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The Kinetics of Cellulose Enzymatic Hydrolysis

Implications of the Synergism Between Enzymes

**BY
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ABSTRACT

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The hydrolysis kinetics of bacterial cellulose and its derivatives by *Trichoderma reesei* cellulases was studied. The cellulose surface erosion model was introduced to explain the gradual and strong retardation of the rate of enzymatic hydrolysis of cellulose. This model identifies the decrease in apparent processivity of cellobiohydrolases during the hydrolysis as a major contributor to the decreased rates. Both enzyme-related (non-productive binding) and substrate-related (erosion of cellulose surface) processes contribute to the decrease in apparent processivity. Furthermore, the surface erosion model allows, in addition to conventional endo-exo synergism, the possibility for different modes of synergistic action between cellulases. The second mode of synergism operates in parallel with the conventional one and was found to be predominant in the hydrolysis of more crystalline celluloses and also in the synergistic action of two cellobiohydrolases.

A mechanism of substrate inhibition in synergistic hydrolysis of bacterial cellulose was proposed whereby the inhibition is a result of surface dilution of reaction components (bound cellobiohydrolase and cellulose chain ends) at lower enzyme-to-substrate ratios.

The inhibition of cellulases by the hydrolysis product, cellobiose, was found to be strongly dependent on the nature of the substrate. The hydrolysis of a low molecular weight model substrate, such as para-nitrophenyl cellobioside, by cellobiohydrolase I is strongly inhibited by cellobiose with a competitive inhibition constant around 20 μM , whereas the hydrolysis of cellulose is more resistant to inhibition with an apparent inhibition constant around 1.5 mM for cellobiose.

Keywords: Acetobacter, Cellobiohydrolase, Cellobiose, Cellulase, Cellulose, Diffusion, Endoglucanase, Hydrolysis, Inhibition, Kinetics, Model, Product, Substrate, Surface, Synergism, *Trichoderma reesei*.

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This thesis is based on the following papers that will be referred to by their Roman numerals:

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- II Väljamäe, P., Sild, V., Nutt, A., Pettersson, G. and Johansson, G. (1999) Acid hydrolysis of bacterial cellulose reveals different modes of synergistic action between cellobiohydrolase I and endoglucanase I. *Eur. J. Biochem.* 266, 327-334.
- III Väljamäe, P., Pettersson, G. and Johansson, G. (2001) Mechanism of substrate inhibition in cellulose synergistic degradation. *Eur. J. Biochem.* 268, 4520-4526.
- IV Väljamäe, P., Kipper, K., Pettersson, G. and Johansson, G. Cellulose synergistic hydrolysis can be described in terms of fractal-like kinetics. (manuscript submitted)
- V Gruno, M., Väljamäe, P., Pettersson, G. and Johansson, G. Inhibition of the *Trichoderma reesei* cellulases by cellobiose is strongly dependent on the nature of the substrate. (in manuscript)

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Abbreviations

BC	bacterial cellulose
BMCC	bacterial microcrystalline cellulose
CBD	cellulose-binding domain
CBH	cellobiohydrolase
CMC	carboxy-methyl cellulose
DP	degree of polymerisation
2-D	two-dimensional
3-D	three-dimensional
EG	endoglucanase
K_i	inhibition constant
NaAc	sodium acetate
<i>T. reesei</i>	<i>Trichoderma reesei</i>

1 Introduction

1.1 The substrate: Cellulose

Every year more than 40 billion tons of carbon are fixed through photosynthesis and incorporated into lignocellulose. Cellulose is a major component of plant cell walls and is the principal carbohydrate produced by plants, constituting up to 50% of the mass in trees. It is a homopolymer of β -1,4 linked D-glucose units. Because successive glucose residues are rotated by 180° relative to each other, cellobiose, rather than glucose, should be regarded as the repetitive unit in the cellulose chain (Fig. 1). The result is a fully extended, straight chain stiffened by intramolecular hydrogen bonds and excellently suited to interact with identical chains to form a highly regular crystalline structure.

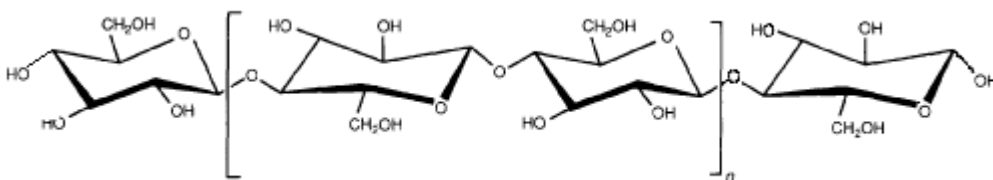


Figure 1. Structure of the single cellulose chain The smallest repeating unit, cellobiosyl moiety, is shown in brackets. Degree of polymerization (DP) is $2(n+1)$ glucose units.

The average degree of polymerization of native cellulose (DP) varies mostly between 1000 and 14 000 glucose units, depending on the source (Fan et al., 1980a). Almost perfect, huge cellulose crystals with a DP as high as 23 000 are produced by certain algae (Sugiyama et al., 1985; Brown, 1996). The cellulose chains have a strict polarity with one end containing a free C₁ semialdehyde group (reducing end) and the other containing a free 4`OH group (nonreducing end). The crystalline structure of cellulose has been one of the most studied structural problems in polymer science (reviewed in Hon, 1994; O`Sullivan, 1997). It was long thought that the cellulose chains in the crystal are oriented in antiparallel array, but in 1974 the groups of Sarko and Blackwell recognized the parallel orientation of chains in native cellulose (cellulose I) (Gardner and Blackwell, 1974; Sarko and Muggli, 1974). Still, the ¹³C CP/MAS solid-

state NMR studies revealed unexpected details in highly crystalline cellulose I which could be explained only by a system consisting of two distinct crystal phases, designated I α and I β (Atalla and VanderHart, 1984). Very recent studies revealed the hydrogen bonding system of the pure I β cellulose (Nishiyama et al., 2002). Native cellulose contains both I α and I β forms, although the relative abundance of these forms varies with the source of the cellulose. Algal and bacterial cellulose is rich in I α , whereas cellulose of plant origin is rich in I β . It has also been shown that the I α component is preferentially hydrolysed by cellulases (Hayashi et al., 1998). Besides the different crystal forms, native cellulose also contains less ordered, amorphous or paracrystalline regions. Native cellulose usually displays about 70% crystallinity. It is generally believed that there exist distinct amorphous parts between crystalline regions in cellulose (Fig. 11 in II).

The cellulose produced by the aerobic bacterium *Acetobacter xylinum* has recently become a substrate of choice for cellulase studies. The advantage of bacterial cellulose as a model substrate is that it is a pure cellulose and is available in never-dried form. The different levels of structural organisation of *Acetobacter* cellulose can be described as follows: glucan chains crystallize into microfibrils, intermicrofibrillar hydrogen bonding holds individual microfibrils into bundles, and these bundles are hydrogen bonded along their surfaces to form ribbons. The microfibrils are 3.0-3.5 nm each and about 50-80 microfibrils form ribbons between 40 and 60 nm wide (White and Brown, 1981). The average degree of polymerization of bacterial cellulose is around 1000-3000 glucose units .

Although the antiparallel orientation of cellulose chains is thermodynamically favored (cellulose II) the parallel orientation of chains stems from the mechanism of cellulose biosynthesis (for recent reviews in cellulose biosynthesis see Delmer, 1999; Reiter, 2002). Cellulose is synthesized by a cellulose synthetase such that two molecules of UDP-glucose are added at a time (Koyama et al., 1997). Cellulose synthetase is situated in the plasma membrane as a multisubunit complex. Each subunit synthesizes one cellulose chain at a time. The chains grow from the reducing end towards the nonreducing end and on the outside of the cell membrane. The aggregation of nascent chains is a non-enzymatic process (cell-directed self assembly) and occurs in parallel with their biosynthesis (Haigler et al., 1980). The number of synthetase subunits in a complex depends on the organism and can vary from rosettes with 36 subunits in higher plants to as many as 1000 subunits in some algae. The formation of crystalline structures is possible because the aggregation of synthetase subunits in the plasma membrane is such that the nascent chains are close to each other in space (Arioli et al., 1998). If substances that can

hydrogen bond and intercalate with cellulose chains, like the fluorescent brightener Calcofluor White ST, are added during the process of cellulose biosynthesis the formation of cellulose crystals is impaired and the product is amorphous, disordered cellulose (Haigler et al., 1980). The formation of highly crystalline cellulose also requires that the individual synthetase subunits work in a concerted manner. Sato et al., (2001) have proposed the existence of a membrane-bound endoglucanase which performs a so-called proofreading, i.e., if the polymerization of some chains becomes somehow restricted, then those chains will be hydrolytically cleaved to eliminate strain and enable the further polymerization of nearby chains. The crystalline areas of cellulose form tight arrays, which shield many of the glucosidic bonds from enzymatic attack. In addition, the presence of other components (i.e., hemicelluloses, pectin and xyloglucans) along with cellulose makes the plant cell wall very compact and a poorly accessible substrate (for review, see Brett, 2000).

1.2 The enzymes: Cellulases

The highly complex structure of plant cell walls has challenged microorganisms throughout evolution to develop systems that could handle cell walls efficiently. Organisms capable of processing cellulose-containing materials usually produce complex extracellular or membrane-bound cellulolytic systems comprising a combination of several enzymes. The complete degradation of cellulose to glucose requires the action of at least three types of enzymes: endo-1,4- β -glucanase, exo-1,4- β -glucanase (cellobiohydrolase) and β -glucosidase (reviewed in Beguin, 1990; Leschine, 1995; Beguin and Lemaire, 1996).

A large number of saprophytic soil bacteria, rumen bacteria and plant pathogens produce cellulolytic enzymes, but relatively few can utilize crystalline cellulose as a carbon source (for reviews on microbial cellulose utilization see Beguin and Aubert, 1994; Lynd et al., 2002). Over time, two different systems evolved to degrade cellulose; the `complete` systems are designed to exploit cellulose as a carbon source, whereas the `incomplete` systems effect relatively limited and localized hydrolysis. The incomplete systems can be found in plant pathogenic bacteria and symbiotic nitrogen-fixing bacteria (Gilbert and Hazelwood, 1993; Tomme et al., 1995a). In general, the total mass of cellulase secreted by cellulolytic bacteria is less than that secreted by filamentous fungi. However, most of the cellulase genes cloned to date are from bacterial sources, probably due to the relative ease of the cloning process (Beguin et al., 1992; Wood, 1992; Wilson and Irwin, 1999).

Microbial cellulose degradation in nature is performed in two quite different environments. In the anaerobic environment, the cellulolytic process is driven by a complexed system which is organized as a multienzyme associated with the cell surface of the microorganism. In aerobic environments, most microorganisms have non-complexed systems whereby they secrete a set of individual cellulases into the external milieu. Despite the fact that cellulose is the most abundant polymer on earth and is a major food source for many animal species, most omnivores and herbivores do not produce cellulases themselves. Ruminants, for example, utilize a highly specialized mixture of bacteria, fungi and protozoa to degrade cellulose under anaerobic conditions. The first animal cellulase was cloned from the termite *Reticulitermes speratus* (Watanabe et al., 1998). An endoglucanase gene has recently been cloned and the corresponding protein characterized from the blue mussel, *Mytilus edulis* (Xu et al., 2000; Xu et al., 2001).

