciable effect on the adsorption capacity of Avicel for $CBD_{Cex}$. At both temperatures (4 and 37°C), saturation was achieved with about 2.7 µmol of protein/g of Avicel. The experimental data were fitted to a Langmuir-type adsorption model:

$$[B] = [N_o]K_a[F]/1 + K_a[F]$$

in which $[B]$ and $[F]$ are the concentrations of bound (mol/gram) and free (mol/liter) protein in the supernatant, respectively. $N_o$ is the initial concentration of binding sites (mol/gram) and $K_a$ is the affinity constant (liters/mol). Table 3 presents the kinetic parameters for these two isotherms. The kinetic values also reveal that Solka Floc had a lower adsorption capacity (12% at 4°C) for the $CBD_{Cex}$ while exhibiting the same affinity ($K_a$) for the protein as Avicel.

We pretreated Avicel with saturating amounts of $CBD_{Cex}$ hoping that adsorption of this protein onto Avicel would defibrillate the substrate as observed by Din et al. *(20)* and enhance the hydrolyzability of the substrate. However, within the constraints of this study, this hypothesis could not be proven, and, in fact, the opposite was observed. As shown in Figs. 2 and 3, presaturation of Avicel with $CBD_{Cex}$ at both 4 and 37°C initially hindered the hydrolysis reaction as manifested by lower reaction rates within the first 24 h. After the first day, however, the reaction rates were restored and hydrolysis yields similar to those of controls were achieved. It seems reasonable to assume that this phenomenon is due to the irreversible binding of $CBD_{Cex}$ onto the substrate. Tomme et al. *(30)* have shown that the adsorption of $CBD_{Cex}$ to BMCC is irreversible. We also tried desorbing this protein from Avicel (results not shown) by either diluting or replacing the supernatant (at equilibrium) with buffer, but no desorption could be observed.

