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Hydrolysis of Steam-Pretreated Lignocellulose

Synergism and Adsorption for Cellobiohydrolase I and Endoglucanase II of Trichoderma reesei

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Abstract

The mechanism of hydrolysis of cellulose is important for improving the enzymatic conversion in bioprocesses based on lignocellulose. Adsorption and hydrolysis experiments were performed with cellobiohydrolase I (CBH I) and endoglucanase II (EG II) from *Trichoderma reesei* on a realistic lignocellulose substrates: steam-pretreated willow. The enzymes were studied both alone and in equimolar mixtures. Adsorption isotherms were determined at 4 and 40°C during 90-min reaction times. Both CBH I and EG II adsorbed stronger at 40 than at 4°C. The time course of adsorption and hydrolysis, 3 min to 48 h, was studied at 40°C. About 90% of the cellulases were adsorbed within 2 h. The hydrolysis rate was high in the beginning but decreased during the time course. Based on adsorption data, the hydrolysis and synergism were analyzed as function of adsorbed enzyme. CBH I showed a linear correlation between hydrolysis and adsorbed enzyme, whereas for EG II the corresponding curve leveled off at both 4 and 40°C. At low conversion, below 1%, EG II produced as much soluble sugars as CBH I. At higher conversion, CBH I was more efficient than EG II. The synergism as function of adsorbed enzyme increased with bound enzyme before reaching a stable value of about 2. The effect of varying the ratio of CBH I:EG II was studied at fixed total enzyme loading and by changing the ratio between the enzymes. Only a small addition (5%) of EG II to a CBH I solution was shown to be sufficient for nearly maximal synergism. The ratio between EG II and CBH I was not critical. The ratio 40% EG II:60% CBH I showed similar conversion to 5% EG II:95% CBH I. Modifications of the conventional endo-exo synergism model are proposed.

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Index Entries: *Trichoderma reesei*; cellulase; cellulose hydrolysis; lignocellulose; cellobiohydrolase; endoglucanase; synergism; adsorption.

Introduction

Cellulose is one of the most abundant biopolymers on the earth. Utilizing this energy source efficiently would give a positive environmental effect: cellulose combustion does not give a net increase in atmospheric CO_2, i.e., no greenhouse effect. One way to utilize cellulose is to hydrolyze it to glucose and then ferment the glucose to ethanol for use as liquid fuel. Cellulose can be hydrolyzed both with acids and with enzymes. Successful attempts have been made to steam pretreat willow to facilitate the enzymatic hydrolysis *(1)*. The drawback so far with enzymatic hydrolysis of cellulose is that it is a slow process, even with optimized substrates. Speeding up the hydrolysis would make the ethanol production from cellulose more economical, and, therefore, increased knowledge of the mechanism of cellulases is essential.

Cellulases are classified as endoglucanases (EGs), which hydrolyze internal β-1,4-bonds, and cellobiohydrolases (CBHs), which hydrolyze the cellulose chain from an end and produce cellobiose as the main product. *Trichoderma reesei*, which has one of the most well-studied cellulase systems, produces at least seven genetically different cellulases *(2,3)*: two cellobiohydrolases (CBH I and CBH II; EC 3.2.1.91) and five endoglucanases (EG I, EG II, EG III, EG IV, and EG V; EC 3.2.1.4). The domain structure for the different cellulases of *T. reesei* is similar. The cellulases consist of a catalytic domain and a cellulose-binding domain (CBD) that are connected with a linker region. The structure of the catalytic domain of CBH I and CBH II is known *(4,5)* as well as the structure of the CBD of CBH I *(6)*. CBH I and CBH II both have the active site in a tunnel. The structure of the catalytic domain of EG I has recently been published *(7)*. The active site of EG I is an open cleft instead of a tunnel. This explains why EG I is able to hydrolyze internal β-1,4-bonds in the cellulose chain. EG III is an exception to the common domain structure; it consists of only a catalytic domain.

The cellulases from *T. reesei* have been shown to hydrolyze cellulose cooperatively; that is, they act in synergism *(8–10)*. There are two kinds of synergism: exo-exo, which is observed between CBH I and CBH II *(11,12)*; and endo-exo, which can be seen between an EG and a CBH. For the latter, it is believed that the EG by cleavage of the cellulose chain produces new free ends to which the CBH can bind and start to hydrolyze from either a reducing end (CBH I) or a nonreducing end (CBH II) *(2,13)*.

We have developed an experimental approach to follow adsorption and hydrolysis simultaneously. We use fast protein liquid chromatography (FPLC) to monitor enzyme adsorption and high-performance liquid chromatography (HPLC) to analyze soluble sugars liberated from the substrate. Two binary enzyme mixtures, CBH I–CBH II *(9)* and CBH I–EG II *(14)*, have so far been investigated for adsorption and hydrolysis properties on microcrystalline cellulose (Avicel).

We studied adsorption and hydrolysis of CBH I and EG II from *T. reesei* on steam-pretreated willow (SPW). The aim of our studies was to more thoroughly understand the enzymatic hydrolysis of this substrate. We were particularly interested in the adsorption and hydrolysis properties of the enzymes. Furthermore, we were interested in getting a more detailed view of the synergism between CBH I and EG II during the hydrolysis of SPW. SPW is a lignocellulosic substrate that has been treated to facilitate the hydrolysis. This substrate has been used for total hydrolysis experiments with culture filtrates from *T. reesei (1)* and is thus a putative substrate for producing ethanol from wood.

