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Adsorption and Activity of *Trichoderma reesei* Cellobiohydrolase I, Endoglucanase II, and the Corresponding Core Proteins on Steam Pretreated Willow

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Abstract

The adsorption and the hydrolytic action of purified cellulases of *Trichoderma reesei*, namely, cellobiohydrolase I (CBH I), endoglucanase II (EG II), and their core proteins, on steam-pretreated willow were compared. The two enzymes differed clearly in their adsorption and hydrolytic behavior. CBH I required the cellulose-binding domain (CBD) for efficient adsorption and hydrolysis, whereas EG II was able to adsorb to steam pretreated willow without its CBD. Absence of the CBD decreased the hydrolysis of cellulose by EG II, but the decrease was less pronounced than with CBH I. A linear relationship was observed between the amount of enzyme adsorbed and the degree of hydrolysis of cellulose only for CBH I. EG II and EG II core appeared to be able to hydrolyze only 1 to 2% of the substrate regardless of the amount of protein adsorbed.

Index Entries: Adsorption; cellulose hydrolysis; cellobiohydrolase; endoglucanase; *Trichoderma reesei*.

Introduction

Trichoderma reesei is one of the most thoroughly studied cellulolytic fungi. It produces a complete set of enzymes for efficient degradation of cellulose. Several commercial cellulase preparations are produced using

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T. reesei. Efficient degradation of cellulose requires the synergistic action of endoglucanases (EGs) (EC 3.2.1.4), which cut the cellulose chain, randomly producing glucose and cellooligosaccharides, as well as cellobiohydrolases (CBHs) (EC 3.2.1.91), which mainly liberate cellobiose units from the chain ends. The cellulase system of *T. reesei* contains at least five endoglucanases (EG I–V) and two CBHs (CBH I and II) *(1,2)*. CBHs are currently thought to attack predominantly the crystalline regions in cellulose, whereas EGs degrade mainly the more disordered and amorphous cellulose *(3)*.

Four of the EGs (EG I, II, IV, and V) and both the CBHs of *T. reesei* have a multidomain structure, like many other fungal and bacterial cellulases, consisting of a large core domain and a small cellulose-binding domain (CBD) *(1)*. The active site of the enzyme is located in the core domain, which is often also called the catalytic domain *(4–6)*. The core domain and the CBD are connected by a highly glycosylated linker peptide, which probably acts as a flexible connection between the two domains and ensures the optimal distance between them *(7)*.

In addition to being a part of native enzymes, the separate core domains also exist separately in culture filtrates produced by *T. reesei (6,8,30)*. Core domains of CBH I and II can also be produced from the corresponding native enzymes by limited papain digestion *(4)*.

Cellulose is the most abundant biopolymer in nature. Glucose obtained from cellulose can be used for the production of ethanol by fermentation *(9)*. Hydrolysis of cellulose to glucose is a challenging task because of its crystalline structure and poor degradability. However, complete hydrolysis of cellulose is necessary for feasible ethanol production. Steam pretreatment of lignocellulosic materials is an efficient method to render the cellulose more accessible to enzymes *(10,11)*.

Adsorption of cellulases on the cellulosic substrate is an essential step in hydrolysis. On the other hand, nonspecific binding may limit the amount of enzyme available for hydrolysis. Hydrolysis and adsorption studies have been performed with a variety of substrates *(12–19)*. Experiments with proteolytically cleaved core domains of CBHs from *T. reesei* have been conducted by several groups *(4,5,16,20)*. Differences in adsorption mediated by CBD have been shown to be explained by amino acid differences at the binding surface of CBD *(21)*. CBD has been demonstrated to enhance the binding and the hydrolytic capacity of CBHs in the hydrolysis of crystalline bacterial cellulose *(4,20)*. The binding of *T. reesei* cellulases on cellulose was believed to be irreversible *(16)*. However, recently it was shown that both the CBD of CBH I *(22)* and whole CBH I *(23)* bind reversibly on cellulose. The exact roles of the different parts of cellulases in the adsorption and their specific roles in the hydrolysis of the substrate are still unclear.