1.3 *Trichoderma reesei* cellulases

Trichoderma reesei is a non-wood degrading filamentous fungus which appears to degrade plant material in the soil, plant litter in its natural environment. Culture filtrates from *T. reesei* contain a multitude of cellulolytic enzymes due to the great number of isoforms and products of partial hydrolysis (for review, see Kubicek, 1992). The amount of cellulases produced by *T. reesei* can be so huge that more than 20% w/w of the total carbohydrate consumed is secreted as cellulases (Esterbauer et al., 1991). Hitherto, seven genes encoding cellulolytic enzymes have been cloned: cellobiohydrolases CBH I (Cel 7A, according to the new designation scheme by Henrissat et al., 1998) (Shoemaker et al., 1983; Teeri et al., 1983) and CBH II (Cel6A) (Chen et al., 1987; Teeri et al., 1987); endoglucanases EG I (Cel7B) (Penttilä et al., 1986; van Arsdell et al., 1987), EG II (Cel5A) (Saloheimo et al., 1988), EG III (Cel12A) (Ward et al., 1993), EG IV (Cel61A) (Saloheimo et al., 1997) and EG V (Cel45A) (Saloheimo et al., 1994) and two β -glucosidases (Barnett et al., 1991; Takashima et al., 1999).

All *T. reesei* cellulases except EG III have a multidomain structure based on a catalytic domain (core) and a cellulose-binding domain (CBD) (Gilkes et al., 1991). CBD is connected to the core protein by a highly glycosylated linker peptide. This gives the whole molecule an elongated tadpole shape (180 Å -long in the case of CBH I) (Abuja et al., 1988a,b). The catalytic and binding domains can be separated following proteolytic cleavage with papain. Some properties of *T. reesei* cellulases are listed in Table 1.

Table 1
Some properties of the cellulases from *Trichoderma reesei*

Name	Designation	Molecular weight (kDa)	Isoelectric point (pI)	Position of CBD
CBH I	Cel7A	57	3.9	C
CBH II	Cel6A	53	5.9	N
EG I	Cel7B	55	4.5	C
EG II	Cel5A	50	5.5	N
EG III	Cel12A	25	7.5	lack of CBD
EG V	Cel45A	36	2.9	C

1.3.1 CBH I (Cel 7A)

CBH I is regarded as the key enzyme in the degradation of crystalline cellulose by *T. reesei*. It comprises about 60% of the total cellulolytic proteins of *T. reesei* and homologous enzymes are also abundant in other cellulolytic fungi, like CBH 58 (Cel7D) in *Phanerochaete chrysosporium* (Uzcategui et al., 1991). CBH I alone is able to hydrolyze crystalline cellulose extensively, although the rate of degradation is slow. The main product of hydrolysis is cellobiose (Fägerstam and Pettersson, 1980). CBH I is a glycoprotein containing 6% carbohydrate. The isoelectric point of CBH I is 3.9.

The crystal structure of the catalytic domain of CBH I revealed a β -sandwich structure with a 50 Å -long substrate-binding tunnel formed by the inner β -sheets and the extensive loops covering the outside (Divne et al., 1994; Divne et al., 1998). A hypothetical model of CBH I acting on the crystalline cellulose is depicted in Fig. 2. Ten glucosyl-unit binding subsites were defined along the active site tunnel named -7 to +3. The hydrolysis proceeds via a double-displacement mechanism resulting in retention of configuration at the anomeric C₁ carbon (Knowles et al., 1988; Claeysens et al., 1990). Site-directed mutagenesis identified the Glu212 as nucleophile and Glu217 as proton donor-acceptor (Ståhlberg et al., 1996). The catalytic pathway involves a covalent enzyme-glycosyl intermediate and cellobiose is the first product. It has been shown that the hydrolysis of cellooligosaccharides proceeds from the reducing end (Barr et al., 1996; Biely et al., 1993). The same was found to be true also for cellulosic substrates (Nutt et al., 1998; Imai et al., 1998). Despite some controversial evidence (Schmid and Wandrey, 1990; Ståhlberg et al., 1993), CBH I is regarded as a strict exoenzyme. Although there is no good method available to determine the processivity of cellulases, structural considerations and indirect results indicate that CBH I is a highly processive enzyme (Divne et al., 1994; Teeri, 1997; Boisset et al.,

2000), meaning that an already bound enzyme will not leave the cellulose chain before its complete degradation. However, Medve et al., (1998) estimated a processivity index of only 10-20, based on the ratio of produced cellobiose and glucose+cellotriose. The k_{cat} values for CBH I acting on cellooligosaccharides increases with the DP of the substrate and are 4.0 s^{-1} and 9.5 s^{-1} for cellotetraose and cellohexaose, respectively (Nidetzky et al., 1994a). In parallel with increasing k_{cat} values, the K_{m} decreases with increasing substrate DP (around $7 \text{ }\mu\text{M}$ and $3 \text{ }\mu\text{M}$ for cellotetraose and cellohexaose, respectively): the higher the DP of the cellooligosaccharide the higher is the specificity of CBH I for that substrate (Nidetzky et al., 1994a). Similar trends were found also for the homologous cellobiohydrolase from *Humicola insolens* (Schou et al., 1993). All of the above results are consistent with the multiple-subsite-tunnel active site topology of cellobiohydrolases. To date, there is very little information about limiting initial rate-based k_{cat} values for CBH I acting on crystalline cellulose. Due to the spatially heterogeneous nature of the cellulose substrate and the different binding modes of cellulase (productive and nonproductive) it is, regardless of the model used for interpretation, difficult to achieve conditions where the enzyme is truly saturated with substrate. This makes estimation of the true catalytic constant a difficult task. Also, since the catalysis involves a covalent intermediate it is not known whether k_{cat} represents the true glucosidic bond hydrolysis, the hydrolysis of the enzyme-glycosyl intermediate or the release of the first product (Sinnot, 1998). The failure of attempts to perform active site titration using model substrates with good leaving groups (Dr. Jerry Ståhlberg, personal communication) indicates that the hydrolysis of the enzyme-glycosyl intermediate is not rate limiting. Hydrolysis of para-nitrophenol cellobioside by CBH I is strongly inhibited by cellobiose with a competitive inhibition constant around $20 \text{ }\mu\text{M}$ (van Tilbeurgh and Claeysens, 1985; Claeysens et al., 1989; Vonhoff et al., 2000). Direct measurements of cellobiose binding or experiments based on competition between cellobiose and various ligands also result in dissociation constants around $20 \text{ }\mu\text{M}$ for cellobiose (van Tilbeurgh and Claeysens, 1985; Henriksson et al., 1999b). The inhibition by cellobiose of the hydrolysis of cellulosic substrates by purified CBH I has not been studied quantitatively, but qualitative data indicate that the action on native substrates is more resistant to inhibition.

1.3.2 CBH II (*Cel6A*)

CBH II, the other exoglucanase produced by *T. reesei* (around 20% of the total cellulase) has a similar structural organization to CBH I, with the small cellulose-binding domain connected to the catalytic domain via

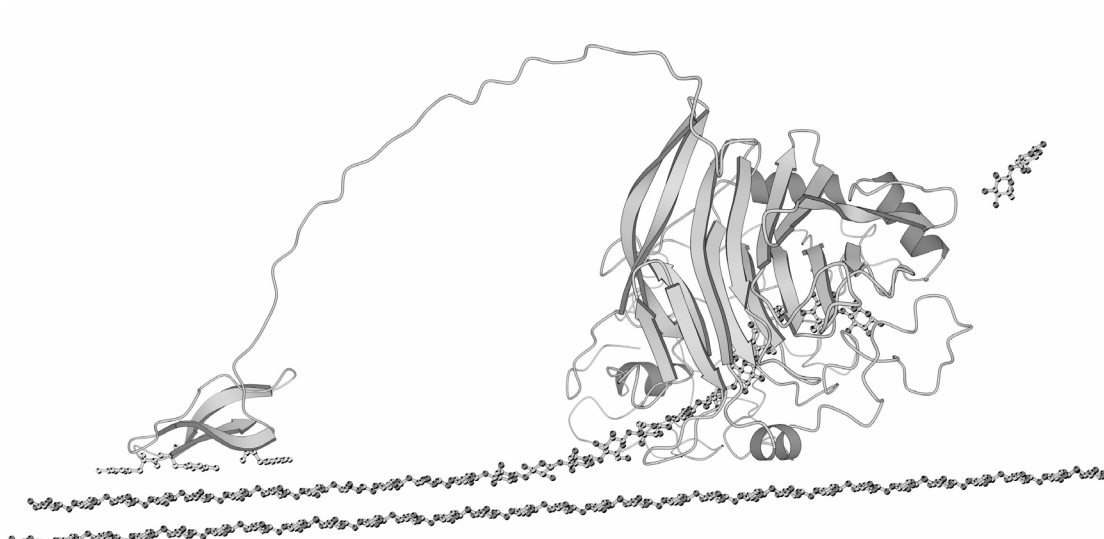


Figure 2. A hypothetical model of CBH I acting on the crystalline cellulose The picture was done with MolScript and kindly provided by Dr. Jerry Ståhlberg with permission from Dr. Christina Divne (© Christina Divne 1998).

a highly glycosylated linker region (Abuja et al., 1988b). Unlike CBH I, CBH II prefers to act on the cellulose chain from the nonreducing end and it is also thought to be less processive (Chanzy and Henrissat, 1985; Nutt et al., 1998; Boisset et al., 1998). The hydrolysis product is mainly cellobiose and hydrolysis proceeds via a single-displacement mechanism resulting in the inversion of configuration at the anomeric C₁ carbon (Knowles et al., 1988; Claeysens et al., 1990). It has been shown that hydrolysis by CBH II has some endo-character, placing the enzyme somewhere between the strict exoglucanases and endoglucanases according to its mode of action (Teeri, 1997; Boisset et al., 2000). The three-dimensional structure of the catalytic domain of CBH II revealed an α/β protein with a fold totally different from that of CBH I (Rouvinen et al., 1990). The fold is similar, but not identical, to the frequently observed (β/α)₈ barrel in triosephosphate isomerase topology (TIM-barrel). Two extensive loops at the carboxyl-terminal end of the barrel form an almost perfectly enclosed 20Å-long tunnel. These loops can undergo movements, leading to the closing or opening of the tunnel roof (Zou et al., 1999; Varrot et al., 1999) Apparently, these movements are responsible for the observed endoactivity and lower processivity of CBH II. The tunnel contains four clear binding sites for glucosyl units that are defined as -2 to +2. Two catalytically important aspartate residues have been identified, of which Asp 221 acts as general acid

catalyst. Recently, the role of Asp 175 in electrostatic stabilization of positively charged transition state was identified (Koivula et al., 2002). The k_{cat} values for CBH II acting on cellooligosaccharides increases with increasing DP of the substrate and the maximum k_{cat} of 12-14 s⁻¹ is observed with cellohexaose (Harjunpää et al., 1996). This observation is in accordance with the presence of two additional glucosyl-unit binding sites in CBH II; namely, +3 and +4 (Zou et al., 1999). Site-directed mutagenesis of tryptophan W 272 in site +4 did not alter the specificity constants of CBH II for the hydrolysis of cellooligosaccharides, indicating that binding to this site is not necessary for transition state stabilisation (Koivula et al., 1998). However, the activity of W272A and W272D mutants on BMCC was drastically reduced, whereas the binding was not altered, indicating a crucial role of W 272 in crystalline cellulose degradation. Since the activity value for cellulose degradation was found to be at least an order of magnitude less than that for soluble oligosaccharides, the authors suggested that the bottleneck in the hydrolysis of cellulose by CBH II is not the hydrolysis of glycosidic bond per se (Koivula et al., 1998).

