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A Model Explaining Declining Rate in Hydrolysis of Lignocellulose Substrates with Cellobiohydrolase I (Cel7A) and Endoglucanase I (Cel7B) of *Trichoderma reesei*

Torny Eriksson, Johan Karlsson, and Folke Tjerneld*

Department of Biochemistry, Lund University, PO Box 124, S-221 00 Lund, Sweden, E-mail: Folke.Tjerneld@biokem.lu.se

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Abstract

It is commonly observed that the rate of enzymatic hydrolysis of solid cellulose substrates declines markedly with time. In this work the mechanism behind the rate reduction was investigated using two dominant cellulases of *Trichoderma reesei*: exoglucanase Cel7A (formerly known as CBHI) and endoglucanase Cel7B (formerly EGI). Hydrolysis of steam-pretreated spruce (SPS) was performed with Cel7A and Cel7B alone, and in reconstituted mixtures. Throughout the 48-h hydrolysis, soluble products, hydrolysis rates, and enzyme adsorption to the substrate were measured. The hydrolysis rate for both enzymes decreases rapidly with hydrolysis time. Both enzymes adsorbed rapidly to the substrate during hydrolysis. Cel7A and Cel7B cooperate synergistically, and synergism was approximately constant during the SPS hydrolysis. Thermal instability of the enzymes and product inhibition was not the main cause of reduced hydrolysis rates. Adding fresh substrate to substrate previously hydrolyzed for 24 h with Cel7A slightly increased the hydrolysis of SPS; however, the rate increased even more by adding fresh Cel7A. This suggests that enzymes become inactivated while adsorbed to the substrate and that unproductive binding is the main cause of hydrolysis rate reduction. The strongest increase in hydrolysis rate was achieved by adding Cel7B. An improved model is proposed that extends the standard endo–exo synergy model and explains the rapid decrease in hydrolysis rate. It appears that the processive action of Cel7A becomes hindered by obstacles in the lignocellulose substrate. Obstacles created by disordered cellulose chains can be removed by the endo activity of Cel7B,

*Author to whom all correspondence and reprint requests should be addressed.

which explains some of the observed synergism between Cel7A and Cel7B. The improved model is supported by adsorption studies during hydrolysis.

Index Entries: Cellulase; cellulose; lignocellulose; hydrolysis; *Trichoderma reesei*; synergism; adsorption; Cel7A; Cel7B.

Introduction

Much research effort has been directed to use cellulose materials effectively for energy production by conversion into fermentable sugars. Pretreatments, such as steam explosion and partial acid hydrolysis, have greatly increased the rate and extent of enzymatic hydrolysis of cellulose substrates *(1)*. However, the rate declines markedly as hydrolysis proceeds, requiring large quantities of enzymes to efficiently convert cellulose. The dramatic drop in hydrolysis rate has been observed in several studies using commercial enzyme mixtures *(2,3)* as well as for purified enzymes *(4,5)*. The reduced hydrolysis rate can be explained either by decreased enzyme hydrolytic activity or by increasingly recalcitrant substrate *(6)*. Products of enzymatic hydrolysis, that is, glucose, and, particularly, cellobiose, inhibit cellulases *(7)*; however, adding β-glucosidase, which reduces cellobiose inhibition, does not entirely solve the problem of the rate decline *(5)*. In studies of purified cellulose substrates hydrolyzed with endocellulases, substrate heterogeneity could explain the observed decline in hydrolysis rate because easily hydrolyzed cellulose reacts first *(5)*. However, studies on pretreated lignocellulose indicate that substrate reactivity declines only modestly as conversion increases, and that substrate transformation is not the main factor *(3)*; thus, the mechanism for the declining hydrolysis rate has not been clarified.

The aerobic filamentous fungi *Trichoderma reesei* produces, like other cellulose-degrading fungi, a set of enzymes that cooperate synergistically in the degradation of cellulose. The total set of cellulases of *T. reesei* comprises two exoglucanases Cel7A (CBHI) and Cel6A (CBHII), and at least four endoglucanases Cel7B (EGI), Cel5A (EGII), Cel12A (EGIII), and Cel45A (EGV) *(8,9)*. All the *T. reesei* cellulases, except Cel12A, have a similar domain structure. The enzymes consist of a catalytic domain and a cellulose binding domain connected with a highly glycosylated linker. The structures of the catalytic domains of Cel7A *(10)* and Cel7B *(11)* are known and have two general active site topologies: the tunnel of the cellobiohydrolases and the cleft of the endoglucanases *(12)*.

The purpose of this study is to obtain information about the mechanisms that decrease lignocellulose hydrolysis rates. We have examined the hydrolysis mechanisms of steam-pretreated spruce (SPS) by two major cellulases produced by *T. reesei*: the exoglucanase Cel7A (CBHI) and the endoglucanase Cel7B (EGI). SPS was used as substrate because of its relevance to producing ethanol from lignocellulose *(13)*. The majority of earlier studies on enzymatic lignocellulose hydrolysis have used enzyme mixtures *(3,14)*, but we have chosen to work with purified, well-character-

ized enzyme components. Our study focuses on Cel7A because it is the most important sugar-producing enzyme in *T. reesei (15)*. Cel7A is about 60–75% of the cellulases in a *T. reesei* culture filtrate, and Cel7B—the dominating endoglucanase—comprises about 6–10% *(16,17)*. Cel7A and Cel7B are related both by gene sequence and structure, they belong to Family 7 of the glycosyl hydrolases *(18)*. Cel7A is the best-characterized *T. reesei* cellulase. From both structural *(10)* and biochemical *(19,20)* studies it is known that Cel7A hydrolyzes a cellulose chain from the reducing end, in contrast to Cel6A (CBHII) which hydrolyzes from the nonreducing end *(20)*. Structure determination shows 10 subsites for glycosyl units in the tunnel-shaped Cel7A active site *(21)*, and the substrate is oriented to cleave each second glucose residue. Cel7A is thought to hydrolyze cellulose processively, that is, Cel7A starts to hydrolyze the cellulose chain from the reducing end and moves along the same cellulose chain producing cellobiose as the reaction proceeds *(21–23)*. Generally accepted models *(24)* that describe the enzymatic degradation of cellulose substrates often include two sequential steps. First, the endoglucanases cleave cellulose chains randomly with preference for the amorphous parts of cellulose. This is followed by cellobiohydrolases, which act as exoglucanases releasing cellobiose from the newly formed cellulose chain ends. Hence the observed synergistic effect between endoglucanases and cellobiohydrolases is traditionally explained by the endoglucanase ability to create new substrate sites for cellobiohydrolase. The aim of this study is to reveal mechanisms involved in the observed synergistic effect of Cel7A and Cel7B. Knowledge of how endo- and exoglucanases cooperate during hydrolysis will give important information needed to explain the causes for declining hydrolysis rates.