Materials and Methods

Steam-Pretreated Willow

The substrate, SPW, was a kind gift from Prof. Guido Zacchi, Chemical Engineering I, Lund Institute of Technology. It was prepared according to Réczey et al. *(15)* by preimpregnation with 1% (w/w) SO_2 followed by pretreatment at 206°C for 6 min. SPW contains 57.7% cellulose and 40.6% lignin, calculated on dry material. A substrate concentration of 17.3 g/L calculated on dry SPW was used, which corresponds to 10 g/L of cellulose and 7.1 g/L of lignin.

CBH I and EG II

For the experiments with no addition of β-glucosidase, CBH I and EG II were purified according to Medve et al. *(16)*. For all experiments in which β-glucosidase was present, Dr. Maija Tenkanen at VTT, Espoo, Finland, kindly provided the CBH I and EG II *(17)*. The enzyme concentrations were determined with a spectrophotometer by measuring the absorption at 280 nm using the following molar absorption coefficients: 78,800 $M^{-1}\cdot cm^{-1}$ CBH I, 78,000 $M^{-1}\cdot cm^{-1}$ EG II *(18)*. The enzyme concentration is given as micromoles of enzyme per gram of cellulose.

β-*Glucosidase*

β-Glucosidase was purified from *A. niger* Novozyme (Novo, Bagsvaerd, Denmark) in two chromatography steps. The first was an anion-exchange step with Source Q (Pharmacia, Uppsala, Sweden), using 20 m*M* piperazine (pH 5.0) as buffer A and 20 m*M* piperazine plus 1 *M* NaCl (pH 5.0) as buffer B. β-Glucosidase was eluted with a linear gradient of buffer B. The second step was gel filtration on a Hiload™ 16/60 (Pharmacia) using 0.1 *M* Na-acetate plus 0.2 *M* NaCl (pH 5.2) as buffer. The amount of β-glucosidase needed in the hydrolysis experiments was determined by experiments with two concentrations of CBH I applying increasing amounts of β-glucosidase. The CBH I concentrations were 2 and 0.1 μmol/g. The substrate (SPW) was hydrolyzed for 90 min at 40°C, and the reaction was stopped by filtering the nonhydrolyzed substrate. The hydrolysate was then analyzed for soluble sugars, glucose, cellobiose, and cellotriose.

Adsorption and Hydrolysis Studies

The adsorption and hydrolysis experiments were performed and analyzed according to Medve et al. *(14,16)* with the exception that Turbochrome 4 (Perkin-Elmer, Norwalk, CT) was used to evaluate the chromatograms. The experimental setup was as follows: the hydrolysis was performed in 1.8-mL screw-cap tubes in a tailor-made mixing system. The reaction was terminated by filtering off the nonhydrolyzed substrate (with bound enzyme) from the soluble sugars and free enzymes. The filtrate was then analyzed for soluble sugars, glucose, cellobiose, and cellotriose, with an HPLC system (Pharmacia) using an HPX-87H column (Bio-rad, Hercules, CA) and 2.5 m*M* H_2SO_4 as eluent. Cellulases, EG II, and CBH I were quantitatively determined by anion-exchange chromatography in an FPLC system (Pharmacia) with a Mono Q column. We could then calculate the amount of adsorbed enzyme and conversion of the substrate.

Isotherms for Adsorption and Hydrolysis

Experiments were performed in either a cold room (4°C) or a water bath (40°C). The enzyme concentrations were between 0.04 and 2.5 μmol/g. The reaction time was 90 min. The experiments were performed both with and without β-glucosidase present (1 nmol/g of substrate). CBH I and EG II were added alone and in equimolar mixtures.

Time Course for Adsorption and Hydrolysis

Experiments were performed at 40°C, with an enzyme concentration of 0.15 μmol/g. The reaction time was varied from 3 min to 48 h. The experiments were performed with β-glucosidase present (1 nmol/g of substrate). CBH I and EG II were added alone and in equimolar mixtures.

Optimal Ratio for Synergy and Hydrolysis

For studies to determine the optimal ratio between CBH I and EG II, the enzymes were added alone and in mixtures with different molar ratios. The percentage ratios of CBH I:EG II were 100:0, 95:5, 90:10, 75:25, 60:40, 30:70, 10:90, and 0:100. The experiments were performed at three different total enzyme loadings (0.08, 0.4, and 1.2 μmol/g). The reaction time was 90 min and the temperature was 40°C. The β-glucosidase concentration was 1 nmol/g.

Synergism

The degree of synergism is defined as the hydrolysis when the enzymes are together divided by the sum of the hydrolysis for the enzymes alone. Thus, this is a way of showing the degree of cooperativity for a given set of enzymes. The synergism was determined both as a function of added total enzyme and as a function of adsorbed enzyme. The latter case was based on adsorption and hydrolysis data from experiments with enzymes alone and in equimolar mixtures (*see* Results).

Table 1
Effect of Addition of β-Glucosidase
on Sugar Production by CBH I During Hydrolysis of SPW[a]

Enzymes		Sugars produced	
CBH I (μmol/g)	β-Glucosidase (nmol/g)	Cellobiose (m*M*)	Glucose (m*M*)
0	0	0	0
0	1	0	0.02
0	2	0	0.01
0.1	0	0.24	0.12
0.1	1	0.01	0.62
0.1	2	0.01	0.66
2	0	1.84	0.57
2	1	0.21	3.97
2	2	0.10	3.97

[a]SPW, 10 g/L of cellulose, was hydrolyzed with different amounts of CBH I and β-glucosidase. The reaction time was 90 min and the reaction temperature was 40°C.