1.3.3 Endoglucanases

T. reesei produces at least four endoglucanases EG I, EG II, EG III and EG V. One of the most abundant endoglucanases is EG I (Cel7B) which accounts for 5-10% of the total cellulase (Bhikabai et al., 1984). The catalytic domain of EG I is structurally homologous to that of CBH I, with 45% identity (Penttilä et al., 1986; van Arsdell et al., 1987). The 3-D structure of the catalytic domain of EG I shows a very similar fold to that of CBH I. However, four loops that form the tunnel in CBH I are partly or fully deleted in EG I, resulting in an open groove-shaped active site rather than the long enclosed tunnel of CBH I (Kleywegt et al., 1997). This active site topology is consistent with the endo-type mode of action of EG I (Teeri, 1997). EG I acting on cellulose produces nearly equal amounts of glucose and cellobiose together with some cellotriose (Karlsson et al., 2002). Besides the activity on cellulose, EG I has a significant xylanolytic activity (Lawoko et al., 2000). Another *T. reesei* endoglucanase, EG II (Cel5A), also accounts for 5-10% of the total cellulases. EG II belongs to the glycosyl hydrolase family 5 and hydrolysis of glycosidic bond occurs via a double-displacement mechanism. The 3-D structure of EG II is not known to date. The hydrolysis products comprise equal amounts of glucose and cellobiose together with traces of cellotriose (Medve et al., 1998; Karlsson et al., 2002). An endo-processive character, as in the case of CBH II, of EG II in the hydrolysis of bacterial cellulose and its cellulose II derivative with

antiparallel orientation of chains (Kuga et al., 1993) was recently proposed (Amano et al., 2002).

A third endoglucanase, EG III (Cel12A) is a small 25 kDa protein. In contrast to other *T. reesei* cellulases, EG III does not have a CBD. Recently, the 3-D structure of EG III was determined (Sandgren et al., 2001). It has been proposed, based on studies of the EG III analogue from *Phanerochaete chrysosporium* (EG 28) that, owing to its small size, EG III can initiate the cellulolytic attack through the disintegration of cellulose fibers (Henriksson et al., 1999a). An expansin-like activity of EG III was also demonstrated (Yuan et al., 2001), but the mechanism of this type of activity is not yet known. Recent comparative studies of the *T. reesei* low-molecular-weight endoglucanases EG III and EG V (Cel45A), together with the well-studied EG I and EG II, revealed turnover numbers of 118 s^{-1} , 65 s^{-1} and 14 s^{-1} on cellopentaose for EG I, EG II and EG III, respectively, whereas EG V did not hydrolyze the pentaose (Karlsson et al., 2002).

1.4 Enzymatic hydrolysis of cellulose: synergism between enzyme components

It was Reese and his coworkers who first suggested a mechanism for the enzymatic breakdown of cellulose which involved a C_1 and C_x components (Reese et al., 1950). In essence, the conversion of native cellulose to soluble sugars was pictured to be a two-step process. The C_1 component was believed to "activate" or disaggregate the cellulose chains so that enzymes classified as C_x could carry out the depolymerization. They proposed that microorganisms that could grow only on soluble forms of cellulose, such as carboxymethyl cellulose (CMC), synthesized only the C_x component, whereas microorganisms capable of growing on highly ordered forms of cellulose produced both C_1 and C_x . Due to inability to produce culture filtrates active against crystalline cellulose, the early studies were focused on the C_x components. However, the discovery in 1964-1965 that culture filtrates prepared from certain strains of *T. viride* and *T. koningii* were capable of extensive hydrolysis of native cellulose was a turning point in the study of cellulases: First then could the search for a C_1 component really begin. In 1972, three independent research groups made the important discovery that the C_1 component was, in fact, a hydrolytically active enzyme, cellobiosyl hydrolase (cellobiohydrolase) (Pettersson et al., 1972; Nisizawa et al., 1972; Wood, 1972; Wood and McCrae, 1972). Cellobiohydrolase was found to act synergistically with the C_x components to degrade crystalline cellulose. It was therefore proposed that C_x (CMC-ase) acts as an endoglucanase to produce available chain ends on cellulose which are substrates for

cellobiohydrolase (Avicelase). It turned out to be the C_x component that initiates the cellulose breakdown rather than the C_1 proposed by Reese et al., (Pettersson et al., 1972; Nisizawa et al., 1972; Wood, 1972; Wood and McCrae, 1972). Still, the hunt for the hydrolytically inactive C_1 analogue continued and the concept of amorphogenesis was introduced. Amorphogenesis has been attributed to hydrogen peroxide produced by some enzymes, some iron-containing proteins in fungal filtrates, or even to CBH I produced by *T. reesei* (Chanzy et al., 1983). An 11 kDa non-hydrolytic fibril-forming protein was isolated from culture filtrate of *T. reesei* and its adsorption properties to cellulose were characterized (Banka et al., 1998; Banka and Mishra, 2002). Another non-hydrolytic protein from *T. reesei*, called swollenin, was also characterized and found to be similar to the plant cell-wall-extending enzymes, expansins, but unlike expansins, swollenin has a putative CBD connected via a linker region to the expansin-like domain (Saloheimo et al., 2002). In 1986, Klyosov et al., suggested that C_1 can be regarded not as component but rather as a property of an enzyme to adsorb to the cellulose. He further elaborated his theory and proposed that the tightly bound enzymes initiate the attack at disturbed regions of the crystalline cellulose and disperse the structure through a mechanochemical action, creating more-accessible areas of attack for the weakly bound, more-mobile enzymes that will carry out the catalytic reaction. Therefore, synergism can be observed between tightly-bound and weakly-bound cellulases (Klyosov, 1990). In 1991, Din et al., put forward the hypothesis that C_1 activity resides not in a system distinct from C_x but in a discrete domain of each enzyme. Cellulose-binding domains initially defibrillate the substrate and render it more susceptible to the action of the catalytic core (Din et al., 1991; Din et al., 1994).

Regardless of whether the initial amorphogenesis is crucial for hydrolysis, it is now realized that efficient hydrolysis of crystalline cellulose requires the cooperative action of three kinds of enzymes (Woodward, 1991; Tomme et al., 1995a): namely, cellobiohydrolases, endoglucanases and β -glucosidase. The role of β -glucosidase is in the hydrolysis of cellobiose, a product inhibitor of the cellobiohydrolases.

The mechanism for so-called endo-exo synergism proposed in 1972 is widely accepted, although the actual situation might be more complicated than this simple sequential attack by endoglucanases and cellobiohydrolases. The observed synergism between endo- and exo-type cellulases is dependent on the relative proportions among the enzyme components (Henrissat et al., 1985; Beldman et al., 1988). Furthermore, synergism was found to be dependent also on the degree of saturation of the cellulose substrate with enzymes, where the highest degree of synergy was found at non-saturating enzyme concentrations (Woodward et al.,

1988a,b). Apart from enzymes, the degree of synergism depends on the nature of the cellulose (Mansfield et al., 1999). Synergism is more pronounced on the semicrystalline substrates with high DP, such as cotton and BC, than on substrates with very high crystallinity, such as *Valonia* cellulose and/or lower DP, such as Avicel and BMCC (Henrissat et al., 1985; Samejima et al., 1998). The synergistic effect has been shown to be reciprocal, i.e., that not only the action of endoglucanase can promote the action by cellobiohydrolase but also *vice versa* (Nidetzky et al., 1994b). It has been proposed that the processive action of cellobiohydrolase creates cellulose crystals with a more disordered surface. An endoglucanase can access the chain from this disordered surface more readily than from a highly ordered regular lattice. Atomic force microscopy observations revealed that CBH I caused tracking of cotton fibers, whereas hydrolysis by EG II resulted in smoothing of the fibre surface (Lee et al., 2000). The existence of a loose complex between EG and CBH on the cellulose surface was also proposed (Wood and McCrae, 1978; Woodward, 1991). The idea was further supported by the observations that some bacterial cellulases form a large complex called a cellulosome. It was suggested that such a complex would have the advantage that components synergistically active would be close to each other on the cellulose surface and the diffusion time for a CBH to reach free chain ends generated by EG would be reduced. In their theoretical study, Fenske et al., (1999) demonstrated that surface dilution of synergistic components can lead to an apparent substrate inhibition, a phenomenon observed already in the 1970-ies (Howell and Stuck, 1975; Okazaky and Moo-Young, 1978) but still not explained mechanistically (Ryu and Lee, 1986; Liaw and Penner, 1990; Huang and Penner, 1991; Ortega et al., 2001). The effect of proximity was demonstrated by fusing together *Clostridium stercorarium* endoglucanase CelZ and exoglucanase CelY from the same organism. The adduct exhibited a higher degree of synergism than did the separate components (Riedel and Bronnenmeier, 1998). Although the close proximity of synergistic components on the cellulose surface seems to be important, the existence of real complexes as a key factor responsible for efficient synergistic degradation is contradicted by the early observation that cellulases from different organisms act synergistically (cross synergism) (Wood, 1969; Wood et al., 1980; Coughlan et al., 1987). It seems unlikely that proteins from different organisms should evolve to have specific interaction sites. Synergism between two cellobiohydrolases has also been reported (Fägerstam and Pettersson, 1980; Henrissat et al., 1985; Niku-Paavola et al., 1986). This type of synergism is more difficult to explain on the basis of mechanism. Wood and McCrae (1986) proposed that different CBH-s can have specificity towards sterically different nonreducing ends (at that

time it was believed that all CBH-s act from the nonreducing end of the cellulose chain). The finding that CBH-s display different directionality on the cellulose chain (CBH I acts from the reducing end, whereas CBH II prefers the nonreducing end) lead to the explanation that the action of a CBH with one directionality will expose buried chain ends that can be acted upon by another CBH with the opposite directionality (Barr et al., 1996; Gilkes et al., 1997). Some authors explained the exo-exo synergism also in terms of a partial complex formation between CBH I and CBH II (Tomme et al., 1990; Kim et al., 1998). When it was shown that some CBH-s (those belonging to the glycosyl hydrolase family 6) are not strict exo-acting enzymes but can occasionally have some endo-character, the conventional mechanism for endo-exo synergism was proposed also to be responsible for the observed synergism between different CBH-s (Penttila et al., 1987; Medve et al., 1994; Varrot et al., 1999; Boisset et al., 2000).

1.5 Kinetics of cellulose hydrolysis

All earlier studies of cellulose hydrolysis kinetics were restricted to crude cellulase mixtures. This made the interpretation of time curves extremely complicated. Regardless of the cellulase used, it was often found that the hydrolysis rate decreased far more rapidly than expected from the total degree of solubilisation. Resultant time curves of hydrolysis exhibited a typical biphasic pattern. The cause of this gradual drop in reaction rates is not fully understood, but it has been postulated that both enzyme- and substrate-related properties contribute to this effect (for review see Mansfield et al., 1999). Conflicting observations have prevented scientists from identifying a single factor as the sole cause of the gradual loss of efficiency during the hydrolysis of cellulose. In general, the proposed explanations fall into three distinct groups.