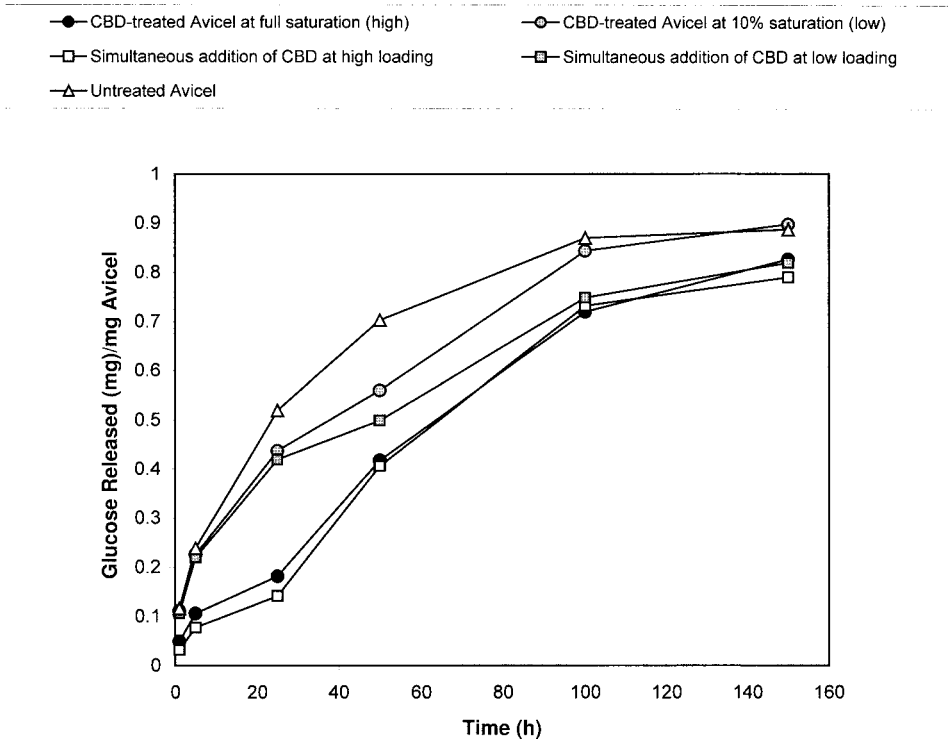
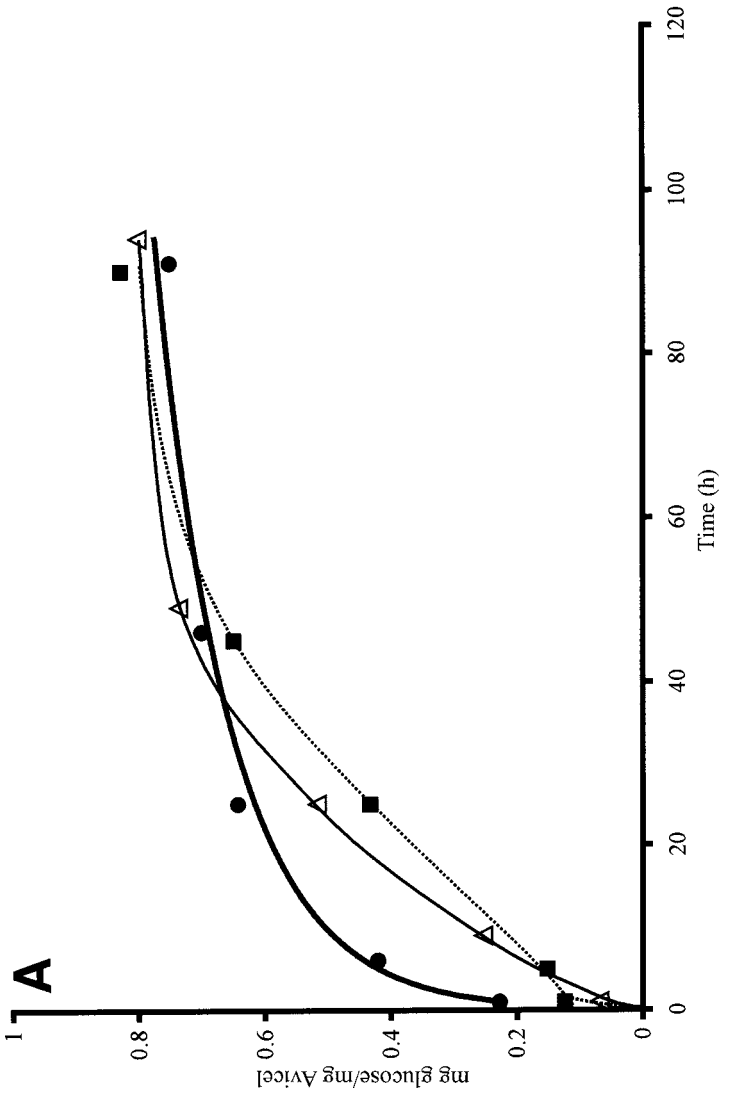



Fig. 2. Rate and extent of glucose production from enzymatic hydrolysis of untreated and $CBD_{Cex}$-treated Avicel (4°C). In simultaneous runs, $CBD_{Cex}$ and hydrolyzing enzymes were added at the same time. Prior to hydrolysis (40 FPU/g of Avicel; CBU:FPU = 2:1) with Celluclast and Novo 188 (Novo) at 45°C, Avicel was incubated at 4°C with enough $CBD_{Cex}$ solution to achieve either full saturation or 10% saturation. Glucose released was measured by HPLC.

We postulate that the presence of $CBD_{Cex}$ on the surface and within the pores limits the accessibility of the substrate by the components of the hydrolyzing enzyme (Celluclast). The gradual movement of $CBD_{Cex}$ proteins, as observed by Gilkes et al. *(29)*, or the competition between Celluclast components and the preadsorbed proteins for the same adsorption sites initiates the hydrolysis reaction although with a slower rate. This initial hydrolysis disperses the substrate and exposes new surfaces that have not been precoated with $CBD_{Cex}$. Once the available surface area is sufficiently large, the proportion of surfaces covered by $CBD_{Cex}$ becomes insignificant and the hydrolysis reaction proceeds with the same rate as with pure Avicel.