Hydrolysis of SPS was performed with Cel7A and Cel7B alone and in reconstituted mixtures. Soluble product formation and enzyme adsorption were followed simultaneously. To investigate mechanisms causing the hydrolysis rate reduction, substrate and enzymes (Cel7A and Cel7B) were added to a substrate previously hydrolyzed for 24 h with Cel7A. An alternative way to present synergism based on hydrolysis rates is presented, which is advantageous when synergism is studied at selected times during hydrolysis. Based on results of lignocellulose hydrolysis and present knowledge of Cel7A and Cel7B structure and function, a model is proposed that shows the role of unproductive binding of exoglucanase for the decreased hydrolysis rate, and extends the standard endo–exo model for synergism between cellulases.

Materials and Methods

Substrate

Steam-pretreated spruce was a kind gift from Prof. Guido Zacchi, Chemical Engineering I, Lund University. The raw material was spruce with a chip size of 2.2–10 mm. The chips were impregnated with 2.5–3% SO_2 (w/w, based on the water content in the raw material). The pretreatment was performed at 210–215°C for 3–5 min. The pretreatment method is

described in more detail elsewhere *(25)*. After the pretreatment, the material was washed in distilled water to remove soluble material. The cellulose content in the material, determined by the Hägglund method *(26)*, was about 50%. The remainder is lignin because hemicellulose and extractives are solubilized during the pretreatment.

Enzymes

Cel7A was a kind gift from Dr. Maija Tenkanen, VTT Biotechnology, Espoo, Finland, and was purified from a culture filtrate devoid of Cel7B and Cel5A. In the strain of *T. reesei* used to produce Cel7A, the genes for Cel7B (EGI) and Cel5A (EGII) had been deleted. The purification protocol, described in *(27)*, was optimized to purify Cel7A from other enzymes with endo activity: Cel12A (EGIII) and Cel45A (EGV). Cel7B was purified from a culture filtrate of *T. reesei* QM9414. This filtrate was obtained from Dr. Göran Pettersson at the Department of Biochemistry, Uppsala University, Uppsala, Sweden. The culture filtrate was desalted by gel filtration with a 5-mL Sephadex G25 column (Pharmacia Biotech, Uppsala, Sweden) using 20-m*M* ammonium acetate buffer, pH 7.0. Cel7B was purified by repeated anion-exchange steps on a fast protein liquid chromatography (FPLC) system (Pharmacia Biotech). The first anion-exchange purification step was performed using a 30-mL Source Q column (Pharmacia Biotech) with a linear ionic strength gradient from 20-m*M* to 1-*M* ammonium acetate, pH 7.0. A second anion-exchange chromatography step was performed at pH 6 with a linear ionic strength gradient from 20-m*M* to 1-*M* ammonium acetate. The purity of the Cel7B fraction was confirmed by isoelectric focusing (IEF) and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). IEF was performed using the PhastSystem™ (Pharmacia Biotech) and precast phast gels (PhastGel IEF 3-9) stained with silver as described by the manufacturer. SDS-PAGE was performed with the NOVEX™ NuPAGE electrophoresis system (NOVEX™, Novel Experimental Technology, San Diego, CA) using precast 10% NOVEX™ NuPage Bis-Tris gels, stained with NOVEX™ SilverExpress™ kit according to the manufacturer's directions. The identity of Cel7B was confirmed by Western blotting using anti-Cel7B antibodies. The monoclonal anti-Cel7B-antibodies was a kind gift from Dr. M. Penttilä, VTT Biotechnology, Espoo, Finland. The blotting procedure was performed according to Amersham International (Amersham, UK) description for Hybond™-C extra supported nitrocellulose membrane. Goat anti-mouse IgG alkaline phosphatase conjugated antibody (BioRad, Richmond, CA) was used as secondary antibody and BCIP/NBT (5-bromo-4-chloro-3-indol phosphate *p*-toluidine salt and *p*-nitroblue tetrazolium chloride) (BioRad) was used to develop color.

Hydrolysis of Steam-Pretreated Spruce

All hydrolysis experiments were performed at 40°C in 50-m*M* Na-acetate buffer, pH 4.8. The enzyme concentrations were determined by

measuring the absorbance at 280 nm (ε_{Cel7A} = 78,800 M^{-1} cm^{-1}, MW_{Cel7A}= 63.5 kDa, ε_{Cel7B} = 67,000 M^{-1} cm^{-1}, MW_{Cel7B} = 52.5 kDa) *(28)*. The hydrolysis reactions were initiated by mixing the enzyme solution with the substrate in 1.8 mL screw-cap tubes. Hydrolysis experiments were performed in 1-mL buffer using 20 mg/mL substrate (10 mg/mL cellulose). Enzyme concentrations were 0.5–1.5 µ*M*. The experiments were performed in a thermostated waterbath at 40°C with gentle agitation. The hydrolysis was terminated by filtering the mixture through a 0.22-µm syringe filter (Millipore, Bedford, MA). The filtration separated nonhydrolyzed substrate with bound enzyme from soluble sugars and free enzyme. All hydrolysis experiments were performed in triplicate and mean values and standard deviations are presented. The filtrates were analyzed for soluble sugars (glucose, cellobiose, and cellotriose) in a DX 500 high performance anion exchange chromatography system with pulsed amperiometric detection (HPAEC-PAD) (Dionex, Sunnyvale, CA) using a CarboPac™ PA100 analytical column (Dionex). For elution, 100-m*M* NaOH was used as an isocratic eluent.

Enzyme Adsorption

Adsorption was determined by measuring free enzyme concentration in solution by either ion-exchange chromatography or radioactive labeling. A FPLC system was used to measure Cel7A and Cel7B adsorption *(29,30)*. The column was an anion-exhange column (Mono Q HR, Pharmacia Biotech). Cel7A adsorption was measured by adding 0.1-µ*M* tritium-labeled Cel7A to the enzyme mixture. Labeled Cel7A was quantified radiometrically (Beckman liquid scintillator LS 1801, Beckman Instruments, CA). Sample (100 µL) was added to 7 mL Beckman ReadySafe scintillation cocktail. The tritium labeling of Cel7A was performed as described in *(31)*. Tritium-labeled Cel7A was compared with the unlabeled enzyme in terms of both hydrolytic and adsorption properties using pure cellulose (Avicel). No significant differences were found between labeled and unlabeled enzyme.

Calculation of Hydrolysis Rates

Hydrolysis rates were calculated using regression analysis with a curve-fitting program (CurveExpert 1.34, Microsoft, Redmond, WA). The following equation

$$P = \frac{ab + ct^d}{b + t^d}$$

gave a good fit to the data points from the hydrolysis experiments. In this equation, P is the amount of formed product, t is the hydrolysis time, and a, b, c, and d are constants adjusted by the program to fit the data points. The fitted curve was differentiated to obtain hydrolysis rates at the selected times.

Thermal Stability of Cel7A and Cel7B

The thermal stability of Cel7A and Cel7B was measured by incubating 40-µ*M* Cel7A and 5-µ*M* Cel7B in 50-m*M* Na-acetate, pH 4.8 at 40°C with gentle agitation in a water bath. Activity was measured at selected times during the 96-h incubation in the following way: 10-µL samples were incubated with 90-µL 3-m*M* *p*-nitrophenyl β-D-cellobioside in 50-m*M* Na-acetate, pH 4.8. Cel7A was incubated for 2 h at 40°C and Cel7B at 25°C for 30 min. The reaction was terminated by adding 100 µL of 12.5% Na_2CO_3. Formation of *p*-nitrophenol was detected by measuring absorbance at 405 nm.