- a) Bulk cellulose substrate contains several regions that differ in susceptibility to enzymatic attack. Consumption of the small amount of easily degradable parts in cellulose causes the rate to fall.
- b) Strong inhibition of cellulases by the reaction product, cellobiose.
- c) Inactivation of the cellulase

The simplest model for the hydrolysis kinetics is based on the assumption that cellulose consists of many different regions, each with its own rate constant for enzymatic hydrolysis, and that the hydrolysis proceeds as a first-order reaction with respect to the concentration of each region (Sattler et al., 1989; Nidetzky and Steiner, 1993). Since cellulose is known to consist of amorphous and crystalline regions, it has long been suggested that the high initial rates of hydrolysis are due to the amorphous regions: As the reaction proceeds and the amorphous regions

become depleted, the overall rate slows to the value corresponding to the hydrolysis of crystalline regions (Fan et al., 1980; Wald et al., 1984). The above hypothesis seems to be especially relevant in the case of EG-s. They exhibit a very low and abruptly decreasing activity toward crystalline substrates, whereas the activity toward amorphous substrates is far higher than that of CBH-s. Pretreatment of crystalline cellulose with EG leads to a strong decrease in the rate of a subsequent hydrolysis with the same EG after washing of the pretreated cellulose (Nidetzky et al., 1994b; Zhang et al., 1999). These results directly indicated that cellulose becomes more resistant to enzymatic attack as the hydrolysis proceeds. However, prehydrolysis of cellulose with cellobiohydrolases did not cause a dramatic decrease in the rate of a subsequent hydrolysis (Nidetzky et al., 1994b). Several investigators have also found no significant changes in substrate crystallinity as the hydrolysis proceeds, but one can speculate whether such dramatic changes should be expected (Ohmine et al., 1983; Lenz et al., 1990). Since native cellulose crystals contain both I α and I β forms and it has been shown that the I α component is more susceptible to enzymatic attack (Hayashi et al., 1998) one can ascribe the decrease in the hydrolysis rate to exhaustion of the I α form. Since the decrease in hydrolysis rate was often seen to be proportional to the rate itself it was suggested that the enzyme somehow becomes inactivated during the process (Howell and Mangat, 1978; Ohmine et al., 1983; Desai and Converse, 1997). The adsorption of cellulase to cellulose is a prerequisite step for hydrolysis. Many investigators found that the hydrolysis rate was proportional to the specific surface area of cellulose. Much of the specific surface area of wood-derived cellulose is within the pores and capillaries. In native unpretreated cellulose only a small fraction of the pores are accessible to cellulase. Grethlein found that only 20% of the pore volume was accessible to a solute with a diameter of 51 Å. Pretreatment of cellulose with acids or at high temperature leads to changes in pore size distribution so that more of the surface area within the pores becomes accessible to cellulases of a certain size (Stone et al., 1969; Grethlein, 1985). It was suggested that the inactivation of the enzyme is due to diffusion into and entrapment of enzyme molecules in the small pores present in cellulose (Tanaka et al., 1986; Tanaka et al., 1988; Converse et al., 1988). The so-called “tethering” hypothesis was put forward by Desai and Converse. According to this hypothesis, the adsorbed enzyme, after catalyzing the hydrolysis of few bonds, remains tethered at a site from which it cannot reach potentially active bonds. Hence, freshly adsorbed enzyme would be more active than previously adsorbed enzyme (Desai and Converse, 1997). Recently, the declining rate of lignocellulose hydrolysis was explained in terms of inactivation of *T. reesei* CBH I due

an increase in the fraction of non-productively bound enzyme with time (Eriksson et al., 2002).

Strong inhibition of CBH-s by the reaction product, cellobiose, has also been reported as a major factor responsible for the decline in rate (for review of early studies, see Holtzapple et al., 1990). Whereas β -glucosidase can hydrolyse cellobiose into glucose, thus relieving the product inhibition, that enzyme itself is inhibited by glucose. Most of our knowledge about the strength of the product inhibition is based on studies with low molecular weight chromogenic model compounds, such as para-nitrophenyl cellobioside (pNPC). Hydrolysis of pNPC by CBH I is inhibited by cellobiose with a competitive inhibition constant of 20 μ M (van Tilbeurgh and Claeysens, 1985; Claeysens et al., 1989; Vonhoff et al., 2000). Such a strong product inhibition would appear to be a drawback from a biological point of view, limiting the levels of soluble sugars that can be produced. The inhibition of cellulases acting on native substrates is more difficult to assess and the obtained results are often controversial. In most cases, competitive or noncompetitive inhibition has been observed (for review, see Holtzapple et al., 1990). As pointed out by Gusakov and Sinitsyn (1992), the reasons for such confusion may be the following.

1. In a product inhibition study, a crude cellulase complex is usually considered as one single enzyme. The observed inhibition thus reflects the inhibition of the enzyme component that is rate limiting. The rate-limiting enzyme component in the synergistic cellulase mixture can depend on the nature of the substrate, the ratio of different components in the mixture as well as on the enzyme to substrate ratio.
2. Cellulosic substrates are insoluble and may differ in structure, crystallinity, and specific surface area. All of those parameters can affect the hydrolysis kinetics.
3. In a product inhibition study, when a significant amount of the product is added to the reaction system at the start of hydrolysis, an accurate determination of the initial rate of the same product formation at this high background is very difficult.

Several attempts have been made to overcome the last problem by monitoring the initial rates of dye release from dyed cellulose derivatives (Holtzapple et al., 1984; Gusakov et al., 1985; Holtzapple et al., 1990). However, this approach fails for cellobiohydrolases where the tunnel-shaped active sites cannot accommodate bulky dye groups. The activity of endoglucanases as well might be influenced by the dye groups. To date there is a lack of quantitative information about product inhibition based on initial rate measurements by single cellulase components. At the same time, many qualitative studies that are based on comparison of the

hydrolysis rates by cellulases with and without supplemented β -glucosidase have been made. From these studies the conclusion can be drawn that although the cellobiose is a strong inhibitor of the CBH-s acting on low molecular weight substrates the hydrolysis of cellulose is more resistant to inhibition.

1.6 Adsorption of cellulase to cellulose: the roles and function of cellulose-binding domains (CBD-s)

Among the different cellulases, some prefer amorphous substrates whereas others are also able to attack the highly ordered, crystalline cellulose. Comparative studies of many different cellulases have been used to identify common features enabling the enzyme to hydrolyze cellulose crystals (Wilson et al., 1985; Davies and Henrissat, 1995). One of these features is the relatively early observation that tight adsorption is linked with good catalytic activity toward the solid substrate (Klyosov, 1990). Structural characterization of a wide variety of different cellulose-degrading enzymes has shed light into the molecular basis of this important observation. Most, if not all, cellulases that are effective against crystalline cellulose share a modular structure composed of a catalytic domain linked to a distinct cellulose-binding domain (Tomme et al., 1995b). The overall binding efficiency of the enzyme (of both fungal and bacterial origin) is greatly enhanced by the presence of the CBD and the enhanced binding clearly correlates with better activity towards insoluble cellulose (van Tilbeurgh et al., 1986; Tomme et al., 1988; Gilkes et al., 1988; Reinikainen et al., 1992, 1995; Ståhlberg et al., 1993; Kruus et al., 1995). Single point mutations of the CBD of *T. reesei* CBH I have been shown to produce mutants with gradually descending affinities and subsequently decreased activities toward crystalline cellulose (Reinikainen et al., 1995). Despite their apparent importance in crystalline cellulose degradation we still lack a decisive answer as to how the CBD-s work as a part of hydrolytic enzymes (Linder and Teeri, 1997). It is now well established that the removal of the CBD has little influence on activity of cellulases toward soluble substrates and amorphous cellulose. It has been suggested that the CBD enhances the enzymatic activity of cellulase merely by increasing the effective enzyme concentration at the substrate surface (Ståhlberg et al., 1991) or possibly by promoting the solubilisation of single glucan chains off the cellulose surface (Knowles et al., 1987; Teeri et al., 1987; Reinikainen et al., 1995; Tormo et al., 1996). At the same time, the binding via CBD can also lead to a population of non-productively bound enzymes (Ståhlberg et al., 1991; Srisodsuk et al., 1993). Recently, it was shown that CBDs can promote the enzyme activity toward different regions in crystalline

cellulose, thus determining the substrate- or more precisely, sub-substrate specificity (Carrard et al., 2000). However, a targeting function alone does not seem likely as a general answer concerning the CBD function in all different cellulases. This is because the cellulase catalytic domains have very different modes of action and roles in the total hydrolysis process, and the properties of their respective CBDs must have been optimized according to these different demands (Couthino et al., 1992; Irwin et al., 1994; Linder et al., 1995a,b; Tomme et al., 1995b,c). It has been often found that CBDs appear to bind irreversibly to cellulose (Ong et al., 1989; Nidetzky et al., 1994; Millward-Sadler et al., 1994).

However, the enzymes should undergo a dynamic process of adsorption and desorption of both domains, allowing processive hydrolysis or relocation to new enzymatically accessible sites on the cellulose. Indeed, more recently it has been shown that the binding of the *T. reesei* CBH I CBD as well as intact CBH I to crystalline cellulose is fully reversible (Linder and Teeri, 1996; Palonen et al., 1999). It has been suggested that the cooperative binding of both domains can give a rise to lateral diffusion of the enzyme on the cellulose surface. The non-productively bound enzymes can thus be regarded as having “stand-by” status. Surface diffusion rates for a fluorescence-labelled bacterial cellulase and its separate CBD from *Cellulomonas fimi* on crystalline *Valonia* cellulose have been measured by direct observation of the process in a confocal microscope (Jervis et al., 1997). One must always bear in mind that the binding can occur through both of the domains, simultaneously and/or separately. Cooperative binding of domains was demonstrated by following the binding of a double CBD construct to crystalline cellulose (Linder et al., 1996). At the same time, the cellulose surface can be regarded as a continuous array of overlapping binding sites (Gilkes et al., 1992; Sild et al., 1996). All of those factors can lead to an apparent irreversibility of binding. However, using improved experimental approaches, Carrard and Linder (1999) demonstrated that, contrary to the situation with CBH I CBD, the binding of *Trichoderma reesei* CBH II CBD to crystalline cellulose was, indeed, irreversible. At the same time, the binding of intact CBH II was found to be only partly irreversible (Palonen et al., 1999).

One of the early suggestions concerning the role of the CBDs was that they might help to loosen individual cellulose chains from the cellulose surface prior to its actual hydrolysis, comparable to that of the C₁ component proposed by Reese et al., 1950, (Knowles et al., 1987; Teeri et al., 1987, 1992). It has been shown that at least some family II CBDs (bacterial CBDs) can penetrate into cellulose fibrils and loosen up the fibril structure (Din et al., 1991; Din et al., 1994). Simultaneous addition of separated family II CBD and catalytic domain resulted in synergy

between these domains in the hydrolysis of cotton cellulose (Din et al., 1994). Similar results have not so far been obtained with CBDs from other families, and even with family II CBD the effect is revealed only with cotton as substrate and not on microcrystalline cellulose (Ståhlberg et al., 1991; Wilson et al., 1995; Tomme et al., 1995c; Esteghlalian et al., 2001). However, it was reported that simultaneous action of CBH I CBD and EG I from *Penicillium janthinellum* resulted in synergistic degradation of Avicel cellulose (Gao et al., 2001).

1.7 Possible applications of cellulases

1. Production of ethanol and other commodity products from the cellulosic biomass: The central technological impediment to more widespread utilization of this important resource is the general absence of low-cost technology for overcoming the recalcitrance of cellulosic biomass. To achieve total competitiveness, a 10-fold increase in specific activity- or production efficiency of cellulase is required (Himmel et al., 1999; Lynd et al., 2002).
2. In the paper industry: cellulases are used in pulp refinement and in paper recycling, e.g., removal of ink from recycled paper.
3. In the textile industry: cellulases are well established in textile wet processing as agents for fibre and fabric surface modification. The most known applications are the ageing of fabric surfaces, like the stone-washed look of denim garments, and also the cleaning and renewing of fabric surfaces from microfibrils, fuzz and loss fibres.
4. In pharmacy: cellulases have been used in the separation of enantiomers from racemic mixture of drugs.

2 Purposes of the study

The main purpose of this study was to explain the rate retardation in cellulose enzymatic hydrolysis in terms of mechanism. The study was, more specifically, focused on synergistic cellulose degradation.

3 Present investigation

3.1 Materials and assays

3.1.1 Cellulose substrates

Bacterial cellulose (BC) Cellulose produced by *Acetobacter xylinum* or its derivatives was used throughout this study. Acetobacter cellulose is a

commercially available product under the trademark CHAOKOH[®] coconut gel in syrup (Thep. Padung Porn Coconut Co. Ltd, Bangkok, Thailand). For laboratory use the commercial product (ca. 1 mL cellulose cubes) was washed thoroughly with tap water to remove soluble sugars. Cellulose was further purified by boiling it (1 mg/mL for cellulose) in 1% NaOH under vigorous stirring for 1-2 days (the hydroxide solution was replaced 3-4 times during this procedure). After alkaline treatment the cellulose cubes were washed with distilled water until neutral and homogenized with a Waring blender until it was possible to pipet the resulting suspension (usually a few minutes). The slurried BC was washed with distilled water and finally with 50 mM NaAc buffer, pH 5.0, by repeated centrifugations (3000 g, 5-10 min) and re-suspension steps. The BC was stored in 50 mM NaAc buffer, pH 5.0 at 5° C until use. *Bacterial microcrystalline cellulose (BMCC)* was prepared from BC as follows: the solvent was replaced by 1M HCl and the suspension (around 2 mg/mL for cellulose) was boiled under continuous stirring (500 r.p.m.) for 5 h. Acid-treated cellulose was neutralized with NaOH and washed thoroughly with distilled water and buffer as described for the preparation of BC above.