Results

Addition of β-Glucosidase

Cellobiose is a strong inhibitor of CBHs *(19)*. Cellobiose is hydrolyzed to glucose by β-glucosidase. To avoid product inhibition during the hydrolysis studies, β-glucosidase was added. The influence of the β-glucosidase concentration was studied to determine the lowest amount that gave a sufficient decrease in cellobiose accumulation. A reaction time of 90 min and a temperature of 40°C were used, and CBH I was at a concentration of 0.1 and 2 μmol/g (g of cellulose in SPW) (*see* Table 1). With a concentration of 2 μmol/g of CBH I, the cellobiose concentration was 1.84 m*M* without β-glucosidase compared with 0.21 m*M* with the addition of 1 nmol/g of β-glucosidase. For CBH I at 0.1 μmol/g, the minimum concentration of β-glucosidase used was 1 nmol/g. Above this level an increase in β-glucosidase did not show a decrease in cellobiose accumulation (Table 1). It was always possible to detect a low amount of cellobiose even when high concentrations of β-glucosidase were used. The cellobiose: glucose molar ratio could never be reduced below 0.015 even when β-glucosidase was included. Without β-glucosidase present, this molar ratio was between 2 and 3. Based on these results, we chose to include β-glucosidase in the hydrolysis experiments at a level of 1 nmol/g of cellulose. β-Glucosidase has a weak adsorption to SPW, but the CBH I and EG II concentrations used in the experiments were between 40 and 2500 times higher than the β-glucosidase concentration. We therefore regard the β-glucosidase adsorption as negligible. Studies of isotherms for adsorption and hydrolysis were performed both with and without β-glucosidase present in order to observe the qualitative effect of cellobiose, which is the main product in

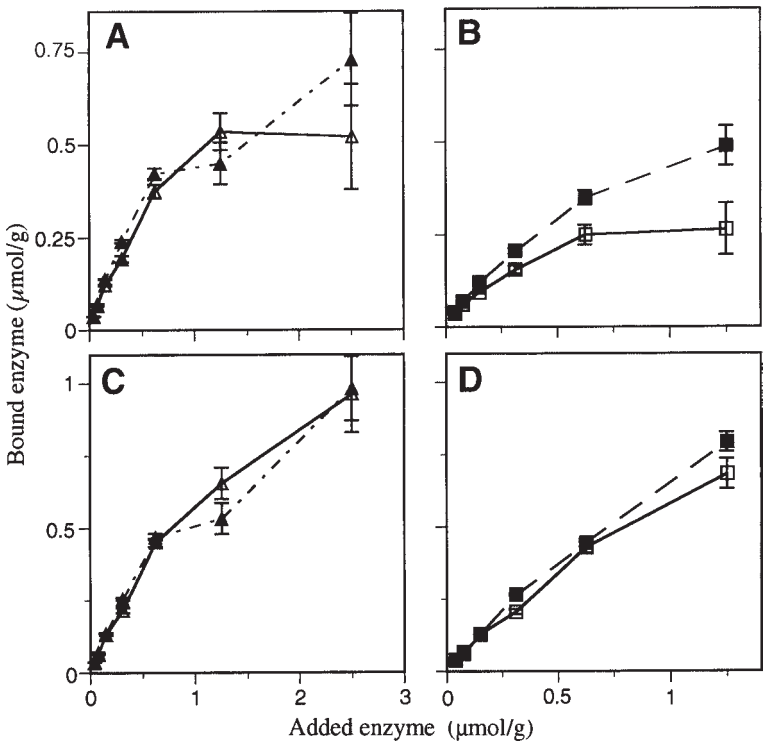

Fig. 1. Adsorption isotherms of CBH I and EG II on SPW, 10 g/L of cellulose, with a reaction time of 90 min. The enzyme concentrations were from 0.04 to 2.5 μmol/g, and the enzymes were added alone and in equimolar mixtures. **(A)** CBH I at 4°C; **(B)** EG II at 4°C; **(C)** CBH I at 40°C; **(D)** EG II at 40°C. ▲, CBH I alone; ■, EG II alone; △, CBH I in an equimolar mixture with EG II; □, EG II in an equimolar mixture with CBH I.

cellulose hydrolysis by *T. reesei* cellulases. In the majority of the presented experiments, β-glucosidase was added.

Adsorption of CBH I and EG II

Isotherms for adsorption were determined by increasing the enzyme concentration and keeping a fixed concentration of SPW. The adsorption was studied at 4 and 40°C, and the enzymes were added alone and in equimolar mixtures. An increase in adsorption with an increase in temperature was observed for both enzymes (*see* Fig. 1A–C and Fig. 1B–D, respectively). The effect was more pronounced for EG II, although the adsorption of CBH I was clearly affected as well. The same observation was also made without the presence of β-glucosidase (data not shown). The adsorption of EG II was reduced by the presence of CBH I at 4°C, but was only slightly affected at 40°C. CBH I was not affected by the presence of EG II at either temperature, with the exception of the highest concentration at 4°C. A higher amount of CBH I was bound compared to EG II at 4°C, whereas both enzymes were bound equally at 40°C.