Results and Discussion

Cel7A and Cel7B Hydrolysis of SPS

Cellulose conversion into soluble sugars was measured during 48-h hydrolysis of steam-pretreated spruce (SPS) (Fig. 1). SPS was hydrolyzed with the enzymes alone and in equimolar ratios. The production of soluble oligosaccharides was determined with an ion-exhange chromatography system (HPAEC-PAD). Synergism between Cel7A (CBHI) and Cel7B (EGI) is clearly apparent in Fig. 1A. The sugar production obtained from hydrolysis with a mixture of Cel7A and Cel7B was higher than the sum of sugar production when the substrate was hydrolyzed with Cel7A and Cel7B independently. During the hydrolysis of SPS, Cel7A produced only very small amounts of cellotriose and Cel7B produced no detectable cellotriose. Figure 1B shows the ratio between the major products cellobiose/glucose during Cel7A and Cel7B hydrolysis. The notable higher ratio for Cel7A agrees with a processive action, where the exoglucanase moves along the cellulose chain and releases cellobiose units by successive cleavages. A strictly processive mechanism will release only cellobiose. The glucose production, which has also been experimentally observed in other studies *(29)*, was proposed to originate from cleavages at the start or end of the cellulose chain. Thus, it should be possible to use the cellobiose/glucose ratio for Cel7A to measure the processivity of the enzyme. It is clear from Fig. 1B that the cellobiose/glucose ratio for both enzymes is lower in the early stage of hydrolysis than in later stages. This is particularly prominent for Cel7A and could reflect the initial formation of glucose by Cel7A activity on the chain ends before cellobiose production becomes dominating by the processive action of Cel7A.

Fig. 1. *(opposite page)* Hydrolysis of steam-pretreated spruce with Cel7A and Cel7B. Enzyme concentrations were 1.5 µ*M*; substrate concentration was 20 g/L (cellulose concentration 10 g/L). Temperature was 40°C. Error bars represent standard deviations of triple experiments. **(A)** Soluble sugars (glucose plus cellobiose) produced with Cel7A (○), Cel7B (□) and Cel7A together with Cel7B (▲); the calculated sum of sugars produced by Cel7B and Cel7A alone (×). **(B)** Product formation shown as cellobiose/glucose ratio for Cel7A (○) and Cel7B (□) during hydrolysis of SPS. **(C)** Hydrolysis rates for Cel7A (○), Cel7B (□) and Cel7A together with Cel7B (▲) calculated from curves fitted to the data in A.

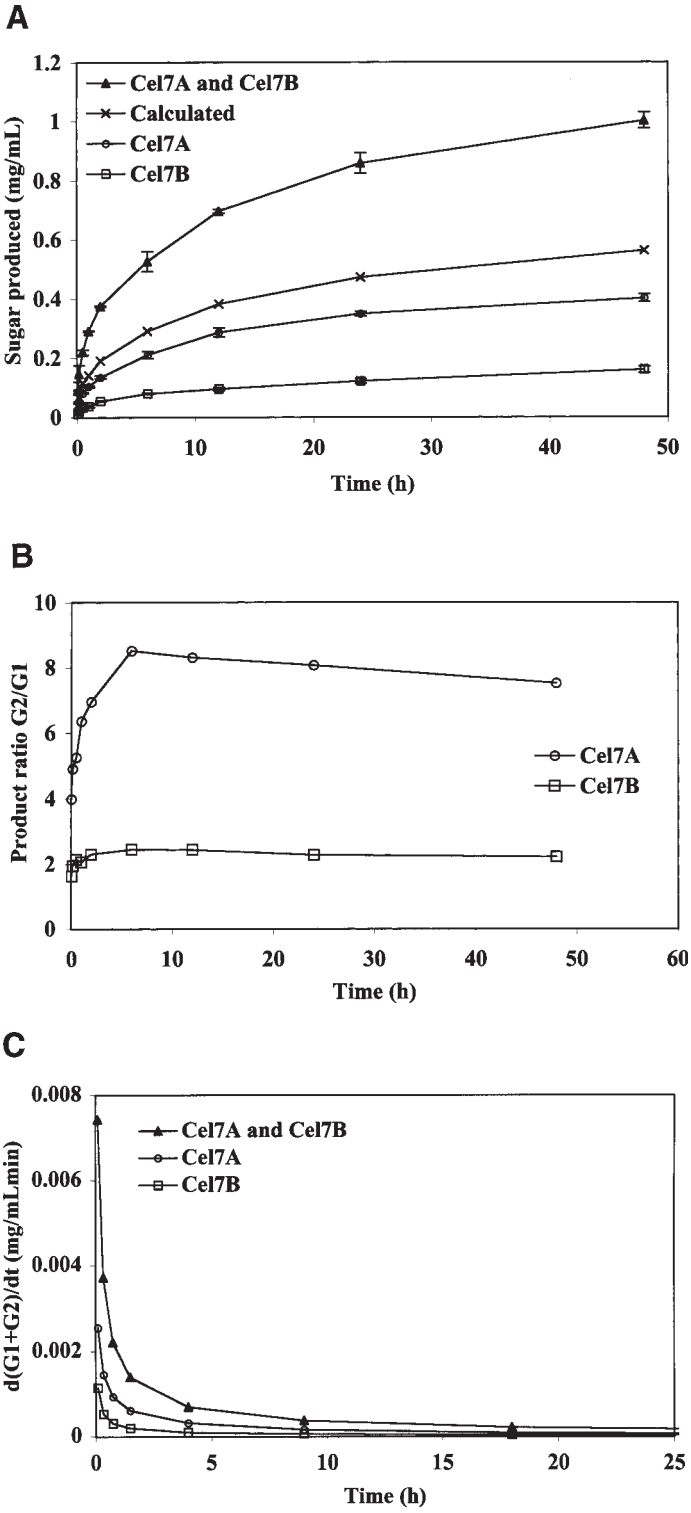
A
Cel7A and Cel7B
Calculated
Cel7A
Cel7B
Sugar produced (mg/mL)
Time (h)
B
Product ratio G2/G1
Cel7A
Cel7B
Time (h)
C
Cel7A and Cel7B
Cel7A
Cel7B
d(G1+G2)/dt (mg/mL.min)
Time (h)