In most studies microcrystalline celluloses, mainly Avicel or bacterial cellulose, have been used as substrates. However, these are not potential substrates for full-scale ethanol production. Steam pretreatment is a quite efficient method for lignocellulosic materials in order to separate cellulose from hemicelluloses and to "open up" the cellulose structure for further

hydrolysis *(10,11)*. The adsorption of intact CBH I and EG II from *T. reesei* on steam-pretreated willow (SPW) was recently studied *(24)*. Since commercial *T. reesei* cellulase preparations contain, in addition to intact enzymes, the core domains of CBHs and EGs *(8)*, the adsorption of CBH I core and EG II core on SPW was studied in this work. In addition, the hydrolytic efficiencies of CBH I, EG II, and their core proteins were studied simultaneously with adsorption. The specific emphasis was on binding and initial hydrolysis by the core domains as compared to the corresponding intact enzymes.

Materials and Methods

Substrate and Enzymes

SPW was produced by the method described by Eklund et al. *(11)* and was kindly provided by Professor Guido Zacchi (Chemical Engineering, University of Lund). The dry weight of the substrate was 44%, of which 57.7% was cellulose and 40.6% lignin *(25)*. The CBH I and EG II of *T. reesei* were purified as described by Rahkamo et al. *(26)*, and the CBH I core and EG II core as described by A. Suurnäkki and colleagues *(30)*. The protein contents of the preparations used were calculated using their molar adsorption coefficients at 280 nm (CBH I = 788,000 $M^{-1} \cdot cm^{-1}$, EG II = 78,000 $M^{-1} \cdot cm^{-1}$, CBH I core = 74,000 $M^{-1} \cdot cm^{-1}$, EG II core = 64,000 $M^{-1} \cdot cm^{-1}$) *(15)*.

Hydrolysis and Adsorption Experiments

The experiments were performed with shaking in 50 m*M* sodium acetate buffer, pH 4.8, at 4° or 40°C for 90 min using a 2.5% suspension of SPW (final cellulose concentration 1%, w/v). The lower temperature was used especially for adsorption studies because hydrolysis was very slow at 4°C. After hydrolysis the samples were centrifuged (30 s, ~2000*g*) and filtered through 0.22-µm pore size filters (Millex-GV4, Millipore, Bedford, MA) before analysis of the free enzyme and hydrolysis products. The filtered samples were divided into two parts, one of which was immediately analyzed for the amount of free enzyme, and the other was analyzed for the solubilized cellooligosaccharides. Hydrolysis was terminated by heating the sample for oligosaccharide analysis in a boiling water bath for 5 min.

Analysis of Hydrolysis Products by High-Performance Liquid Chromatography

The amounts of glucose, cellobiose, and cellotriose were measured by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA). Five millimolar H_2SO_4 was used as eluent with a flow rate of 0.6 mL/min at 65°C. An RI-detector (ERC-7515A, Erma Cr., Tokyo, Japan) and the HPLC Manager program (Pharmacia, Uppsala, Sweden) were used for detection and data

analysis, respectively. The degree of hydrolysis (conversion) was calculated as the total amount of glucose units in the soluble cellooligosaccharides (G_1–G_3) produced from the theoretical amount of glucose moieties in the substrate.

Quantification of Proteins by Fast Protein Liquid Chromatography (FPLC)

The amount of free proteins in the solution was determined by anion-exchange chromatography (Mono Q column, Pharmacia) at pH 7.6 as described previously *(27)*.

Results and Discussion

Adsorption

At 4°C CBH I bound well on the SPW (Fig. 1A). The enzyme was bound almost totally at low concentrations (<4 μ*M*), and 52% of the CBH I was bound even at the highest concentration studied. Adsorption of CBH I was clearly temperature dependent, and 20% less CBH I bound to the substrate at 40°C than at 4°C, with the highest enzyme concentration studied (32 μ*M*). At low enzyme concentrations (below 8 μ*M*) the amount of bound enzyme was only slightly less at 40°C than at 4°C. The adsorption of the CBD of CBH I has also been found to be temperature dependent, with increased affinity at lower temperatures *(22)*.