We removed the $CBD_{Cex}$ from the substrate by proteolysis so that it would not block the substrate pores and limit enzyme accessibility. Our hypothesis was that if $CBD_{Cex}$ adsorption is able to alter the pores and enhance the accessibility of cellulose structure, upon its removal using a noninvasive method such as proteolysis, the substrate should exhibit better hydrolyzability because of the increase in its pore size. However, the


A
mg glucose/mg Avicel
Time (h)
0
0.2
0.4
0.6
0.8
1
0
20
40
60
80
100
120

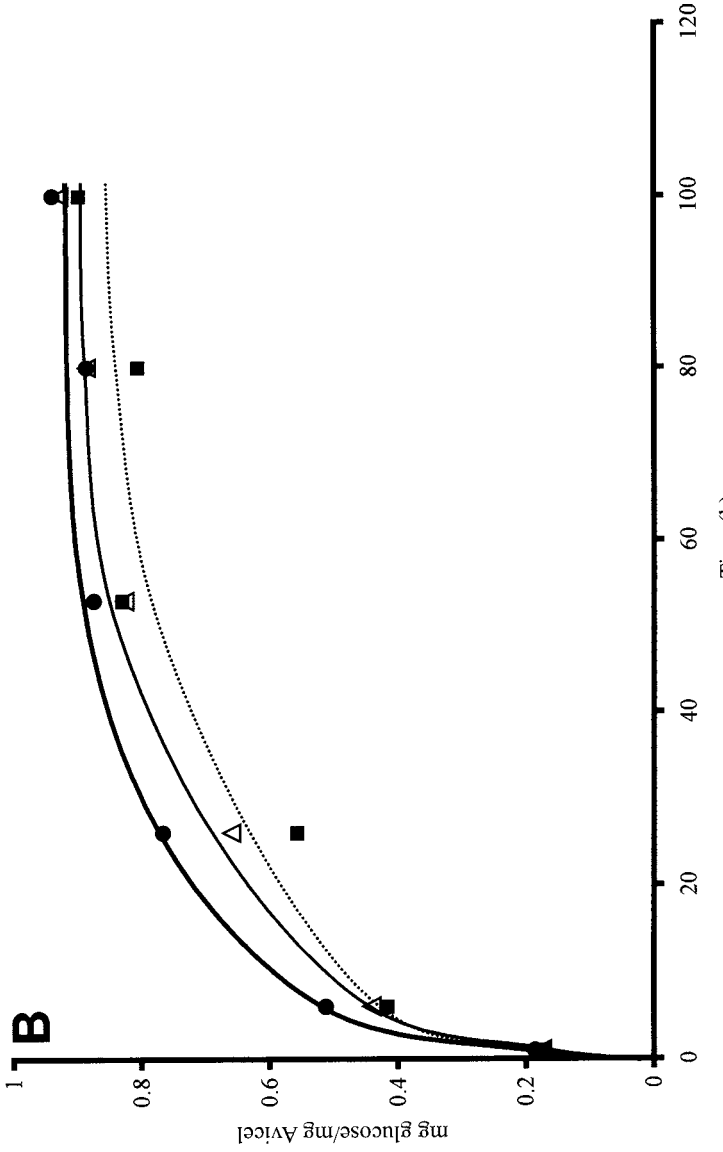



Fig. 3. Rate and extent of glucose production from enzymatic hydrolysis of untreated (——) and $CBD_{Cex}$-treated (- - ■ - - ) Avicel at 37°C. In simultaneous runs (—Δ—), $CBD_{Cex}$ and hydrolyzing enzymes were added at the same time. Prior to hydrolysis (40 FPU/g of Avicel; CBU:FPU = 2:1) with Celluclast and Novo 188 (Novo) at 45°C, Avicel was incubated at 37°C with enough $CBD_{Cex}$ solution to achieve either full saturation **(A)** or 10% saturation **(B)**. Glucose released was measured by HPLC.