Fig. 1

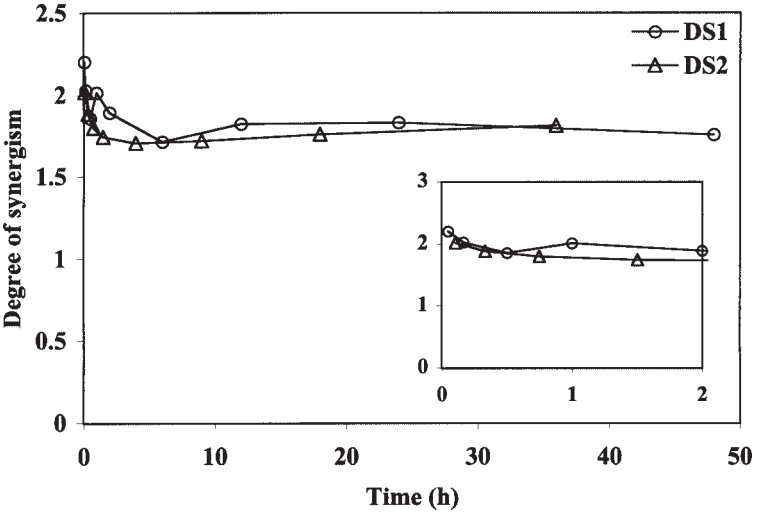

Fig. 2. Synergism between Cel7A and Cel7B during hydrolysis of SPS. The experimental data are from Fig. 1. Synergism based on sugar production (DS_1) and synergism based on hydrolysis rates (DS_2). In the inset the initial phase is enlarged.

Figure 1C shows hydrolysis rates of Cel7A and Cel7B alone and in mixture during hydrolysis of SPS. The rates were calculated from the slopes of hydrolysis curves fitted to the data points presented in Fig. 1A. The hydrolysis rate is defined as $d(G_1 + G_2)/dt$, where G_1 and G_2 are concentrations of glucose and cellobiose, respectively. Production of cellotriose (G_3) is low relative to the main products G_1 and G_2. G_3 production could be neglected without changing the results of the rate calculations. The rate was dramatically decreased during hydrolysis of SPS. The hydrolysis rate was about an order of magnitude lower after 7-h compared with after 10-min hydrolysis. The rate was decreased for Cel7A and Cel7B used independently as well as when the two enzymes were used as a mixture.

The degree of synergism based on product formation is the conventional way to quantify synergism. The degree of synergism DS_1 is defined as the ratio of products formed from the mixture of endo- and exoglucanase enzymes, to the sum of the products of the individual enzymes:

$$DS_1 = \frac{(G_1 + G_2)_{\text{Cel7A and Cel7B}}}{(G_1 + G_2)_{\text{Cel7A}} + (G_1 + G_2)_{\text{Cel7B}}}$$

Figure 2 presents the degree of synergism (DS_1) calculated from data in Fig. 1A as a function of hydrolysis time. Throughout the hydrolysis, the degree of synergism is about 2.0. When synergism is based on accumulated product, it is difficult to follow the synergism with time. This is because early product formation complicates the evaluation of synergism in later stages of the hydrolysis. An alternative is to calculate synergism from hydrolysis *rates* (Fig. 2). Degree of synergism based on hydrolysis rates

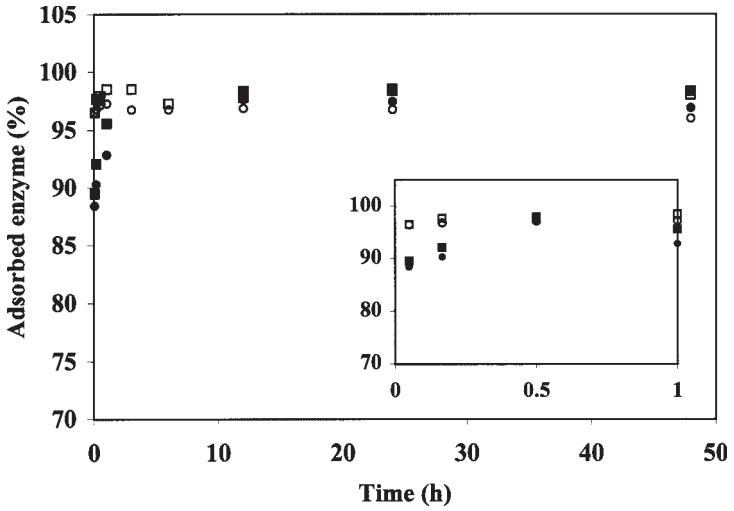

Fig. 3. Time course for adsorption of Cel7A and Cel7B during hydrolysis of steam-pretreated spruce. The hydrolysis was performed as described in Fig. 1. Adsorption was measured with ion-exchange chromatography. Adsorption is shown in percent of added enzyme: (○) Cel7A alone; (□) Cel7B alone; (●) Cel7A in equimolar mixture with Cel7B; (■) Cel7B in equimolar mixture with Cel7A. In the inset the initial phase is enlarged.

(DS_2) is defined as the ratio of the hydrolysis rate of the mixed enzymes to the sum of the hydrolysis rates of the individual enzymes:

$$DS_2 = \frac{[d(G_1 + G_2)/dt]_{\text{Cel7A and Cel7B}}}{[d(G_1 + G_2)/dt]_{\text{Cel7A}} + [d(G_1 + G_2)/dt]_{\text{Cel7B}}}$$

This method of presenting synergism makes it possible to determine the synergism at selected times during the hydrolysis. It is clear from Fig. 2 that the Cel7A–Cel7B synergism is highest in the beginning (i.e., after a few minutes) of the hydrolysis than in later stages. The synergism is about 2.0 in the initial stage of the hydrolysis, and then drops to a fairly stable value at 1.7–1.8. Values of around 2.0 in endo–exo synergism, when calculated based on product formation, was observed earlier for *Trichoderma* enzymes on complex substrates like SPS. Karlsson and coworkers *(32)* reported values around 2.0 for Cel7A and Cel5A (EGII) on steam-pretreated willow.

Adsorption During Hydrolysis

Enzyme adsorption was determined during the hydrolysis to gain more information about mechanisms involved in Cel7A and Cel7B hydrolysis of SPS. Adsorption of enzyme to the substrate was analyzed by measuring the concentration of free enzyme in the hydrolysis solution. During the hydrolysis with Cel7A and Cel7B alone, 96–98% of the added enzymes remained bound to the substrate (Fig. 3). Adsorption was fairly

constant at this level from 3 min to 48 h. However, the adsorption was lower in the beginning of the hydrolysis when the two enzymes were used in combination: 89% was reached after 3 min and 90–92% at 10 min. The same effect was observed for Cel7A and Cel5A (EGII) adsorption to Avicel *(29)*. The conclusion was that the enzymes competed for binding sites and a longer time was needed to reach equilibrium compared with when the enzymes were added alone. It is clear that most of the added enzymes are adsorbed to the substrate within just a few minutes of hydrolysis and that enzyme adsorption remains at a high level during the 48-h hydrolysis.