Amorphous cellulose was prepared from BMCC or BC by the following procedure: Freeze-dried cellulose was dissolved in a solution of 10 % (w/v) LiCl in dry N,N-dimethylacetamide to give a cellulose concentration of 5mg/mL. After dissolution, the sample was diluted with N,N-dimethylacetamide to a LiCl concentration of 1%. An equal volume of 98% ethanol was then added dropwise to the cellulose solution under vigorous stirring. Finally, 1/3 the volume of water was added dropwise and the suspension was left under stirring overnight. After regeneration, the cellulose was washed thoroughly with water and reaction buffer.

[³H]-cellulose. Labelling of the reducing ends on cellulose with [³H] was carried out as described in Nutt et al., 1998. Labelled cellulose was stored in glycerol and washed thoroughly with water and reaction buffer before use. The specific activity of labelled cellulose was 29400 CPM/mg and 33400 CPM/mg for [³H]-BC and [³H]-amorphous cellulose, respectively. Labelled cellulose samples were completely solubilized by a mixture of *T. reesei* cellulases prior to analysis of specific activity.

3.1.2 Cellulases

Cellulases were purified from culture filtrates of *Trichoderma reesei* strain QM 9414. CBH I, CBH II, EG I and EG II were purified essentially as described in Bhikhabhai et al., 1984; Saloheimo et al., 1988. EG III was purified according to Håkansson et al., 1978. CBH I core proteins were obtained by papain cleavage of the purified intact enzymes and

isolated as described in van Tilbeurgh et al., 1986. The catalytically inactive E212Q mutant of CBH I (Ståhlberg et al., 1996) was a generous gift from Dr. Jerry Ståhlberg (Department of Molecular Biology, University of Uppsala/ Agricultural University, Sweden). If necessary, CBH I, CBH II and EG II were further purified on a Superose-12 column connected to the HPLC solvent delivery system (LKB) as follows: the column (104 mL total volume) was equilibrated with 0.5M ammonium-sulphate in 100 mM NaAc buffer, pH 5.0. 700 μ L of concentrated protein solution (up to 100 absorbance units at 280 nm) was applied to the column and eluted isocratically with equilibration solution at a flowrate of 1 mL/min. Elution was followed by monitoring the absorbance at 280 nm and fractions were collected. The enzymatic activity in fractions was followed by assays for endoglucanase (using CMC as substrate) and cellobiohydrolase (using BMCC as substrate). The purity of the enzymes was confirmed by SDS/PAGE.

3.1.3 Hydrolysis kinetics

Enzymatic hydrolysis was carried out in 1.5 mL Eppendorff tubes by incubating cellulose suspensions in 0.05M NaAc buffer, pH 5.0, with enzyme at 25 °C without agitation. The reaction was initiated by addition of enzyme or a mixture of enzymes followed by rapid Vortex mixing and stopped after defined times by addition of 1.0 M methylamine to a final pH of 11.5 or 1.0 M NaOH to a final pH of 12.5. The total volume of the reaction mixture was 0.5 - 1.0 mL. The cellulose residue was pelleted by centrifugation (16,000 g, 5 min) and the concentration of cellobiose in the supernatant was determined using different assays for cellobiose. Zero data points were made by adding the cellulase shortly after the alkali and were otherwise treated similarly.

3.1.4 Analytical procedures

Cellobiose (I) was quantified specifically as follows: the sample (450 μ L) in acetate buffer at pH 5.5 was mixed with 0.5 mL 70 μ M iodine solution in 0.3M KI and the reaction was initiated by addition of 50 μ L of 2 μ M cellobiose dehydrogenase. After incubation for 60 min at 50° C the change in absorbance at 350 nm was measured and the cellobiose concentration was calculated from calibration curves made with cellobiose as standard.

The cellobiose concentration (as total sugar) was determined by the following procedure: 600 μ L supernatant aliquots were neutralized by addition of 41 μ L of 1.56 M acetic acid to obtain pH 5.0 followed by addition of 2.0 mM 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid)

(ABTS) and a mixture of β -glucosidase, glucose oxidase and peroxidase and incubated in a water bath at 25 °C overnight. The final concentrations of the reagents in the assay were 0.2 mM for ABTS, 0.5 U/mL for β -glucosidase, 2.0 U/mL for glucose oxidase and 0.5 U/mL for peroxidase. Units of enzyme activity were as stated by the manufacturer. The oxidation of ABTS was followed by the increase in absorbance at 420 nm. Calibration curves were made using cellobiose as standard, including the addition of methylamine and acetic acid in order to mimic the sample treatment. The assay sensitivity allowed measurements from 0.5 μ M cellobiose in the hydrolysis mixture. Since β -glucosidase can also hydrolyze higher celooligosaccharides to glucose the above assay gives us the concentration of total sugar when cellulase action on pure cellulose is studied.

It must be noted here that the alkaline pH used for termination of the reaction can influence the assay. A time-dependent reduction in the assay signal was observed when the cellobiose was incubated in the presence of CBH I at a pH values above 12 before the assay. This effect was, however, negligible at lower pH values and pH 11.5 was found to be optimal for rapid termination of hydrolysis.

The cellobiose concentration (as total sugar, higher concentrations) (II, III and V) was determined by the anthrone/sulphuric acid method (Hörmann and Gollwitzer 1962). Samples (100 μ L) were mixed with 1 mL of reagent (2mg/mL anthrone in sulphuric acid water (100/40 v/v)) and heated in a smoothly boiling water bath for 12 min. The samples were cooled rapidly in an ice bath and, after about 1h at room temperature, the change in absorbance at 585 nm was measured. Calibration curves were made using glucose or cellobiose as standard.

The cellulose concentration was determined by the anthrone sulphuric acid method described above and expressed as (mg glucose/mL). If necessary, the cellulose samples were solubilized completely by a mixture of *T. reesei* cellulases prior to analysis of total sugar.

3.2 Results and discussion

3.2.1 Hydrolysis kinetics, individual enzymes (I)

Characteristic of enzymatic hydrolysis of insoluble cellulose is the pronounced decrease in the reaction velocity already during the very initial stage of the process. Clearly, this decrease is not proportional to the amount of solubilized bulk substrate (Fig .3).

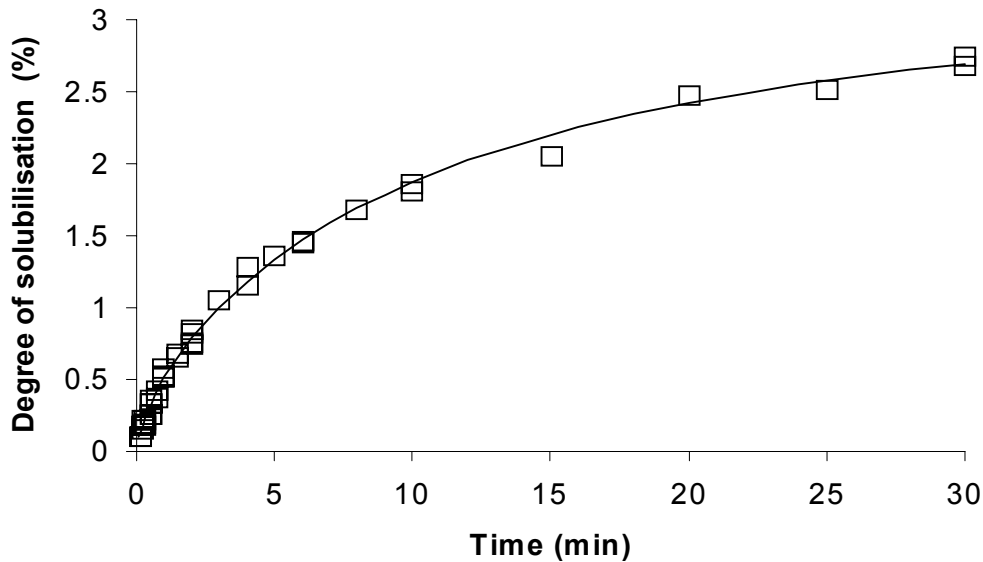


Figure 3. Typical pattern of the degree of solubilization as a function of time in enzymatic hydrolysis of cellulose Bacterial cellulose (0.5 mg/mL) was incubated with 0.5 μ M CBH I in 0.05M NaAc buffer, pH 5.0 at 25° C. Solid line is according to Eq. 1.

Explanations for the phenomenon found in the literature generally fall into one of the three following groups:

1. Bulk cellulose substrate contains several regions that differ in susceptibility to enzymatic attack. Consumption of the small amount of easily hydrolysable parts in cellulose causes the rate to fall.
2. Strong inhibition of cellulases by the reaction product, cellobiose.
3. Inactivation of cellulase during the hydrolysis.

The role of substrate transformation in the gradual decrease of the hydrolysis rate (hypothesis 1) can be tested most directly in prehydrolysis (also called resuspension) experiments. For that purpose, cellulose is treated with enzyme (pre-hydrolysis) to some certain degree of conversion, whereafter the reaction is stopped and the residual cellulose is thoroughly washed and used as a substrate in a second hydrolysis. If the rate is controlled solely by changes in the cellulose structure, then the initial rate of the second hydrolysis should be the same as the instantaneous rate at the time when the prehydrolysis was interrupted. The data in I, Fig. 2 show that the prehydrolysis indeed leads to lowered rates in the following hydrolysis. However, the initial rates of the second hydrolysis are far higher than those the rates when prehydrolysis was interrupted. These results can be explained either by, a) changes in cellulose structure are only partly responsible for the decline

in hydrolysis rates or b) changes in cellulose structure are, at least partly, of reversible nature and easily accessible parts can recover during the washing of cellulose for the second hydrolysis.

In paper I we put forward a mechanism whereby both substrate- and enzyme-related factors are responsible for rate retardation. The central idea of this mechanism is that the hydrolytic action of a processive enzyme can be sterically hindered by obstacles in its way along the cellulose chain. Processivity of the enzyme is here defined as the average number of hydrolytic events (hydrolytic cleavage of glycosidic bonds) per one productive binding (product sites are occupied by the cellulose chain). After the hydrolysis of one cellobiose unit from the chain end is completed the remaining chain, still bound in the substrate-binding sub-sites, has two possible fates.

a) snapping back toward upstream substrate-binding sub-sites. At the extreme this should lead to complete dissociation of the cellulose chain from the active-site tunnel.

b) moving forward toward product sites, enabling a new hydrolytic event to occur. This alternative requires that the product of the previous cleavage event, cellobiose, has been released from product sites.

If something on the cellulose surface causes the forward movement to be sterically hindered the balance of the fates *a* and *b* will be changed in favour of *a* and the result can be seen as a lower processivity. We call it the apparent processivity to distinguish it from the true or intrinsic processivity, which is an idealized parameter determined only by the balance of probabilities of the enzyme to move back and forth at zero concentration of the hydrolysis product in the solution and in the absence of any sterical hindrance. As can be demonstrated by Monte-Carlo simulation the hydrolysis of crystalline cellulose with an initially homogeneous structure by a processive exoenzyme leads to erosion of the crystal surface (Fig. 4 C, D). Remaining solitary chain residuals in the surface layer can serve as obstacles to the processive hydrolysis of the next layers resulting in a decreased apparent processivity of the enzyme. The substrate thus becomes gradually more resistant to further hydrolysis as the hydrolysis proceeds. It must be noted here that the latter hypothesis is empirically indistinguishable from the hypothesis 1. Both hypotheses assume that cellulose becomes less susceptible to enzyme during hydrolysis. The only difference is that hypothesis 1 assumes an initial heterogeneity of the substrate.