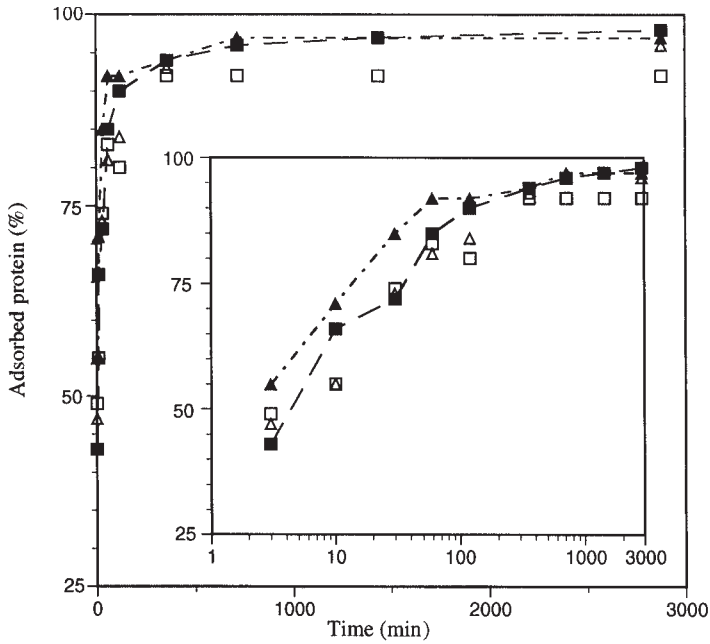

Fig. 2. Time course for adsorption of CBH I and EG II to SPW at 40°C. Enzyme concentrations were 0.15 μmol/g and cellulose concentration was 10 g/L. Reaction times were from 3 min to 48 h. Inset is identical to the major figure except for the logarithmic *x*-axis. Adsorption was determined for the enzymes alone and in equimolar mixtures. ▲, CBH I alone; ■, EG II alone; △, CBH I in an equimolar mixture with EG II; □, EG II in an equimolar mixture with CBH I.

The time course for adsorption of CBH I and EG II to SPW was studied during substrate hydrolysis. In the time course experiments, a fixed enzyme concentration (0.15 μmol/g), and reaction temperature (40°C) were used when the reaction time was varied from 3 min to 48 h. The enzymes were studied alone and in equimolar mixtures (Fig. 2). After 3 min only 50% of the enzymes was adsorbed. Initially CBH I bound stronger than EG II, but after 2 h the adsorption was quite similar at about 90% for both of the enzymes alone. The adsorption of CBH I was not affected by the presence of EG II, whereas EG II showed a slightly decreased maximal bound amount (from 95 to 90%) when CBH I was present.

Hydrolysis as Function of Adsorbed Enzyme

For each sample, both the amount of adsorbed enzyme and the hydrolysis of the substrate were determined (*see* Fig. 3). For CBH I, a linear relationship between conversion and bound enzyme at 4 and 40°C was observed (Fig. 3A,C). The same observation could be made in experiments without β-glucosidase, with the difference that the slope of the fitted line was lower when compared with experiments in which β-glucosidase was present. When comparing the 4 and 40°C experiment, a much steeper slope was obtained at 40°C. The corresponding data for EG II were significantly

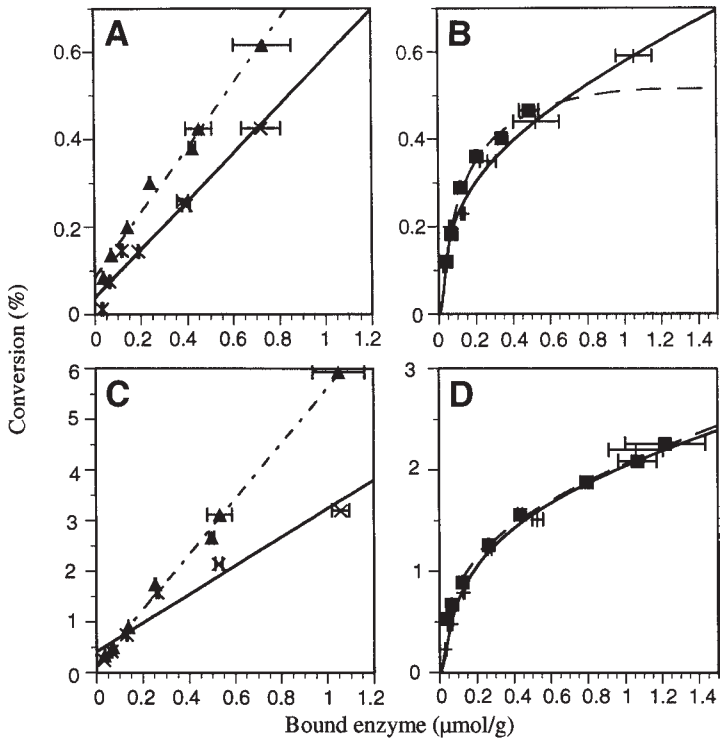

Fig. 3. Hydrolysis of SPW per adsorbed CBH I and EG II with and without β-glucosidase present, with a reaction time of 90 min. The CBH I and EG II concentrations added varied from 0.04 to 2.5 μmol/g, the cellulose concentration was 10 g/L, and the β-glucosidase concentration was 1 nmol/g. **(A)** CBH I at 4°C; **(B)** EG II at 4°C; **(C)** CBH I at 40°C; and **(D)** EG II at 40°C. ▲, CBH I with β-glucosidase; ■, EG II with β-glucosidase; ×, CBH I without β-glucosidase; +, EG II without β-glucosidase.

different (Fig. 3B,D). The conversion curve for EG II started to level off at a concentration between 0.15 and 0.3 μmol/g at both temperatures. The presence of β-glucosidase did not significantly influence the hydrolytic activity of EG II. When comparing the two enzymes at 4°C, EG II was slightly more efficient in hydrolyzing cellulose than CBH I. The same comparison at 40°C showed that EG II was more efficient than CBH I at low conversion, below 1%, but CBH I was the more efficient enzyme at higher conversion.