At low temperature (4°C) the amount of CBH I core bound was half that of CBH I. Surprisingly, only a small portion of CBH I core adsorbed to the substrate at 40°C. Thus, it seems that at 4°C, when hydrolysis is very slow, the CBH I core might be bound to the substrate by its active site and would apparently only "sit" on the substrate. When the temperature is increased to 40°C, CBH I core hydrolyzes itself from the substrate because the major part of the CBH I core is not bound to the substrate.

Previous studies on CBH I core (produced by papain degradation of whole CBH I) have shown that the core protein binds on bacterial cellulose at 4°C *(20)*, on Avicel cellulose at 20°C *(5,14)*, and on filter paper at 50°C *(16)*; but the amount of protein bound is clearly less than that of the intact CBH I. However, the protein concentration used in these experiments was lower than the concentrations used in this study. Nidetzky et al. *(16)* used 1.25 μ*M* solutions of CBH I and CBH I core with 20 g/L of filter paper. The low amounts of CBH I core also adsorbed in our experiments at 40°C, but the amount of protein adsorbed did not increase by increasing the CBH I core concentration above about 4 μ*M* (Fig. 1A). Similar behavior was also observed by Reinikainen et al. *(20)* on bacterial cellulose.

The amount of EG II bound correlated almost linearly with the amount of the enzyme applied to SPW (Fig. 1B). At high protein concentrations, the adsorption was clearly higher at the higher temperature, which is opposite to the adsorption of CBH I. This result is in accordance with the results of earlier studies on the adsorption on EG II of SPW *(24)*.