However, as already noted, the effect of prehydrolysis had only a moderate effect on the second hydrolysis. In paper I we ascribed this, again, to the decrease in apparent processivity during hydrolysis. Since most cellulases are composed of two domains, the binding via CBD only can give a rise to a population of non-productively bound enzymes.

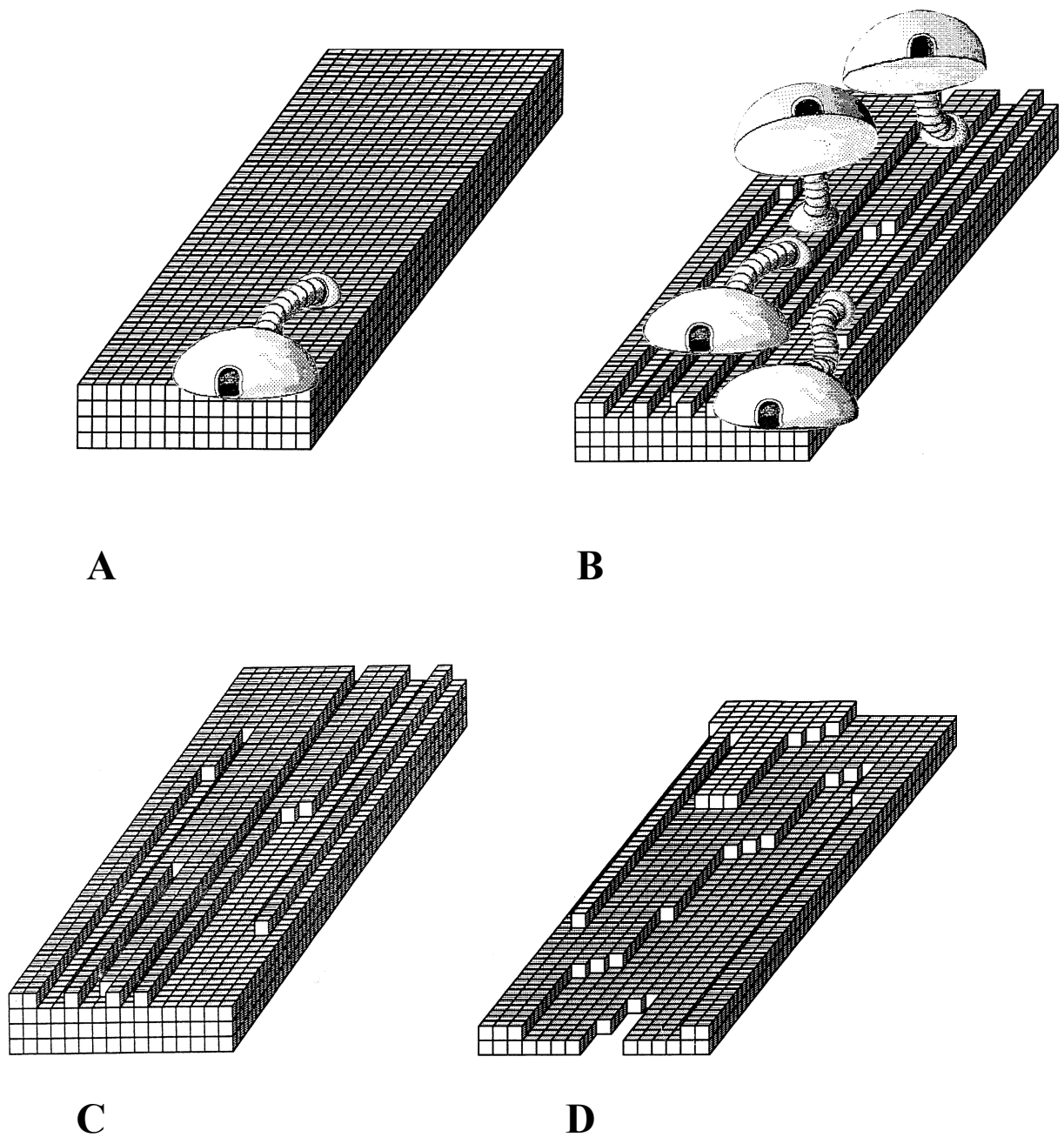


Figure 4. Pictures of typical modeled cellulose surface erosion patterns A) At the beginning of the adsorption of cellobiohydrolase B) after limited hydrolysis by a processive cellobiohydrolase, adsorption equilibrium has been reached C) the same as in B but enzymes are not shown C) after extended hydrolysis by a processive cellobiohydrolase, enzymes are not shown.

These enzymes on the cellulose surface can serve as obstacles to the processively hydrolyzing enzyme, just like the chain residuals described above. Since the adsorption of CBH I takes at least a few minutes to reach the steady state, the first among productively bound enzymes encounter less obstacles in their way and have, therefore, a higher apparent processivity than those that find their chain ends after the steady state of binding has been reached and most of the surface is covered with non-productively bound enzymes (Fig 4 A, B). Since the overall binding capacity is little affected during the initial stage of hydrolysis, the later effect persists also in the hydrolysis of pretreated celluloses.

In paper I we also report that factors included in hypothesis 2 and hypothesis 3, i.e., the strong inhibition by cellobiose and inactivation of the CBH I, can be ruled out as main rate controlling factors during the early stages of the hydrolysis. Addition of cellobiose at concentrations comparable to the released product under the studied time intervals did not affect the activity of the CBH I to the necessary extent (Fig. 6 in I). The addition of an amount of "fresh" substrate at the point in the hydrolysis time course where the rate retardation was clearly evident caused a new burst of activity, indicating that the enzyme was still active (Fig. 7 in I).

3.2.2 Synergism between cellulases (II, III)

High synergism between endoglucanases (EG) and cellobiohydrolases (CBH) is common for cellulolytic enzymes. The conventional rationale is that randomly-acting EG creates new chain ends, mainly on the amorphous parts of the cellulose, upon which CBH can act. Consistent with this, the observed synergism between EG I and CBH I is dependent on the crystallinity of the cellulose substrate and/or the DP of the substrate (Table 2; Table 1 in II). Whereas the synergy factor on native bacterial cellulose is 7 or more, the synergism on BMCC, i.e., acid pretreated BC, is strongly reduced with a synergy factor of 1.5-2. Similar results were obtained also by Samejima et al with *T. reesei* CBH I and EG II (Samejima et al., 1998). The synergy factor (SF_p) is defined as the ratio of the activity of combined enzymatic action (p_{mix}) to the sum of the activities of individual components ($\sum p_i$):

$$SF_p = p_{\text{mix}} / \sum p_i$$

High synergy factors can thus be due to both, a) very high activity of combined action or b) very low activities of individual components. From the data presented in paper II and Table 2 one can see that the difference in synergy factors on BC and BMCC is the result of the high activity of combined action on BC rather than to differences in the activities of individual components (see also Fig. 1 in III). The action sites for EG on

Table 2

Activities and synergy factors (SFp) in the hydrolysis of bacterial cellulose (BC) and bacterial microcrystalline cellulose (BMCC) by CBH I together with EG I or CBH II. SFp-s are based on the product formation upon hydrolysis of 1 mg/mL substrate at 25° C for 1h. The concentration of all enzyme components was 1 μ M.

Enzyme or combination of enzymes	BC		BMCC	
	Solubilisation (%)	SFp	Solubilisation (%)	SFp
CBH I	2.8		5.2	
CBH II	2.0		5.4	
EG I	1.5		2.1	
CBH I + CBH II	16	3.3	15.4	1.5
CBH I + EG I	39	9.1	11.3	1.5
CBH II + EG I	5.6	1.6	14.6	1.9

BC are probably the amorphous regions. The same regions are also target sites for heterogeneous acid hydrolysis. After extended acid hydrolysis of BC, there will thus be few sites remaining for endo-type action and synergism is reduced. The picture seems, however, more complicated, since the simultaneous action of EG and CBH is far more effective than is sequential application of the two component, especially on BMCC-type substrates. Saturating pretreatment of BC with EG results in a two-fold increase of the activity of CBH I on pretreated substrate, whereas the the activity of CBH I on BMCC-type substrates was virtually unaffected by pretreatment of the substrate with EG (Fig. 9 in II).

It is interesting to note that there must be some other factor besides the number of chain ends that affects the activity of CBH I on cellulose. If the number of chain ends were the only parameter controlling the activity, then the activity of CBH I toward BMCC would be much higher than that toward BC. Pretreatment of BC with HCl to get a BMCC increases the number of chain ends roughly 10-fold. The observed activity of CBH I on BMCC is only two-fold higher than on BC (Fig. 4 in II). However, if the EG generates new chain ends on BC in the presence of CBH I the substrate is efficiently degraded, whereas the number of EG-generated chain ends apparently never exceeds the number of chain ends present in BMCC. The highly crystalline nature of BMCC itself can not be responsible for the relatively low activity of CBH I on that substrate, because the same crystalline structures are present also in the original BC and are easily degraded upon synergistic action (Table 2; Fig.

5 in II). Only moderate difference in activities in the light of large difference in the number of chain ends in BC and BMCC can be explained by a different "quality" of the chain ends on these substrates. Different "quality" of the chain ends means that the chain ends on BC and those generated by EG on BC are more easily available for CBH I than those on BMCC. Considering the long tunnel-shaped active site of CBH I it seems logical that productive binding can occur only when a considerable number (around 10) of the glucosyl units at the chain end are free from the hydrogen bonding in the crystal lattice. The so-called "loose" chain end should be a better substrate for CBH I than the blunt end that is tightly bound to the bulk crystal via a hydrogen-bonding network. It seems reasonable to assume that EG-generated chain ends that are produced on the amorphous parts will be "loose" end types, whereas the acid attack is more random and after extended treatment probably generates blunt ends. The adsorption capacities for intact CBH I E212Q mutants are similar for BC and BMCC, indicating that the specific surface area is roughly the same for these substrates. Although BMCC contains far more chain ends and has similar specific surface area, the binding capacity of E212Q core protein on BMCC is even lower than on the BC (data not shown). This is consistent with the lower availability, or "quality", of the chain ends in BMCC, assuming that core protein binds mostly to the chain ends. An important conclusion follows here: that although acid pretreatment is helpful for removing noncellulosic components from lignocellulosic materials it can drastically reduce the susceptibility of the residual cellulose to cellulases.