Synergism During Hydrolysis

The conversion of SPW was studied with the enzymes alone and together at different enzyme concentrations and at two different temperatures, 4 and 40°C (Fig. 4). We observed that the sum of conversion for EG II and CBH I alone was significantly lower than the experimentally found conversion when the enzymes were added together; that is, the enzymes acted in synergism. Synergism is defined as the ratio between the experimentally found conversion and the theoretical conversion. Significant synergism was observed at 4°C, with values above 1.5, as well as at 40°C, with values above 2.0. The synergism was clearly higher in the experiment at 40°C.

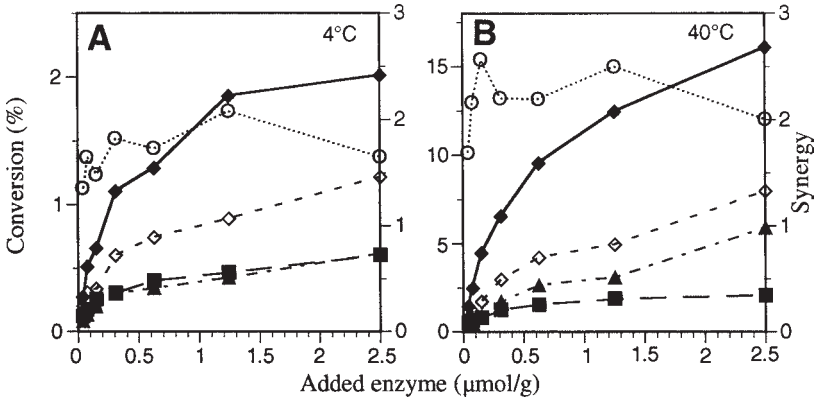

Fig. 4. Hydrolysis and synergism for CBH I and EG II, calculated for added enzyme. The enzyme concentrations were from 0.04 to 2.5 μmol/g. The reaction time was 90 min and cellulose concentration was 10 g/L. Experiments were performed at **(A)** 4°C and **(B)** 40°C. ▲, CBH I alone; ■, EG II alone; ◇, CBH I + EG II calculated; ◆, CBH I + EG II experimental; ○, synergism.

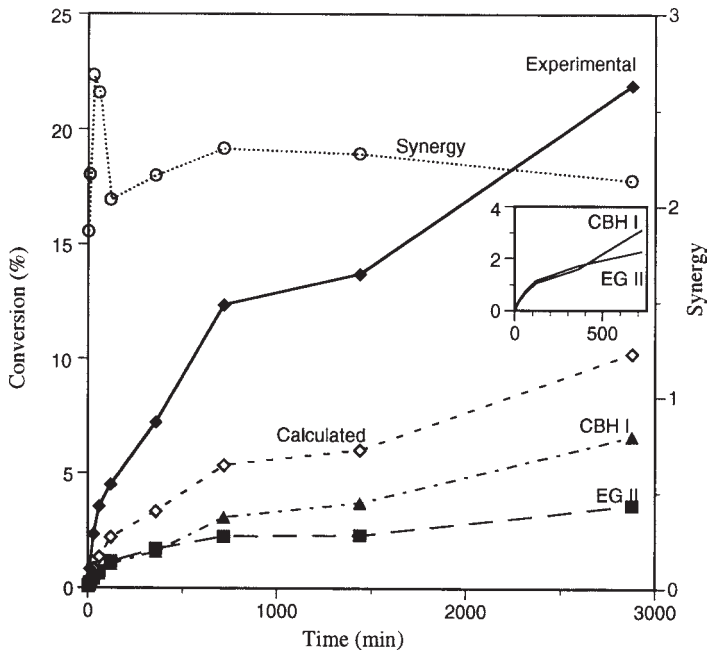

Fig. 5. Time course of hydrolysis by CBH I and EG II at 40°C. The enzyme concentrations were 0.15 μmol/g and the substrate concentration was 10 g/L. The reaction times were from 3 min to 48 h. ▲, CBH I alone; ■, EG II alone; ◇, CBH I + EG II calculated; ◆, CBH I + EG II experimental; ○, synergism.

The time course of SPW hydrolysis was also studied (Fig. 5). The enzyme concentration was kept constant at 0.15 μmol/g, and the reaction time was from 3 min to 48 h. A significant synergism was also observed in