Factors Affecting Enzymatic Hydrolysis of Lignocellulose

The decrease in hydrolysis rate observed in Fig. 1C can be explained by one or both of the following:

1. *Enzyme inactivation*: Enzymes might become inactivated due to thermal denaturation during long hydrolysis times (up to 48 h in 40°C). Inactivation may also be caused by product inhibition (i.e., glucose, cellobiose) or lignin components released during hydrolysis. A third possibility for enzyme inactivation is unproductive binding of active enzyme to the substrate.
2. *Substrate transformation*: Increased substrate recalcitrance has been proposed to explain the declining rate *(6)*. Easily hydrolyzed parts of the cellulose substrate might be hydrolyzed in the early stages, leaving recalcitrant parts for later stages. Using purified and well-characterized enzyme components, we have a better possibility of clarifying the role and mechanism of enzyme inactivation in lignocellulose hydrolysis compared with earlier studies using enzyme mixtures. The experiments have been designed to elucidate the factors affecting enzymatic lignocellulose conversion.

Thermal Stability of Cel7A and Cel7B

A possible explanation for the pronounced decrease in hydrolysis rate may be found in enzyme deactivation. To test the thermal stability, under the hydrolysis conditions used in the experiment presented in Fig. 1, a solution of Cel7A (CBHI) and Cel7B (EGI) was incubated at 40°C and activity was measured at selected times during the incubation (Fig. 4). Results show that the activity is retained for both Cel7A and Cel7B throughout the 96-h incubation; thus, the decreased hydrolysis rate observed in Fig. 1B cannot be explained by thermal deactivation of enzymes. The presence of substrate in the hydrolysis experiments could affect the stability of the enzymes; however, adsorption of enzyme to solid substrate is likely to increase rather than decrease enzyme stability. High stability has been demonstrated for cellulase adsorbed on Solka Floc cellulose during continuous hydrolysis for 1200 h *(33)*.

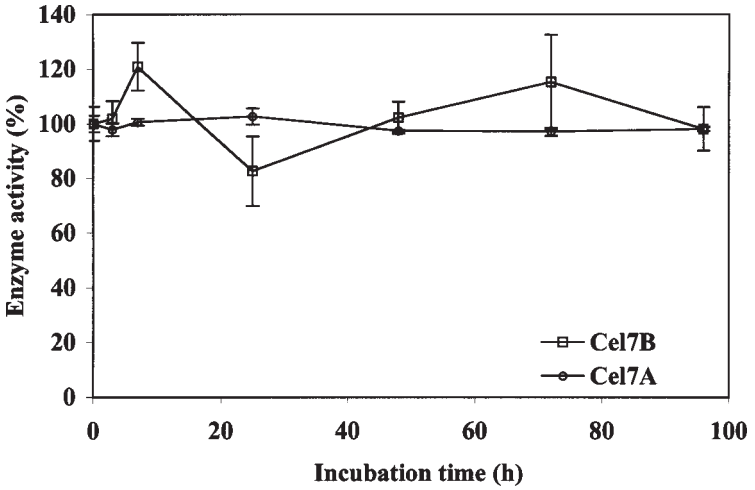

Fig. 4. Thermal stability of Cel7A and Cel7B. The cellulases Cel7A (○) (40 µ*M*) and Cel7B (□) (5 µ*M*) were incubated at 40°C in 50-m*M* Na-acetate buffer pH 4.8. Activity was measured at selected times during the 96-h incubation using *p*-nitrophenyl β-D-cellobioside as substrate.

Product Inhibition

It is well known that the products formed from cellulose hydrolysis (i.e., cellobiose and glucose) can inhibit cellulases during hydrolysis. Cellobiose is a stronger inhibitor than glucose *(7)*. In the hydrolysis experiments presented here, low enzyme concentrations were used; therefore, the hydrolysis resulted in low concentrations of sugars relative to the substrate concentration. However, it is possible that lignin components were released during hydrolysis, which could deactivate the enzymes and decrease the hydrolysis rate observed in Fig. 1C. Product inhibition and the effect of released lignin components were evaluated by hydrolyzing SPS for 24 h with Cel7A and utilizing the posthydrolysis filtrate in a subsequent hydrolysis. The filtrate, containing glucose (0.02 mg/mL), cellobiose (0.39 mg/mL), and liberated lignin components, was compared with a buffer solution in a 24-h hydrolysis of SPS with Cel7A. SPS hydrolyzed with Cel7A in the presence of the filtrate produced 0.37 mg/mL soluble sugars compared with 0.41 mg/mL in buffer. This 10% difference cannot explain the pronounced decrease in hydrolysis rate during Cel7A hydrolysis and thus other mechanisms must be involved in decreasing the rate.

Effects of Addition of Fresh Cel7A and Substrate to Cel7A Hydrolysis

Cel7A is a key enzyme in the hydrolysis of crystalline cellulose *(15)*, and the hydrolysis rate dramatically decreases during hydrolysis of SPS with Cel7A (Fig. 1C). To obtain information about mechanisms involved in decreasing the hydrolysis rate, substrate and enzymes were added after 24-h hydrolysis of SPS with Cel7A (Fig. 5A,B). The substrate (SPS) at a

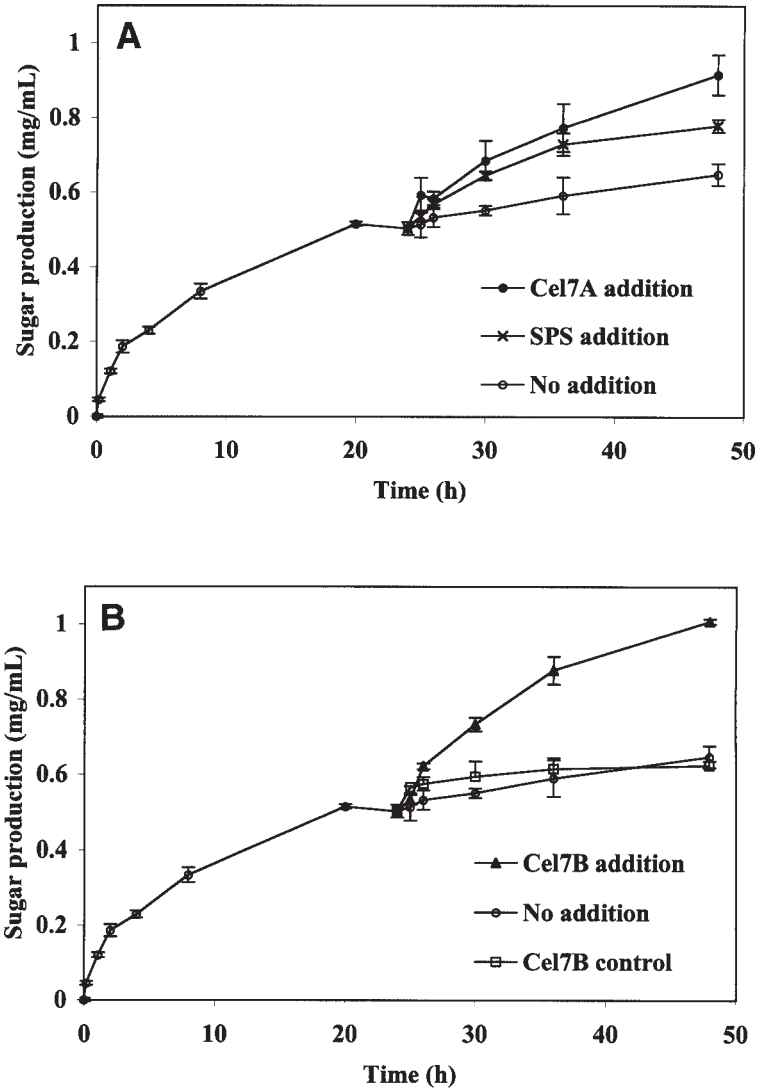

Fig. 5. Addition at 24-h of substrate and enzymes to hydrolysis of SPS with Cel7A. SPS was hydrolyzed at 40°C in 50-m*M* Na-acetate buffer. Initial concentrations were 1.5-µ*M* Cel7A and 20 g/L SPS (cellulose concentration 10 g/L) **(A)** (×) addition of 20 g/L SPS; (●) addition of 1.5 µ*M* Cel7A; (○) no addition. **(B)** (▲) Addition of 0.5-µ*M* Cel7B; (□) addition of 0.5-µ*M* Cel7B to heated samples (i.e., Cel7A activity had been deleted); (○) no addition.