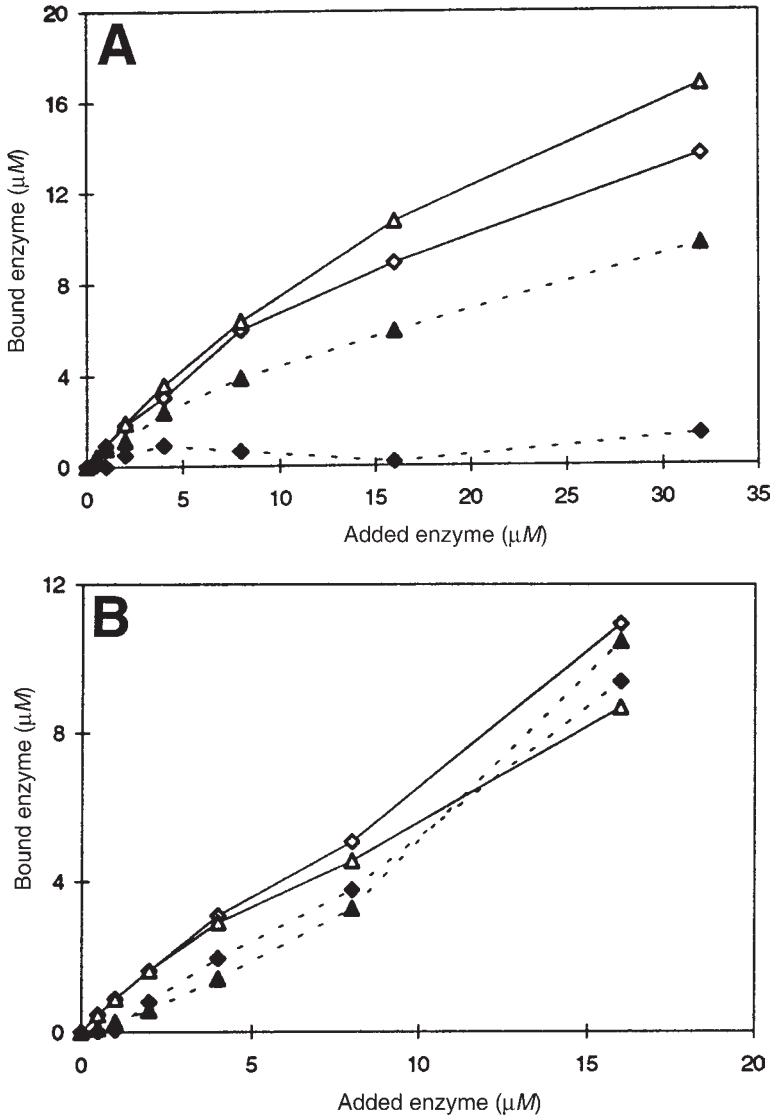

Fig. 1. The adsorption of CBH I and CBH I core **(A)** and EG II and EG II core **(B)** on SPW at 4 and 40°C in 90-min incubation. The final cellulose concentration used in the experiments was 10 g/L. For (A): (—◇—), CBH I 40°C; (– –◆ – –), CBH I core 40°C; (—△—), CBH I 4°C; (– –▲ – –), CBH I core 4°C. For (B): (—◇—), EG II 40°C; (– –◆ – –), EG II core 40°C; (—△—), EG II 4°C; (– –▲ – –), EG II core 4°C.

There appeared to be only small differences in the binding of EG II and EG II core. EG II core behaved clearly differently than the CBH I core: EG II core adsorbed at 4°C to the same extent as at 40°C, and the binding correlated to the amount of the added core protein analogously to the intact enzyme. Absence of the CBD of EG II has previously been reported to have a marked effect on binding of the enzyme to Avicel cellulose at 0°C *(6)* or

to filter paper at 50°C *(16)*. However, the protein concentrations studied were much lower than in our study. In fact, the binding of EG II in low protein concentrations was much lower than that of EG II core in our study as well. The substrate used in our study was not pure cellulose, and it contained a significant amount (40.6%) of lignin, which might bind proteins unspecifically. Earlier studies have shown that endoglucanases of *T. reesei* adsorb to lignin *(12,28)*.

Hydrolysis of Cellulose

The degree of hydrolysis was analyzed from the same samples as the adsorption. After incubation, the hydrolysate was divided in half and the amount of soluble cellooligosaccharides formed was analyzed from one-half of the sample by HPLC. Production of soluble sugars describes the activity of especially CBHs. With EGs it does not measure the whole activity, since the enzyme's function is partially the production of free cellulose chain ends rather than soluble sugars. However, the production of soluble cellooligosaccharides is commonly used to determine the activity of EGs. Often β-glucosidase is added together with CBHs and EGs in order to prevent the end-product inhibition by cellooligosaccharides. In a previous study, it was observed that addition of β-glucosidase enhanced the action of CBH I on SPW but not that of EG II *(24)*. However, β-glucosidase was not added in the present study, because the main aim was to compare the action of the core domains and the corresponding intact enzymes.

The CBH I core and EG II core purified from the culture filtrate of *T. reesei* have the same activity per mole of protein on soluble substrates as intact CBH I and EG II, but they act clearly more slowly on amorphous and microcrystalline cellulose than the intact enzymes *(30)*. For both enzymes, the lack of CBD restricted the hydrolysis of microcrystalline bacterial cellulose more than the hydrolysis of amorphous cellulose.

CBH I produced soluble sugars from the substrate more efficiently than EG II. When 3.2 μmol of CBH I was added per gram of cellulose, about 7% of the cellulose was solubilized during 90 min of hydrolysis at 40°C (Fig. 2A). The amount of cellulose solubilized at 40°C by EG II was less than half that obtained by CBH I (Fig. 2B). However, lack of the CBD had a drastic effect on the action of CBH I, and only about 1% of the substrate was hydrolyzed by CBH I core even when high-protein concentrations were used. The absence of the CBD also had an effect on the action of EG II core, but the effect was much smaller than in the case of CBH I core. CBH I was active also at 4°C, and the degree of hydrolysis was close to that of CBH I core at 40°C.