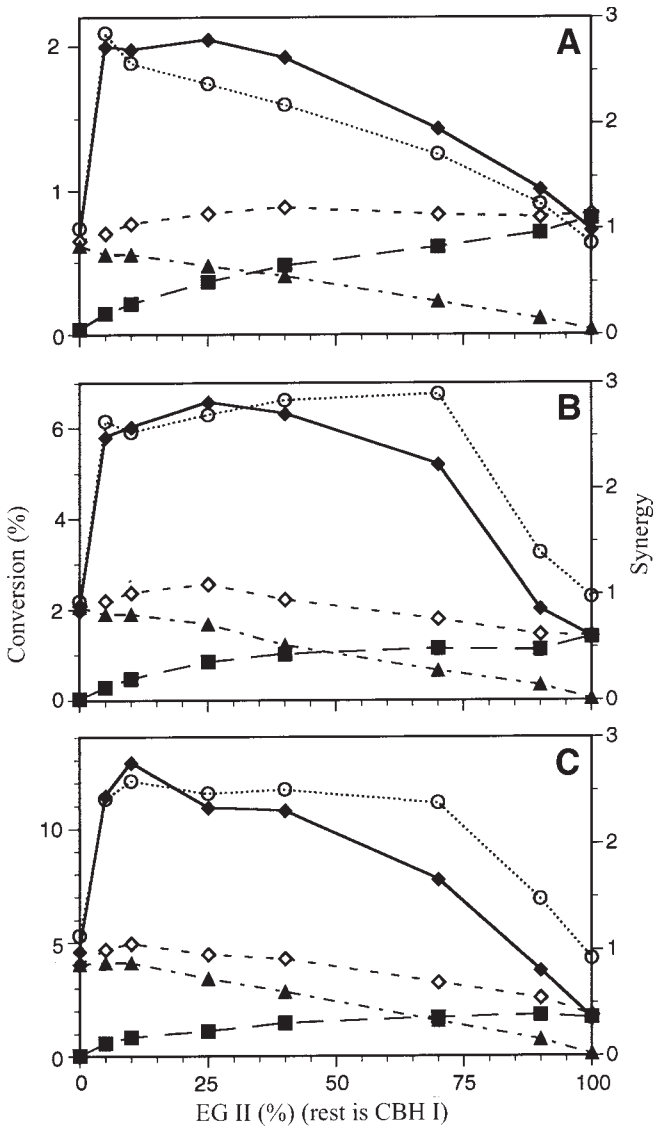

Fig. 6. Hydrolysis of SPW by CBH I and EG II at different molar ratios. The reaction time was 90 min at 40°C and the cellulose concentration was 10 g/L. **(A)** Total enzyme concentration of 0.08 μmol/g; **(B)** total enzyme concentration of 0.4 μmol/g; **(C)** total enzyme concentration of 1.2 μmol/g. ▲, CBH I alone; ■, EG II alone; ◇, CBH I + EG II calculated; ◆, CBH I + EG II experimental; ○, synergism.

these experiments. The synergism was, with the exception of the initial scattering, stable at a value of about 2. The conversion for the enzymes alone showed that EG II was equally efficient in hydrolyzing cellulose as CBH I when the conversion was below 1.5%. The two conversion lines crossed each other at about 1.5%, and above this point the conversion by CBH I was more efficient.

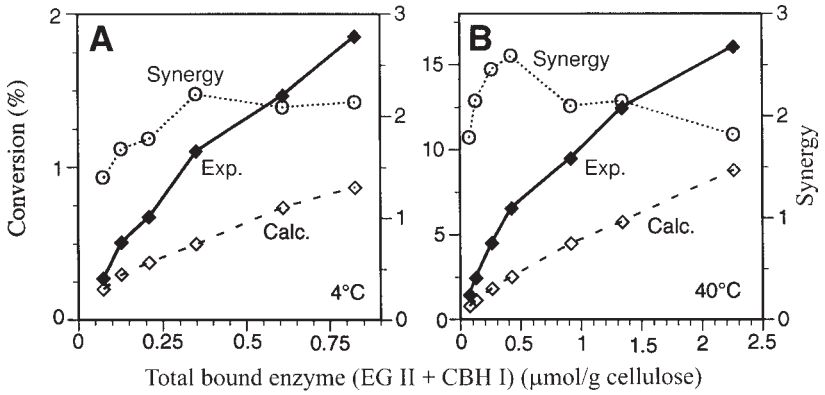

Fig. 7. Conversion of SPW and synergism between CBH I and EG II for total bound enzyme, bound CBH I + bound EG II. The reaction time was 90 min and the cellulose concentration was 10 g/L. Experiments performed **(A)** at 4°C and **(B)** 40°C. ◇, CBH I + EG II calculated; ◆, CBH I + EG II experimental; ○, synergism.

Optimal Ratio Between EG II and CBH I During Hydrolysis

The optimal ratio for the binary mixture of EG II and CBH I during hydrolysis of SPW was studied for three different enzyme concentrations (Fig. 6). The ratio between the enzymes was changed, but the total added enzyme was constant within each experiment (0.08, 0.4, and 1.2 μmol/g). The reaction time was 90 min, the reaction temperature was 40°C, and β-glucosidase was present in all experiments. At a low enzyme concentration (0.08 μmol/g), more than 80% of the added enzyme was adsorbed to the substrate. The corresponding adsorption for the high enzyme concentration (1.2 μmol/g) was that more than 60% of the added enzyme was adsorbed (data not shown). Furthermore, the ratio between EG II and CBH I for the adsorbed enzymes was very close to the ratio of added enzyme.

For all three enzyme concentrations, a small addition of EG II (5%) strongly increased hydrolysis, and the synergism was between 2.5 and 2.7. By increasing EG II to 40%, only a small change in hydrolysis was observed; a further increase in EG II decreased hydrolysis. The theoretical conversion, i.e., the hydrolysis for EG II alone plus CBH I alone, was not a straight line, but had a maximum close to the maximum for the experimental conversion. The synergism decreased with an increase in EG II for the 0.08 μmol/g concentration. For the higher enzyme concentrations, the synergism was fairly stable up to 70% EG II, above which it dropped rapidly.