cellulose concentration of 10 mg/mL was hydrolyzed with Cel7A as the only enzyme in the hydrolysis. After 24-h hydrolysis, the sugar (cellobiose and glucose) production reached 0.50 mg/mL. At this point about 98% of added Cel7A was adsorbed to the substrate (Fig. 3) and the hydrolysis rate was low ($<1 \times 10^{-4}$ mg/mL·min) (Fig. 1C). When the hydrolysis with Cel7A alone was continued without any addition (Fig. 1A), hydrolysis rates

remained low, that is, from 24 h to 48 h only 0.15 mg/mL products (cellobiose and glucose) were formed.

Adding fresh Cel7A (the same amount [1.5 μ*M*] added at the start of the hydrolysis) increased the hydrolysis rate (solid circles in Fig. 5A) resulting in formation of 0.41 mg/mL products from 24 to 48 h. This implies that "productive" substrate sites are available for Cel7A even though the substrate had been hydrolyzed with only Cel7A for as long as 24 h. Thus, increased recalcitrance of the substrate cannot solely explain the rate decline. However, adding fresh enzyme to the hydrolyzed substrate did not lead to the same extent of hydrolysis as when the same amount of enzyme was added to previously unhydrolyzed substrate, that is, adding fresh enzyme at 24 h produced about 82% of the sugars produced in the initial 24 h hydrolysis.

The role of unproductive enzyme binding may be evaluated in experiments where fresh substrate is added to already hydrolyzed lignocellulose. Adding fresh substrate at 24-h hydrolysis with Cel7A (10-mg cellulose, the same amount as initial) increased the total sugar production: 0.27 mg/mL products were formed after the addition from 24- to 48-h hydrolysis. Thus, a fraction of the bound enzymes were able to desorb from the hydrolyzed substrate and adsorb to the newly added substrate. The fresh substrate was hydrolyzed at a significantly lower rate compared to the substrate at the beginning of the hydrolysis. These results show that after 24-h hydrolysis, much of the Cel7A enzymes are unproductively bound to the substrate. The rate increase upon adding new substrate was significantly lower compared to adding fresh Cel7A. However, the results of adding fresh enzyme and substrate to Cel7A hydrolysis indicate that enzyme inactivation or unproductive binding has a more important role than increased substrate recalcitrance for the decrease in hydrolysis rate.

Addition of Endoglucanase to Cel7A Hydrolysis

The strongest effect on the Cel7A hydrolysis of SPS was obtained by addition of endoglucanase Cel7B (EGI). Adding Cel7B (0.5 μ*M*) at 24 h strongly increased the hydrolysis rate to such an extent that after the addition, between 24 and 48 h, 0.50 mg/mL products (cellobiose and glucose) were formed (Fig. 5B). The Cel7B activity had the effect of "restarting" Cel7A hydrolysis leading to sugar production at the same level as for Cel7A alone during the initial 24 h (0.50 mg/mL). In a control experiment, Cel7B was added to the reaction mixture after heating the substrate to 100°C for 10 min and thus inactivating Cel7A. No increase in hydrolysis rate could be observed. The control experiment showed the low cellulose conversion obtained only with the added endoglucanase Cel7B. This implies that the increased hydrolysis rate when Cel7B was added (Fig. 5B) was due to the increased activity of Cel7A caused by Cel7B addition. Thus, a likely explanation is that adding endoglucanase Cel7B activated unproductively bound exoglucanase Cel7A. This cooperation between the enzymes can be understood by an extension (presented in this paper) of the endo–exo synergy model.

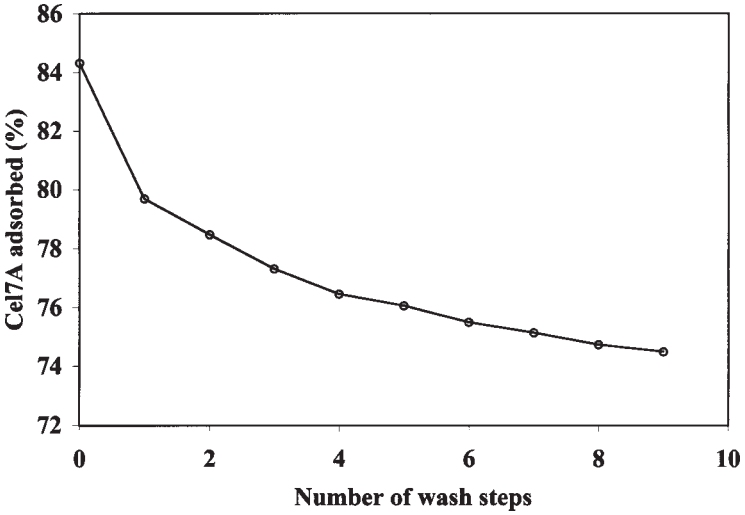

Fig. 6. Cel7A adsorption followed during wash of substrate with buffer solution. SPS was hydrolyzed with 1.4-µ*M* Cel7A and 0.1-µ*M* tritium-labeled Cel7A at 40°C in 50-m*M* Na-acetate buffer pH 4.8 for 24 h. Release of tritium-labeled Cel7A was determined in the repeated wash steps.

Desorption of Cel7A by Addition of Cel7B

To study the synergistic effect between Cel7A and Cel7B in more detail, it was desirable to measure changes in adsorption of Cel7A when Cel7B was added to the hydrolysis. To measure small adsorption changes of Cel7A, a sensitive method to quantify Cel7A was needed. Therefore, Cel7A was tritium-labeled according to the method described by Tack et al. *(31)*. Cel7A could then be quantified radiometrically. Lignocellulose hydrolysis was performed by adding tritium-labeled Cel7A to the hydrolysis mixture. After 24-h hydrolysis of SPS with Cel7A, 7.5% of added Cel7A was detected in free solution, which closely agrees with the adsorption data obtained with ion-exchange chromatography (Fig. 3).