Up to an enzyme concentration of 1 μ*M*, EG II, EG II core, and CBH I core produced approximately as much soluble sugars as CBH I. This might be explained by the assumption that about 1% of cellulose in SPW is in a form that is easily hydrolyzable by cellulolytic enzymes.

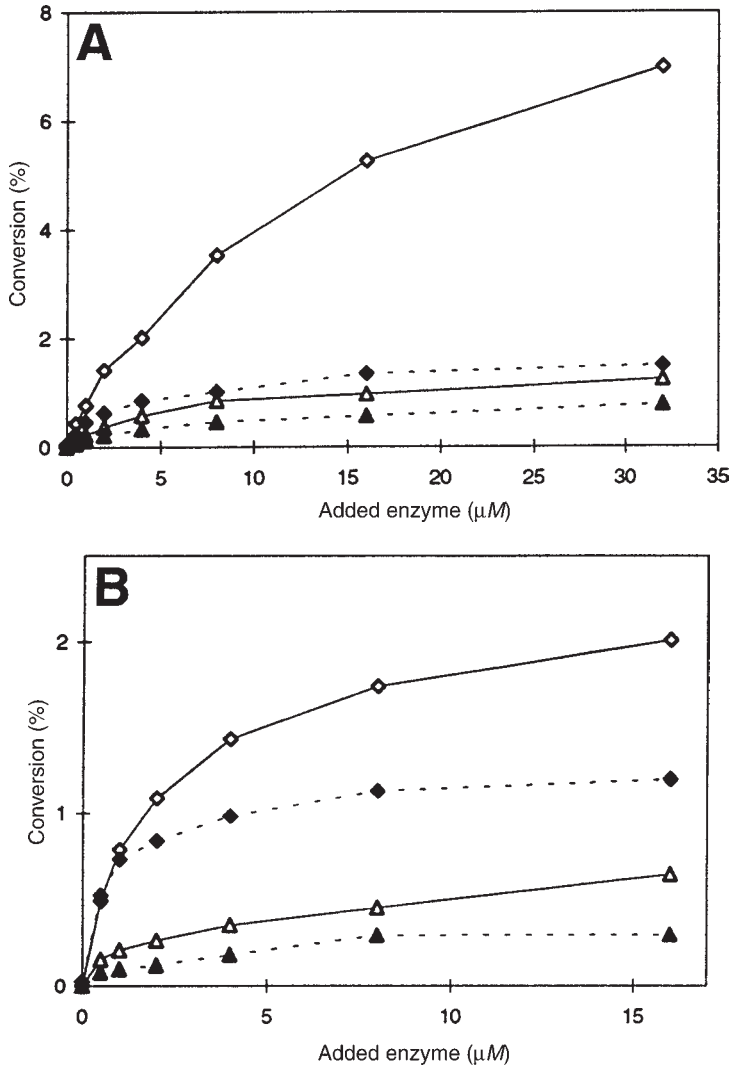

Fig. 2. Hydrolysis of SPW with CBH I and CBH I core **(A)** and EG II and EG II core **(B)** at 4 and 40°C. Hydrolysis time was 90 min and initial cellulose concentration was 10 g/L. For (A): (—◇—), CBH I 40°C; (– –◆– –), CBH I core 40°C; (—△—), CBH I 4°C; (– –▲– –), CBH I core 4°C. For (B): (—◇—), EG II 40°C; (– –◆– –), EG II core 40°C; (—△—), EG II 4°C; (– –▲– –), EG II core 4°C.

Hydrolysis as a Function of Adsorption

CBH I showed a linear correlation between the amount of bound enzyme and the hydrolysis of cellulose (Fig. 3). With CBH I core, the hydrolysis of the substrate was very low, and even the highest amount of bound CBH I core did not hydrolyze the substrate efficiently. However, the small amount of the bound CBH I core seemed to be able to release soluble oligomers more efficiently than the corresponding amount of bound CBH I.