Synergism as a Function of Adsorbed Enzyme

The experimental conversion as a function of bound enzyme was determined by using the adsorption data; this gives the solid line in Fig. 7. To calculate the theoretical conversion, the adsorption isotherms and hydrolysis data for the individual enzymes were used. From the adsorption isotherms (Fig. 1), the amount of adsorbed enzyme was determined,

and from hydrolysis experiments (Fig. 3), the conversion for a certain amount of adsorbed enzyme was obtained. The theoretical conversion for the enzymes added alone to the substrate was calculated. This was done for both CBH I and EG II, and the theoretical conversion for the enzymes together was summed. These data plotted as function of the adsorbed enzyme, CBH I + EG II (i.e., the total bound enzyme) give the theoretical curve (dashed) in Fig. 7. The theoretical conversion curves are almost linear in both experiments, whereas the experimental conversion curves show a tendency to level off. The synergy (i.e., the ratio between the experimental and calculated curves) was also calculated (Fig. 7, dotted line). In this way, were we able to study the synergism as a function of enzyme adsorption to the substrate. At low enzyme loading, the synergy increased up to a total bound enzyme amount of 0.3–0.4 μmol/g of cellulose. At higher total bound enzyme, the synergy reached a stable value at about 2. Similar behavior was observed at both 4 and 40°C.

Discussion

A prerequisite for enzymatic cellulose hydrolysis is adsorption of the enzymes to the substrate. The enzymatic hydrolysis of cellulose is well studied (for a review, *see* refs. *13* and *20*), but the function of the enzymes, alone and in different mixtures, on the substrate is still not known in detail. We have developed an experimental approach to study adsorption and hydrolysis simultaneously. By studying hydrolysis as a function of adsorbed enzyme, important additional information about the cellulases have been obtained *(9,21)*. In the present study, we used a lignocellulosic substrate, SPW, which has been used as a putative substrate for ethanol production from wood *(1)*. It has been reported that some of the EGs of *T. reesei* adsorb to lignin as well as to cellulose although with lower affinity *(22)*. In the present study, we cannot distinguish between adsorption to lignin and adsorption to cellulose. For simplicity, all calculations have been made as though all adsorbed enzyme were adsorbed to cellulose.

Adsorption to SPW

Both EG II and CBH I adsorbed more at 40 than at 4°C (Fig. 1). This is contradictory with what has previously been reported for the *T. reesei* CBD on bacterial microcrystalline cellulose *(23)* and for intact enzymes on Avicel *(9)*. A possible explanation for this observation is that the hydrolysis of SPW during the 90-min incubation at 40°C leads to the release of lignin residues, which reveals new adsorption sites for the enzymes. A lignin release was seen by a noticeable increase in the brown color of the hydrolysis solution after 90 min of incubation for both EG II and CBH I.

EG II bound to a lesser extent when it was in an equimolar mixture with CBH I compared to when it was alone; that is, it was excluded from its adsorption sites by the binding of CBH I (Fig. 1). This competition was most pronounced at 4°C; the difference observed at 40°C was within the margin

of error. CBH I was not affected by the presence of EG II. These observations are in agreement with those reported by Medve et al. *(14)* using Avicel as substrate. The adsorption capacities of the two enzymes were similar when they were in an equimolar mixture; the difference was within the margin of error. This indicates that the CBD makes the dominating contribution to the binding. There is strong sequence homology between the CBDs of CBH I and EG II, and thus similarity in structure is expected *(24)*. The time course data (Fig. 2) show that the adsorption was relatively fast: 65–70% bound in 10 min. EG II was excluded from its binding sites when CBH I was present whereas CBH I was not affected by the presence of EG II. It can therefore be concluded that CBH I has stronger affinity for the common enzyme-binding sites.

Hydrolysis of SPW

CBH I showed a linear increase in conversion when plotted against bound enzyme (Fig. 3A,C), which is in agreement with Medve et al. *(9)* and Nidetzky et al. *(25)*. At 40°C, when β-glucosidase was present, the slope was higher compared to experiments without β-glucosidase. The higher hydrolytic activity with β-glucosidase present is explained by product inhibition by cellobiose *(19)*. The product inhibition was most pronounced at an increased conversion at 40°C.

A possible explanation for the linear increase in the hydrolysis as a function of bound CBH I is that all adsorbed CBH I is productively bound. However, another alternative is that the two-domain structure of CBH I also leads to unproductive binding, i.e., binding only with the CBD. In the second alternative, the linearity of the curve shows that the ratio of productively to nonproductively bound CBH I does not depend on the enzyme concentration.

EG II showed a different behavior. From Fig. 3 it is clear that there was not a linear increase in conversion when plotted against bound EG II. The hydrolysis curve shows a biphasic behavior, because after an initial steep increase the release of soluble sugars was lower per bound enzyme. This finding is in agreement with earlier studies *(14,25)*. The conversion as a function of bound enzyme was quite similar when compared with and without β-glucosidase. If the biphasic behavior were owing to inhibition by cellobiose or cellotriose, we would expect a significant difference in the hydrolysis with and without β-glucosidase; however, this was not the case. The biphasic behavior could therefore not be explained by product inhibition. The transition from a steep increase to slower conversion occurred at close to the same enzyme concentration at both temperatures. This did not depend on the substrate conversion (Fig. 3B,D). The explanation could be that the substrate has easily hydrolyzable sites (e.g., loose ends, disorganized chains) where EG II binds preferably. When these sites are saturated the enzyme starts to bind to sites where it has lower exo activity; that is, at these sites less soluble sugars are produced. EG II may have equally high hydrolytic activity at the second sites but will here have more exclusive

endo activity. We are not analyzing for endo activity on the substrate but only the exo activity by measuring the release of soluble sugars. By measuring both exo and endo activity, it would be possible to test this hypothesis.