Detailed studies of Cel7A desorption resulting from Cel7B addition demand removal of unspecifically bound enzyme. To facilitate these measurements, the substrate after 24-h hydrolysis with Cel7A was washed repeatedly. The samples were centrifuged and the supernatant was removed and new buffer was added, followed by a 15-min incubation to establish a new equilibrium. This was repeated to remove all low-affinity bound Cel7A. Desorption of Cel7A during the repeated equilibrations was followed by measuring release of tritium-labeled Cel7A. The result of the washing procedure is presented in Fig. 6. After nine equilibrations, 75% of the originally added Cel7A was still bound to the substrate. This suggests that Cel7A is bound with different affinities for the substrate. The low-affinity bound enzyme was removed in the repeated wash steps. The substrate with tightly bound Cel7A was used in the studies presented in Fig. 7.

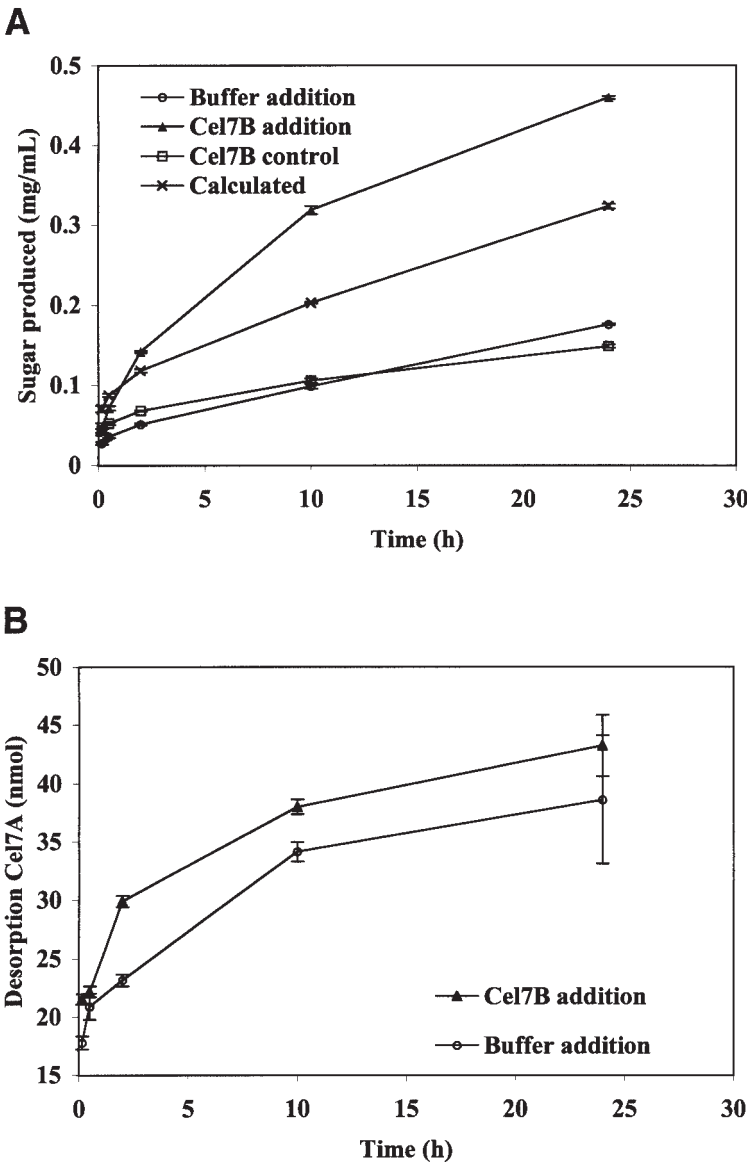

Fig. 7. Addition of endoglucanase Cel7B to hydrolyzed lignocellulose with tightly bound Cel7A after washing procedure presented in Fig. 6. SPS was hydrolyzed with 1.4-µ*M* Cel7A and 0.1-µ*M* tritium-labeled Cel7A at 40°C in 50-m*M* Na-acetate buffer for 24 h. The SPS concentration was 20 g/L (cellulose concentration 10 g/L). **(A)** Production of sugars (glucose, cellobiose) after addition of: (▲) 0.5-µ*M* Cel7B; (□) 0.5-µ*M* Cel7B after heating the substrate and deleting Cel7A activity; (○) buffer. The calculated sum for Cel7A plus Cel7B is shown by (×). **(B)** Desorption of tritium-labeled Cel7A after buffer (○) and Cel7B (▲) addition.

The principle for the experiment in Fig. 5B was followed with substrate that was incubated 24 h with Cel7A and with subsequent washing, as described above (Fig. 6). Adding Cel7B to the substrate hydrolyzed by

Cel7A strongly increased the hydrolysis (Fig. 7A). In a control experiment, where Cel7B was added to the hydrolysis after heating the substrate and thus inactivating Cel7A, there was no increase in hydrolysis compared with Cel7A alone. Thus, the results with the washed substrate were similar to results in Fig. 5 for Cel7A hydrolysis followed by Cel7B addition (Fig. 5B). This further supports the conclusion that endoglucanase activates the adsorbed, but unproductive, Cel7A. Figure 7B presents desorption of Cel7A, measured by detecting tritium-labeled Cel7A, following Cel7B addition. Adding Cel7B significantly increased desorption of Cel7A compared to the control when only buffer was added to the substrate. This result further strengthens an extension of the endo–exo synergy model. The desorption of Cel7A is unexpected within the framework of the classical endo–exo synergy model, where exoglucanase adsorption should increase after endoglucanase addition.

The Obstacle Model for Rate Decrease and Synergism

The observed endo–exo synergism is currently explained by endoglucanase internally cutting the cellulose chain, thereby making new substrate sites for cellobiohydrolase (exoglucanase) *(24)*. Alternative models to the observed endo–exo synergism have been proposed when experimental data have contradicted this model. Ryu et al. *(34)* suggested a model with competitive adsorption between endoglucanases and cellobiohydrolases. They proposed that endoglucanase adsorption facilitates desorption and activation of cellobiohydrolase. However, Nidetzky et al. *(35)* showed that simultaneous action of cellulases is not necessary to observe synergistic effects. Sequential action of endo- and exoglucanases on cellulose also showed synergism, therefore it is unlikely that competitive adsorption can explain endo–exo synergism. Recently, synergism models have been presented where Cel7A processive action is hindered by obstacles. In a surface erosion model, Väljamäe et al. *(4)* proposed that Cel7A processive activity would alter the cellulose surface. The hydrolytic action of Cel7A erodes the substrate where randomly left solitary chains may block hydrolysis of the chains in the layer beneath. Endoglucanase may remove such obstacles, which can explain the observed synergistic effect *(36)*. In complex lignocellulose substrates like SPS, it is probable that obstacles block Cel7A's processive mode of action. Based on experimental data in our laboratory, Karlsson and colleagues *(32,37)* have proposed a model where endoglucanase cleavage of cellulose chains decreases the cellulose chain length and thereby decreases the possibility for Cel7A to become blocked by an obstacle and become unproductively bound to the substrate. Figure 8 shows the extension of the endo–exo model to describe the observed synergism between Cel7A and Cel7B on complex lignocellulose substrates. Cel7A has been proposed to hydrolyze well-ordered or crystalline parts of the cellulose in a processive manner *(21–23)*. Cel7A liberates cellobiose from the reducing end of the cellulose chain *(23)* and the long tunnel-shaped active