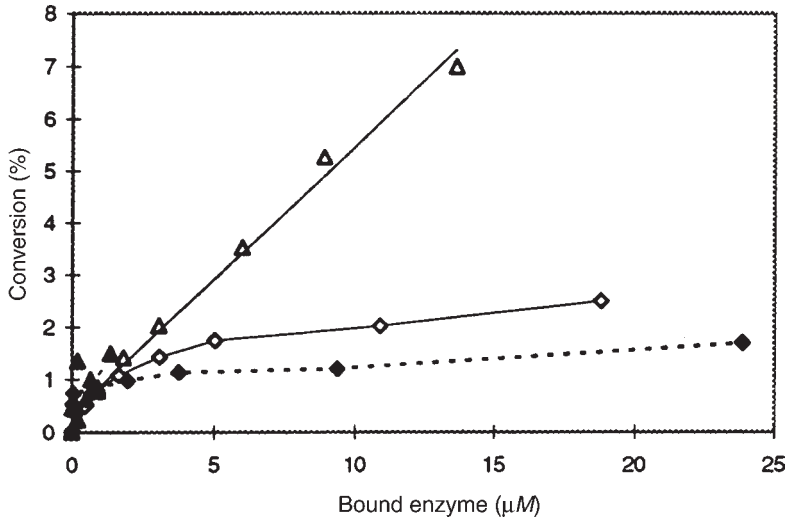

Fig. 3. Hydrolysis of SPW as a function of the amount of enzyme adsorbed. Incubation was at 40°C for 90 min using an initial cellulose concentration of 10 g/L. (—◇—), EG II; (– –◆ – –), EG II core; (—△—), CBH I; (– –▲ – –), CBH I core.

This might be owing to the presence of a small noncrystalline easily hydrolyzable cellulose in SPW that is attacked more readily by CBH I core because it does not have a CBD to target the enzyme to a more crystalline part of cellulose. The crystallinity of SPW was 72.8 as determined by X-ray diffractometry *(29)*. By comparing the results of adsorption and hydrolysis (Figs. 1A and 2A), it seems obvious that CBH I must be bound to cellulose by its CBD for an efficient hydrolysis. This has also been found using papain-produced CBH I core *(4–6,16)*.

With EG II and EG II core, the amount of bound enzyme correlated linearly with conversion of the substrate at 40°C and at low enzyme dosages (0.8 μmol/g). However, an increase in the amount of bound EG II or EG II core did not notably enhance the hydrolysis of cellulose in concentrations of adsorbed protein above about 4 μ*M*. Possibly part of the EG II was nonproductively bound to the substrate. It seems that only 1 to 2% of the cellulose present in SPW was hydrolyzable by EG II, and that additional enzyme therefore did not further hydrolyze the substrate (Figs. 2B and 3). The access of EG II to the amorphous parts of cellulose may be restricted either because of physical barriers (e.g., lignin) or by the crystalline structure of cellulose. The production of soluble sugars does not describe the action of EG in the best possible way. Additional information on the action of EGs could also be obtained by determination of the amount of free chain ends formed in the cellulose or by analyzing the decrease in the degree of polymerization of cellulose. Previously it has been found that up to a degree of hydrolysis of about 1%, EG II is somewhat more efficient in the hydrolysis of SPW than CBH I *(24)*.

Conclusion

Both the enzymes of *T. reesei*, CBH I and EG II, used in this work share a common structure consisting of two separate domains, a catalytic domain and a CBD. CBH I clearly needed a CBD for efficient adsorption to and hydrolysis of cellulose, whereas EG II adsorbed to SPW rather well without its CBD. The lack of a CBD in EG II core decreased the degree of hydrolysis by only about 35%. The enzymes might not only bind to the cellulose in SPW, but also unspecifically to lignin. The binding of these enzymes to lignin and the synergistic action between intact and core proteins in the hydrolysis of SPW should be studied in future work.

Acknowledgments

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