By comparing the hydrolysis capacity of EG II alone and CBH I alone (Figs. 3 and 4), it is clear that EG II produces as much soluble sugars as CBH I at 4°C, when comparing the same amount of added enzyme. At 40°C, EG II produces approximately as much as CBH I when the conversion is below 1%, but when the conversion increases, CBH I is the more effective enzyme. This can also be seen in the initial phase in Fig. 5. Evidently, EG II is as good in hydrolyzing the easily hydrolyzable parts of SPW, which would then constitute about 1% of the available substrate. Knowledge about the properties of SPW is presently limited. Our view is that these easily hydrolyzable parts could be solvated free ends against which EG II, with its presumably open-cleft active site, are highly active.

Optimal Ratio Between CBH I and EG II

It is well established that CBH I and EG II cooperate during cellulose hydrolysis *(21,26)*; they act in synergism. The CBH I–EG II synergy has been thoroughly studied on Avicel *(14)*, and different enzyme mixtures have been studied on Avicel *(12,27)*, bacterial microcrystalline cellulose *(12)*, and filter paper *(12,28)*. Henrissat et al. *(12)* observed an optimum in conversion for a 50:50 mixture of EG II:CBH I on Avicel, but for synergism the optimum was at 25:75. They proposed that a maximal synergism should be observed at an extreme low:high mixture of endo:exo enzymes. In Fig. 6, our findings confirm this in the sense that only a small amount of EG II (5%) is needed to strongly increase conversion and synergism. The data also show all that at three enzyme levels, a mixture with 40% EG II:60% CBH I was approximately equally efficient in hydrolyzing SPW as the mixture with 5% EG II:95% CBH I. Furthermore, the maximum in hydrolysis was moved to lower EG II addition at higher total enzyme loading: the maximum was at 10% EG II at higher enzyme loading (Fig. 6C) compared with 25% for the lower enzyme loading (Fig. 6A). This is in agreement with the results of Nidetzky et al. *(25)* in which the optimal mixtures were found at 18% EG II for low concentrations and 8% for high concentrations. Our results show that for efficient endo-exo synergy, only a small addition of EG to a CBH is needed.

Endo-Exo Synergism

It has been shown that sequential addition of EG II followed by CBH I significantly increased hydrolysis but that adding the enzymes in reverse order did not *(28)*; that is, EG II prepares the substrate for CBH I and not vice versa. The traditional hypothesis for the endo-exo synergism is that the EG, by making random internal cuts, produces new free ends, to which the exoglucanase can bind and start to hydrolyze *(2,13,20)*. The synergism observed should thus be owing to an increase in substrate for the exoglucanase. If this were the case, one would expect to see a significant

increase in adsorption for CBH I when synergism was observed owing to binding of CBH I to new free ends created by EG II activity. Also, one would not expect to observe any synergism at low enzyme loadings, when there is a very small amount of CBH I free in the solution. At low enzyme concentration, CBH I has an excess of substrate, and therefore the increase in free ends (owing to EG II activity) is marginal compared to the amount of free ends that is normally present.

The results presented herein suggest an extension of the traditional endo-exo synergism hypothesis. From Fig. 1 we observe a minor decrease, not an increase, in adsorption for CBH I when EG II is present, and in Fig. 4 we see a significant synergism even at low CBH I concentrations. If EG II produced a large number of free ends to which CBH I would bind, we would expect a significant difference in synergism for low and high CBH I concentrations. However, the synergism observed is rather constant (Fig. 4). Thus, the endo-exo synergism cannot be explained by only an increase in substrate for the exoglucanase. Evidently, EG II prepares the substrate for CBH I, as suggested by Nidetzky et al. *(28)*. The endo activity of EG II will thus produce a substrate that is easier to hydrolyze for CBH I.

We feel there are two possible ways for EG II to prepare the substrate for CBH I. Both will lead to endo-exo synergism. First, the cellulose chains might be physically blocked by, e.g., other cellulose microfibrils, or lignin in the case of lignocellulose. The low activity for CBH I on long cellulose chains should be because CBH I is a processive enzyme *(4)* and will get trapped if the cellulose chain is blocked. The endo activity of EG II would make the cellulose chains shorter. When the cellulose chains become shorter, the probability of CBH I hydrolyzing a complete chain without getting trapped (and nonproductively bound) increases. Second, CBH I could have the ability to bind nonproductively with the CBD to the cellulose fiber with binding strength similar to that for productive binding. The new ends produced by EG II would be "productive sites" for CBH I. If EG II were to make the internal cuts on the chains, CBH I would have more sites for productive binding, and the ratio between productive and nonproductive bound CBH I would then increase. Both of these mechanisms for endo-exo synergy can operate simultaneously.

When studying the synergism as a function of adsorbed enzyme (Fig. 7), the synergism increased initially with an increase in adsorbed enzyme. A similar effect has been observed for the synergism between CBH I and CBH II *(9)*. The initial increase in synergism indicates that EG II and CBH I cooperate better at shorter distances between the enzymes but only to a certain point, up to an enzyme concentration of about 0.4 μmol/g. At higher enzyme concentrations, it is possible that the positive effect for CBH I by the EG II activity is dominated by the competition for the same adsorption sites. During hydrolysis of microcrystalline cellulose (Avicel) at 4°C by EG II and CBH I, no synergism was observed *(14)*, whereas significant synergism was observed at 4°C on SPW. This dis-

crepancy must be owing to the difference between a microcrystalline and a steam-pretreated substrate.

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