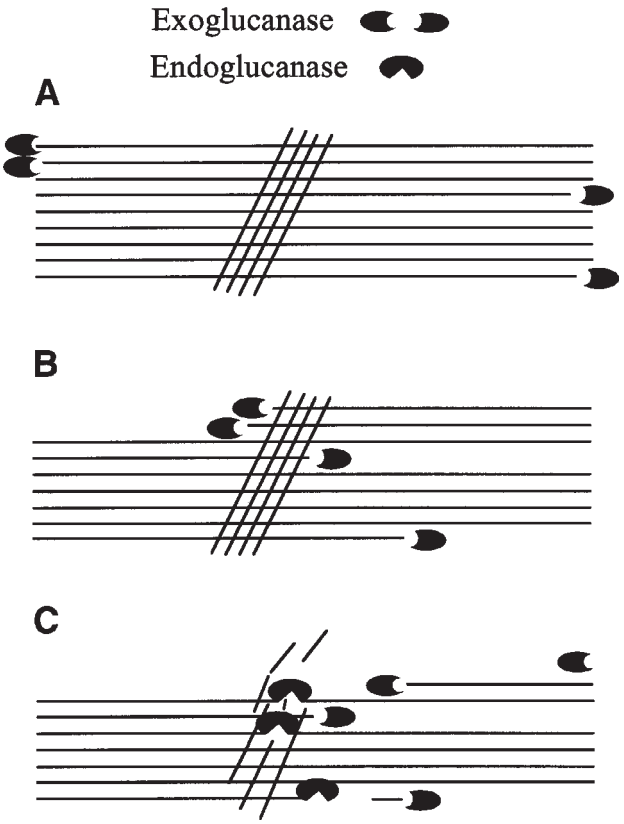

Fig. 8. Model for elucidating hydrolysis rate reduction and synergism during hydrolysis of pretreated lignocellulose. **(A)** Exoglucanases start hydrolyzing cellulose from the chain ends, e.g., Cel7A from the reducing ends and Cel6A from the nonreducing ends. The cellulose chain is hydrolyzed processively, i.e., the enzyme moves along the cellulose chain releasing cellobiose units by successive cleavages. **(B)** The processive action of the exoglucanase (Cel7A) is hindered by obstacles occurring in the substrate. **(C)** Obstacles formed by cellulose chains can be removed by endoglucanase (Cel7B) leading to the synergistic action of exo- and endoglucanases. If the endoglucanase has hydrolyzed the cellulose chain in front of the obstacles, the exoglucanase will reach the end of the cellulose chain before the obstacle. Thus, it will be liberated from the cellulose chain and be able to adsorb at another site.

site with several binding sites causes Cel7A to remain attached to the cellulose chain during the processive hydrolysis. The endoglucanase Cel7B has an open-cleft active site and is able to internally hydrolyze cellulose chains in the substrate *(38)*. Figure 5 shows that during Cel7A hydrolysis of cellulose, the enzymes become unproductively bound to the substrate, that is, the hydrolysis rate is low. One explanation is the presence of obstacles that block the processive action of Cel7A (Fig. 8). After a short hydrolysis time, the obstacles form unproductive binding sites for Cel7A. If the obstacles are less ordered cellulose, the ability of Cel7B to remove these obstacles for the processive action of Cel7A can explain some of the

observed synergism between Cel7A and Cel7B. This synergism mechanism would also operate when endoglucanase (Cel7B) cleaves a cellulose chain where an exoglucanase (Cel7A) is processively hydrolyzing, thus making the chain shorter. If the cellulose chain is hydrolyzed in front of the obstacle, the exoglucanase will come to the end of the cellulose chain before it reaches the obstacle (Fig. 8C), thus the exoglucanase can desorb from the substrate without getting "trapped" at an unproductive binding site.

Two experimental results presented in this paper favor the model in Fig. 8 as an extension to the classical endo–exo synergism model.

1. Figure 5 shows that Cel7A hydrolysis rate is low, and adsorption data show that practically all Cel7A is adsorbed. Thus, Cel7A is inactivated while remaining adsorbed to the substrate. The reason for the inactivation is not the depletion of substrate sites for Cel7A hydrolysis. Adding fresh Cel7A after 24-h hydrolysis of SPS with Cel7A (Fig. 5A) increase the hydrolysis rate showing that there are available substrate sites after 24-h hydrolysis; therefore, it is reasonable that abundant substrate sites exist in the beginning of the hydrolysis, when the hydrolysis rate is high. Endoglucanase production of cellulose chain ends for the exoglucanase cannot be the only explanation for the observed synergism, because low synergism in the initial stage of hydrolysis should be expected because of abundant sites. Synergism is as high, if not even higher, in the beginning (i.e., after a few minutes), as in the later stages of the hydrolysis (Fig. 2). Hence it is unlikely that Cel7A-Cel7B synergism would exclusively come from Cel7B producing new chain ends for Cel7A.
2. In the classical endo–exo synergism model an adsorption increase of Cel7A would be expected when Cel7B is added to a hydrolysis mixture with Cel7A. An increase in free ends, and subsequent increase in hydrolysis, should increase adsorption of the exoglucanase. This is not observed; on the contrary, desorption of Cel7A was observed (Fig. 7B) when Cel7B was added to the hydrolysis. Synergism between the two enzymes was observed simultaneously with desorption of Cel7A, which can be explained within a model where obstacles to Cel7A hydrolysis are reduced by endoglucanase activity.

Conclusions

During hydrolysis of steam-pretreated spruce with exoglucanase Cel7A and endoglucanase Cel7B, the hydrolysis rate rapidly decreases with hydrolysis time. A rapid, high adsorption of enzymes to the substrate was observed in the hydrolysis experiments. Thermal deactivation, product inhibition, or release of inhibiting soluble lignin components during hydrolysis was not the main reason for the decreased hydrolysis rate. Adding fresh substrate to an ongoing Cel7A hydrolysis increased hydrolysis rate, but a larger rate increase was obtained by adding fresh Cel7A. This implies that enzyme inactivation is more important for decreasing

hydrolysis rate compared to increased substrate recalcitrance. Inactivation of Cel7A is caused by unproductive binding of enzyme to the substrate during hydrolysis; an important conclusion that explains the decline in hydrolysis rate. Adding small amounts of Cel7B to previously hydrolyzed lignocellulose activated unproductively bound Cel7A and restarted the hydrolysis. Adding endoglucanase to substrate with unproductively bound Cel7A was followed by desorption of the Cel7A. These observations can be explained by extending the endo–exo synergy model where the effect of endoglucanase activity reduces obstacles to the processive action of Cel7A. The model explains the decline in hydrolysis rate for enzyme systems based on processive exoglucanase activity of cellobiohydrolases, that must have access to the synergistic activity of endoglucanases to lower unproductive binding.

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