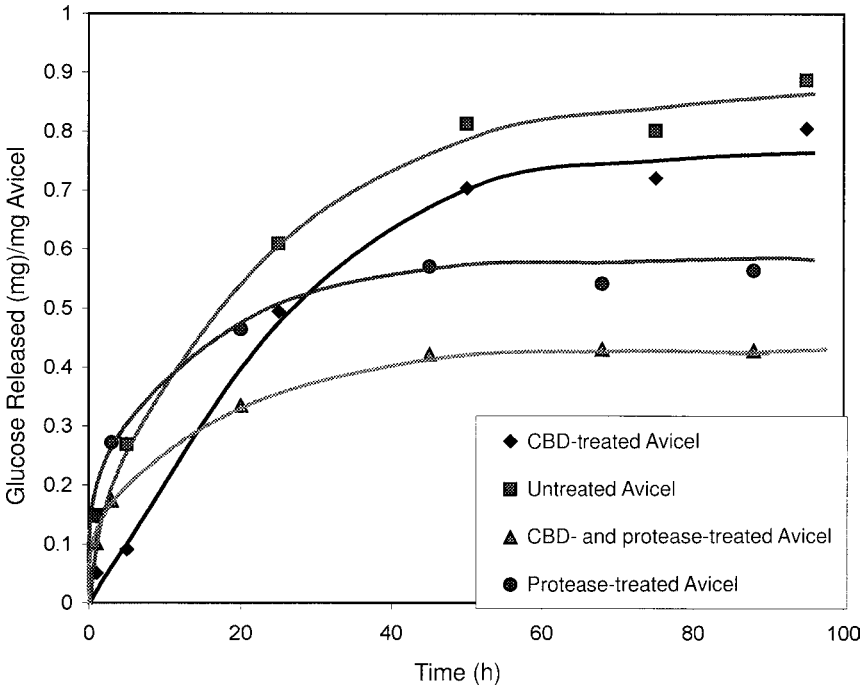



Fig. 4. Hydrolysis of Avicel treated with $CBD_{Cex}$ (20 μ*M*, 37°C, 24 h) and protease.

contrary was observed (Fig. 4). Treatment of both untreated (control) and $CBD_{Cex}$-treated Avicel with Proteinase K reduced the extent of hydrolysis significantly. This seems to be due to the entrapment of protease in the substrate and its subsequent impact on the hydrolyzing enzyme (Celluclast). Multiple washing could not remove the residual protease, and apparently its release during hydrolysis rendered Celluclast less effective. We are currently testing other chemical methods for removing the protein from Avicel to elucidate the impact of $CBD_{Cex}$ adsorption on substrate hydrolyzability.

Extended incubation of small pieces of filter paper with $CBD_{Cex}$ solution according to the protocol suggested by Banka et al. *(19)* did not release any glucose or small particles (as measured by $A_{600nm}$) into the solution. However, we observed a 2.8% weight loss in all filter paper samples treated with $CBD_{Cex}$. It is likely that the amount of fragments separated from the substrate was not significant enough to be detected by spectrophotometery.

## Conclusion

The irreversible nature of $CBD_{Cex}$ adsorption onto Avicel initially reduced the effective interaction between the substrate and Celluclast enzyme. The presence of CBD proteins reduced the rate of hydrolysis reaction within the first 24 h. After 24 h, however, these rates were restored to the control level, perhaps due to the dispersion of Avicel and creation of

new surface areas. Ultimately, the CBD-treated samples achieved near-complete hydrolysis. Removal of $CBD_{Cex}$ by proteolysis could not clearly elucidate the impact of CBD adsorption, because the presence of residual protease, which could not be removed by repeated washing, degraded the hydrolyzing enzymes and produced lower hydrolysis yields even in the control sample (Avicel treated with protease). The question that whether $CBD_{Cex}$ adsorption can enhance substrate porosity and enzymatic hydrolyzability remains unanswered. Complete removal of adsorbed protein from the substrate prior to any further investigation will be necessary. It must also be ensured that the protein removal agent will not affect the hydrolytic activity of the hydrolyzing enzyme.

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