Synergistic degradation of BC by CBH I and EG is interesting in that it shows a phenomenon of apparent substrate inhibition. In a qualitative way, this inhibition was observed to be independent of the nature of the EG component (Fig. 5; Fig. 1 in III). The intact two-domain nature of CBH I was necessary to reveal substrate inhibition, since mixtures of CBH I core protein with EG followed ordinary saturation kinetics (Fig. 2A in III; Fig. 3 in IV). The position of the apparent optimal substrate concentration was distinctly dependent on the concentration of CBH I and the EG component (Fig. 2 in III) as well as on temperature (Fig. 3 in III). Higher CBH I and EG concentrations, but also higher temperatures, all shifted the optimal substrate concentration toward higher values. No substrate inhibition was observed on BMCC. Experiments with BC-s having different chain-end concentrations on their surfaces indicated that the important factor for the higher hydrolysis rates is not only the absolute number of available chain ends on cellulose but also their density on the cellulose surface (Fig. 4 in III). Intact CBH I has been proposed to be able to diffuse on the cellulose surface in a two-dimensional way. Lateral diffusion gives non-productively bound enzyme

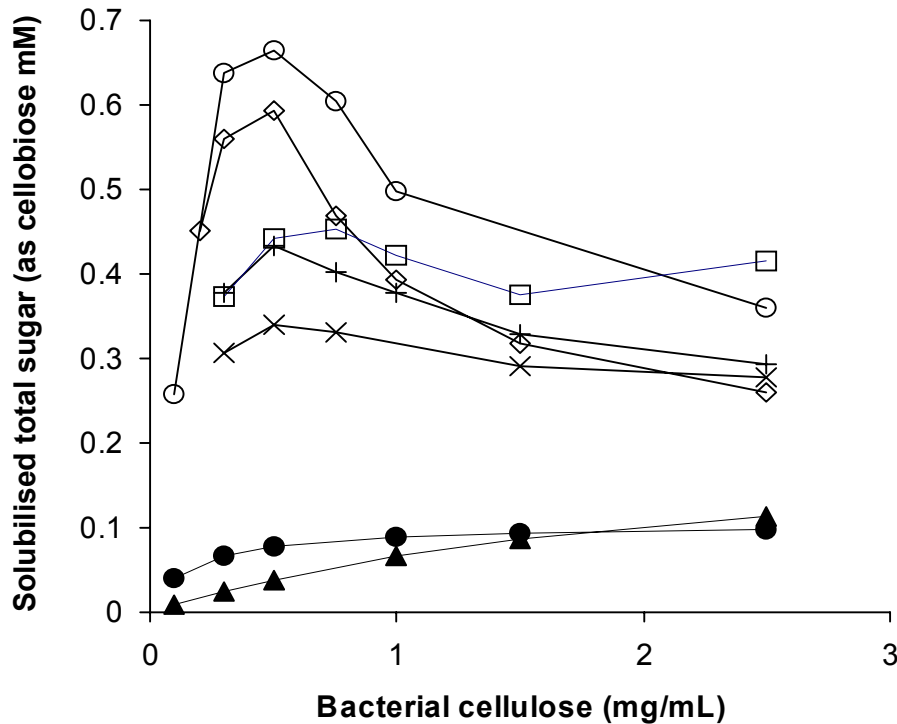


Figure 5. Hydrolysis of cellulose by CBH I in binary mixtures with different endoglucanases and CBH II at different substrate concentrations Bacterial cellulose was incubated with individual CBH I or EG I or with binary mixtures of 1 μ M CBH I and either 0.1 μ M endoglucanase or 1 μ M CBH II in 0.05 M NaAc buffer, pH 5.0, at 25° C for 1h. EG II (○); EG I (◇); EG II core protein (+); EG III (×); CBH II (□). Individual 1 μ M: (●) CBH I and (▲) EG II.

a "standby" status, allowing it to become productive when it encounters a chain end. That possibility is, however, dependent on the number of chain ends in the vicinity of non-productively bound enzyme and also on the rate of lateral diffusion. This gives the rationale for substrate inhibition as proposed by Fenske et al., based on a Monte-Carlo simulation of the process (Fenske et al., 1999). It is more probable that EG-generated chain ends will be in the near vicinity of standby CBH I and will thus be picked up more readily at high surface coverage than at low enzyme to substrate ratios. The higher optimal BC concentrations observed at higher CBH I and EG concentrations are due to the increased surface concentrations of reaction components (CBH I and chain ends). Chain end density is dependent on the EG concentrations and will decrease with substrate concentration at constant EG loading. The same happens with the surface concentration of CBH I. Although a higher CBH I loading will increase the optimal BC concentration, the surface concentration of CBH I calculated from the binding isotherm is same at the position of the optima

(about 1.9 $\mu\text{mol/g}$ at 0.1 μM EG concentration). A higher temperature, on the other hand, should increase the rate of lateral diffusion, again resulting in higher optimal BC concentrations.

The absence of substrate inhibition during synergistic hydrolysis of BMCC (Fig. 1 inset in III) reflects the different modes of synergy on this type of substrate. The same conclusion follows from the fact that far higher EG concentrations are required for efficient synergy with CBH I on BMCC than on BC (Fig. 8 in II). Similarly, efficient synergism between two cellobiohydrolases, CBH I and CBH II, was achieved only at high CBH II concentrations, but in this case on both BC and BMCC as substrates (Fig. 6). Also, the synergistic effect between CBH I and CBH II on BC was only twice as high as on BMCC, whereas the synergism between CBH I and EG is far higher on BC (Table 2). All of these observations indicate that there must be another mechanism apart from conventional endo-exo synergism. Moderate substrate inhibition seen in the hydrolysis of BC by the mixture of CBH I and CBH II (Fig. 5) indicates that the mechanism of the conventional endo-exo synergism can be involved, at least in part. This is consistent with the proposed weak endo-activity of CBH II (Zou et al., 1999; Varrot et al., 1999).

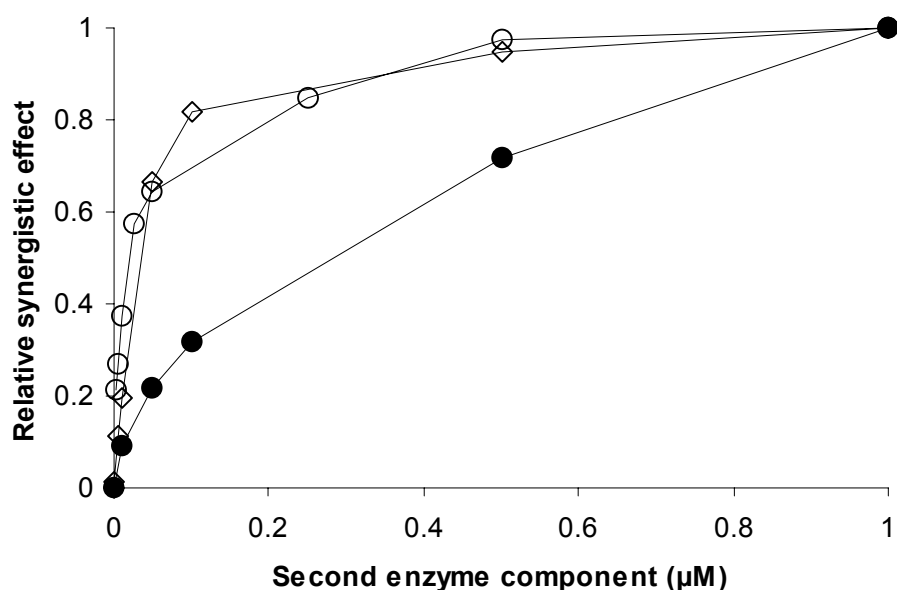


Figure 6. Relative synergistic effects in hydrolysis of bacterial cellulose by binary mixtures of CBH I with second enzyme component Bacterial cellulose (1 mg/mL) was incubated with binary mixture of 1 μM CBH I and (◇) EG I; (○) EG II; (●) CBH II in 0.05M NaAc buffer pH 5.0, at 25° C for 1h.

Apparently, a second mode of synergism operates in parallel with the conventional one and probably involves disruption of the crystal structure of the cellulose surface or removal of the obstacles created by processive exo-enzyme. Doing it in both directions can result in synergism between enzymes that attack cellulose chains from different directions, like CBH I and CBH II. Loose chains on the surface are also more readily attacked by EG type enzyme, so the synergism between CBH and EG is reciprocal, consistent with ours (Fig. 10 in II) and also with earlier observations by others (Nidetzky et al., 1994b). It is obvious that such a mode of synergism does not depend on the surface density of the synergy components, explaining why there is no substrate inhibition in the hydrolysis of BMCC. The conventional mode is predominant in the hydrolysis of BC, whereas the second mode is prevalent in the hydrolysis of the more crystalline BMCC. Also, the second mode of synergism is predominant in the case of synergistic action of two cellobiohydrolases on either BC or BMCC as substrate.

Owing to the fact that the purification of cellobiohydrolases is often not an easy task it must be noted that the phenomenon of substrate inhibition allows a plausible method to assess the possible contamination of some cellobiohydrolases (CBH I and CBH 58 an example) with endoglucanase. The higher activity of a cellobiohydrolase preparation due to contamination with endoglucanase measured at one BC concentration cannot itself reveal contamination without having done measurements with absolutely pure, "reference" cellobiohydrolase in parallel. However, including measurements at different BC concentrations reveals contamination with EG as a qualitatively different pattern (substrate inhibition) that can be detected easily without running a "reference" in parallel. Artificial contamination of 1 μM CBH I stock solutions with only 1 nM EG II was revealed by a substrate inhibition assay using BC as substrate (data not shown). Longer incubations are preferred to reveal substrate inhibition more clearly (Fig. 2A in IV).

The minimum initial rate-based turnover numbers at 25° C and pH 5.0 for CBH I and CBH II on cellulose were also estimated. Assuming that at saturating cellulose concentration all enzyme is productively bound the k_{cat} for CBH I of $1.4 \pm 0.1 \text{ s}^{-1}$, $1.6 \pm 0.2 \text{ s}^{-1}$ and $2.5 \pm 0.4 \text{ s}^{-1}$ on amorphous cellulose, BC and BMCC, respectively, was estimated. The k_{cat} for CBH II on amorphous cellulose was $8.0 \pm 0.2 \text{ s}^{-1}$, a figure close to that observed on cellohexaose (Harjunpää et al., 1996).

3.2.3 *Diffusion limitations, fractal-like kinetics (IV)*

Implicit in traditional chemical kinetics is the assumption that the reaction occurs in dilute solutions that are spatially homogeneous.

However, it is now clear from theory, computer simulation and experiment that elementary chemical kinetics are quite different when reactions are diffusion limited, dimensionally restricted, or occur on fractal surfaces (Kopelman, 1988 and references therein). Under these conditions, the conventional rate law exhibits a characteristic reduction of the rate constant with time. The rate coefficient k is time dependent and is related to the classical rate constant k_1 by $k = k_1 t^{-h}$. In 3-D homogeneous space, $h = 0$, and thus k is a constant. For a typical fractal system h has a value near 1/3 (Kopelman, 1988).

Although the bacterial cellulose forms stable suspensions it is not a homogeneous solution at molecular level. Furthermore, the actual action sites for cellobiohydrolase, the free chain ends, are not homogeneously distributed on the cellulose surface, but are more concentrated at the ends of the cellulose crystals and on the amorphous sites, especially in the presence of endoglucanase. These dimensionally restricted conditions are precisely those in which power-law or fractal-like kinetics can be expected to arise.

In paper IV we made an approach to explaining the kinetics of cellulose synergistic degradation in terms of fractal-like kinetics. It has been shown by several authors that cellulose enzymatic hydrolysis can be described as a pseudo first order reaction or as a sum of several pseudo first order reactions. According to the fractal-like kinetics analogue of a pseudo first order reaction, the product concentration $p(t)$ is equal to:

$$p(t) = [S]_0 [1 - \exp(-k \cdot t^{(1-h)})] \quad (1)$$

where $p(t)$ is the cellobiose concentration (μM); $[S]_0$ is the initial concentration of the cellulose (as cellobiose, μM); t is time (min); and k and h are empirical constants.

Time curves for the hydrolysis of BC by mixtures of 1 μM CBH I or CBH I core protein with 0.1 μM EG II are represented in (Fig. 1 A,B in IV). Non-linear regression of the hydrolysis data according to Eq 1. results in a different pattern of apparent h values for hydrolysis by synergistic mixtures containing intact CBH I or CBH I core protein (Fig. 7). In paper IV we proposed that this is due to the different diffusion modes of intact CBH I and the corresponding core protein. Intact CBH I is confined to the cellulose surface and finds its target substrate via the 2-D diffusion mode, whereas core protein is mostly free in solution and the 3-D diffusion mode is prevalent. Since the 2-D and 3-D diffusion modes are affected in opposite directions by the enzyme to substrate ratio the dependence of apparent h values on substrate concentration is also opposite for synergistic hydrolysis by intact CBH I and its core protein. The effect of CBD is gradually reduced at lower enzyme to substrate

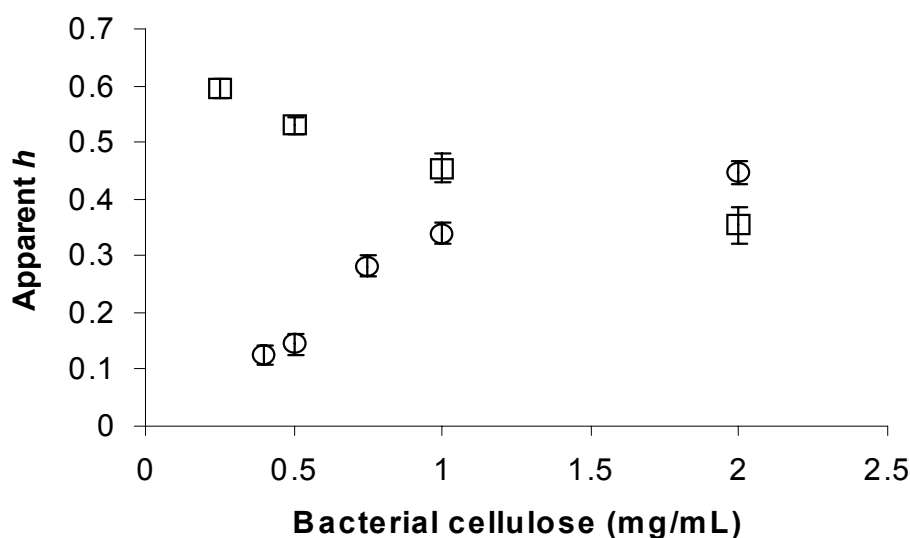


Figure 7. Values of the apparent fractal kinetics exponent h in hydrolysis of bacterial cellulose (BC) by the mixture of EG II and CBH I or CBH I core protein BC was incubated with the mixture of 0.1 μM EG II and 1.0 μM CBH I (\circ) or 1.0 μM CBH I core protein (\square) in 0.05 M NaAc buffer pH 5.0 at 25° C. The apparent h values are from the non-linear regression of hydrolysis data in (Fig. 1 in IV) according to Eq. 1.

ratios and, as can be predicted from the trends of apparent h values (Fig. 7), diffusion limits are further relieved for CBH I core protein and severed for intact CBH I with increasing substrate concentration, meaning that for certain enzyme to substrate ratios the effect of CBD on the overall efficiency of cellulose synergistic degradation will be negative. Therefore, it might depend on the cellulase to cellulose ratio in a particular environment whether it is advantageous for a fungus to secrete intact cellobiohydrolase or a truncated form of the protein. It must be noted that, although empirical, the apparent h values obtained by the fitting of hydrolysis data to Eq. 1 provides a simple way to estimate the strength of rate retardation in time and how it will depend on reaction conditions.

3.2.4 Inhibition of cellulases by cellobiose (V)

In the studies of paper V we investigated the role of product (cellobiose) inhibition in the decrease of hydrolysis rates at a quantitative level. Although our earlier results indicated that product inhibition can be ruled out as a principal cause of the decrease in hydrolysis rate during the initial stage of hydrolysis, there is a lack of quantitative knowledge about the inhibition of cellulases acting on insoluble cellulose. This is

especially true for initial rates-based studies. Difficulties in measuring the inhibition of initial rates by product converge to the problems of measuring low hydrolysis rates in the presence of a high background of supplied product. We escaped this problem by using reducing end [³H]-labelled cellulose as substrate. Fig. 1 in V shows the normalized rate of the release of radioactivity after 10 s of hydrolysis as a function of substrate concentration. Although the conventional Michaelis-Menten formalism is not directly applicable to insoluble cellulose hydrolysis the experimental data were found to fit the Michaelis-Menten equation, thus enabling use of the data for estimation of the limiting velocity (P_{lim} , CPM·mL⁻¹·s⁻¹) and half-saturating substrate concentration ($S_{0.5}$, mg/mL). Since high $S_{0.5}$ values observed for the initial rates of cellulose hydrolysis are difficult to measure precisely because the solution properties of BC set limits on the highest usable concentration, the conventional approach involving the measurements of apparent parameters (P_{lim} , $S_{0.5}$) at different inhibitor concentrations is not applicable. We used an alternative approach based on initial rate measurements at a fixed substrate concentration in the presence of inhibitor at different concentrations and non-linear estimation of parameters according to equations for different types of inhibition.

$$p = \frac{P_{lim} \cdot [S]}{[S] + S_{0.5} \cdot \left(1 + \frac{[I]}{K_i}\right)} + H \quad (2)$$

$$p = \frac{\frac{P_{lim} \cdot [S]}{\left(1 + \frac{[I]}{K_i}\right)}}{[S] + S_{0.5}} + H \quad (3)$$

Where $[S]$ and H stand for the cellulose concentration (mg/mL) and the background radioactivity (CPM·mL⁻¹·s⁻¹), respectively. $S_{0.5}$ was fixed at a value listed in Table 3.

Relative activities (based on release of radioactivity after 10 s of hydrolysis) as a function of added cellobiose concentration are shown in Fig. 8 and the values for the inhibition constants of different enzymes are listed in Table 3. Despite the considerable error of the measurements, the main conclusion follows here that, whereas cellobiose is a strong inhibitor of CBH I acting on low-molecular weight model substrates, like para-nitrophenyl cellobioside (K_i around 20 μM) (van Tilbeurgh and Claeysens, 1985; Claeysens et al., 1989; Vonhoff et al., 2000), the

action of CBH I on cellulose is more insensitive to inhibition, with an apparent K_i around 1.5 mM. The hydrolysis of cellulose by endoglucanases was even more resistant to inhibition (Table 3).

Table 3

Estimated substrate concentration ensuring half-limiting release rate of label ($S_{0.5}$) and apparent inhibition constant (K_i) values for the hydrolysis of [^3H]-labelled celluloses by *Trichoderma reesei* cellulases. The apparent inhibition constants (K_i) were calculated according to Eq. 2. The substrate was [^3H]-BC in case of CBH I and [^3H]-amorphous cellulose in case of EG I, EG II and EG III.

Enzyme	$S_{0.5}$, mg/mL	K_i , mM
CBH I	2.2 ± 1.0	1.6 ± 0.5
EG I	5.8 ± 1.2	10.7 ± 3.4
EG II	5.6 ± 0.7	33.6 ± 6.3
EG III	4.1 ± 0.3	activation

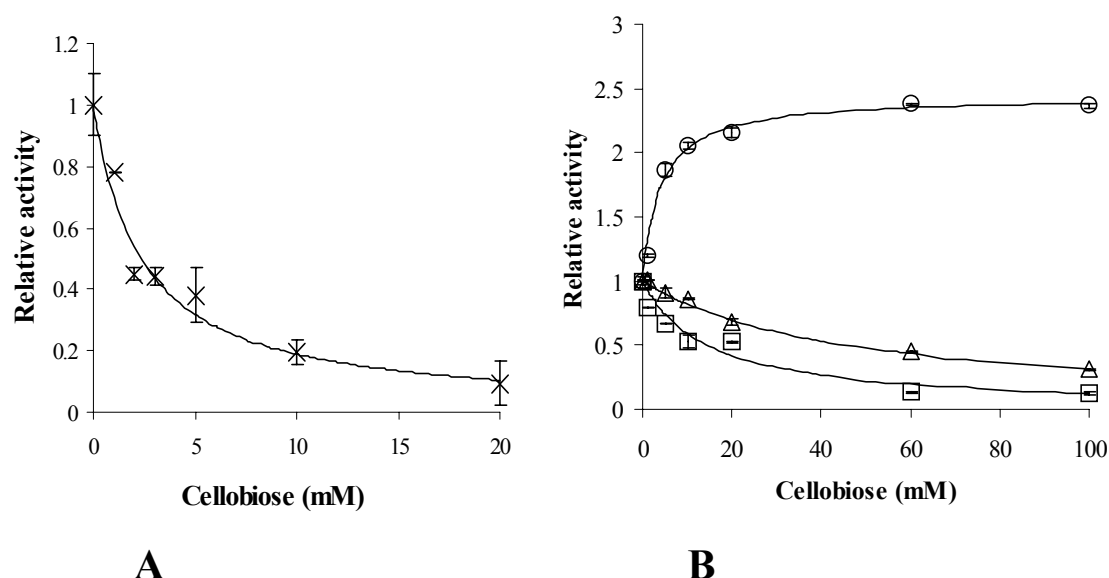


Figure 8. Inhibition of the initial rates of hydrolysis of [^3H]-labelled celluloses by cellobiose [^3H]-labelled cellulose was hydrolysed with enzyme in 0.05 M NaAc buffer, pH 5.0, at 25 °C for 10 s in the presence of externally supplied cellobiose (0-100 mM). (A) the concentration of CBH I (\times) was 1 μM and [^3H]-BC 1 mg/mL and (B) concentrations of EG I (\square), EG II (Δ) and EG III (\circ) were 1 μM and substrate [^3H]-amorphous cellulose 2 mg/mL. The solid lines are based on Eq 2. on the normalized scale ($p_I/p_{I=0}$).

Hydrolysis of [³H]-amorphous cellulose by EG III revealed, most interestingly, that cellobiose had an apparent activating effect on the release of label. All cellulases are, in principle, capable of transglycosylation. All of the enzymes studied here have a retaining hydrolysis action (Henrissat and Davies, 1997) involving a covalent enzyme-glycosyl intermediate which normally is split by water. At sufficiently high cellobiose concentrations its free 4'-hydroxyl group can possibly compete with water as nucleophile, partly due to its optimal positioning. The probability for the transglycosylation reaction may be enhanced by a high ratio between the primary product release rate and the covalent intermediate hydrolysis rate. A similar activating effect of cellobiose on CBH I possibly occurs at cellobiose concentrations above 30 mM, as reflected by a slight increase in the release of label which is inconsistent with Eq. 2. and 3. (data not shown). The transglycosylating activity of EG III proposed here may be related to the recent observation that this enzyme can cause plant cell wall expansion, an activity that may involve transglycosylation (Yuan et al., 2001).

It must be noted here that exact knowledge about the type of inhibition is not crucial to reveal the strength of inhibition under our experimental conditions using substrate concentrations around $1/2 S_{0.5}$. Based on structural features of cellulases with an active site containing multiple glucosyl-unit binding sites, the only plausible mode besides competitive- is pure non-competitive inhibition, whereas uncompetitive inhibition seems unlikely. Analysis of the hydrolysis data according to the equation for pure non-competitive inhibition (Eq. 3.) resulted in higher apparent K_i values which, however, did not differ from the competitive inhibition constants by more than \pm the standard error. It must be noted here also that different units used for the cellobiose concentration and $S_{0.5}$ do not affect the resulting apparent K_i values.

4 Conclusions

The cellulose surface-erosion model was introduced to explain the gradual and strong retardation in the rate of enzymatic hydrolysis of cellulose. This model identifies the decrease in apparent processivity of cellobiohydrolases during the hydrolysis as a major contributor to the decreased rates. Both enzyme-related (non-productive binding) and substrate-related (erosion of cellulose surface) processes contribute to the decrease in apparent processivity. Furthermore, the surface erosion model allows, apart from conventional endo-exo synergism, the possibility of different modes of synergistic action between cellulases. The second mode of synergism operates in parallel with the conventional one and was

found to be prevalent in the hydrolysis of more crystalline celluloses and also in the synergistic action of two cellobiohydrolases.

A mechanism of substrate inhibition in the synergistic hydrolysis of bacterial cellulose was proposed whereby the inhibition is a result of surface dilution of reaction components (bound cellobiohydrolase and cellulose chain ends) at lower enzyme to substrate ratios.

An approach was made to describe the hydrolysis of cellulose in terms of fractal-like kinetics. Non-linear regression of hydrolysis data according to the expression for a fractal-like kinetics analogue of a pseudo-first order reaction allowed an easy method for assessing the strength of rate retardation and its dependence on reaction conditions.

The inhibition of cellulases by the hydrolysis product, cellobiose, was found to be strongly dependent on the nature of the substrate. The hydrolysis of a low molecular weight model substrate, such as para-nitrophenyl cellobioside by cellobiohydrolase I is strongly inhibited by cellobiose, with a K_i around 20 μM , whereas the hydrolysis of cellulose is more resistant to inhibition, with an apparent K_i around 1.5 mM for cellobiose.

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