Carbohydrate Polymers **10** (1989) 1–14

Decrease in Reactivity and Change of Physico-chemical Parameters of Cellulose in the Course of Enzymatic Hydrolysis

A. P. Sinitsyn, O. V. Mitkevich, A. V. Gusakov

Department of Chemistry, MV Lomonosov Moscow State University, Moscow 117234, USSR

&

A. A. Klyosov

AN Bach Institute of Biochemistry, 33 Leninsky Pr., Moscow 117071, USSR

(Received 20 January 1988; revised version received and accepted 27 April 1988)

ABSTRACT

As enzymatic hydrolysis progresses, cellulose and hydrocellulose become less susceptible to enzymatic attack. Other properties, such as the crystallinity index, the specific surface area accessible to enzymes, and the degree of polymerization are variously affected by the degree of hydrolysis. The effects of the composition of the multienzyme cellulase complex and the properties of the enzymes on the reactivity and structural parameters of cellulose are discussed.

INTRODUCTION

The reactivity of cellulose in enzymatic hydrolysis depends on the following physico-chemical and structural parameters (Shewale & Sadana, 1979; Kelsey & Shafizadeh, 1980; Klyosov & Sinitsyn, 1981; Plotnikov, 1981; Lee & Fan, 1982; Charpuray *et al.*, 1983; Lee *et al.*, 1983):

Crystallinity index (CI),
Specific surface area accessible to enzyme molecules (SSA),
Degree of polymerization (DP).

Carbohydrate Polymers 0144-8617/89/$03.50 —

The rate of enzymatic cellulose hydrolysis is proportional to SSA and inversely proportional to CI (Klyosov & Sinitsyn, 1981; Lee & Fan, 1982; Charpuray *et al.*, 1983; Lee *et al.*, 1983). Before hydrolysis can take place, the enzyme must be adsorbed on the surface of the celulose; it is clear that a higher SSA value will result in a greater amount of adsorption and a higher rate of hydrolysis (Klyosov & Sinitsyn, 1981; Lee *et al.*, 1983). The explanation for the relationship between CI and the reaction rate is that the glucoside linkages in cellulose molecules are much more accessible to enzyme attack in amorphous regions than in tightly-packed crystalline regions.

It has been reported that the degree of polymerization does not influence the efficiency of cellulose hydrolysis (Tsao & Lee, 1975; Knappert *et al.*, 1980). In these cases, however, cellulose was hydrolyzed by enzyme complexes containing both depolymerizing enzymes (*endo*-1,4-β-glucanases) and those that attack cellulose molecules from the non-reducing end (cellobiohydrolases). From the properties of the latter enzymes, it can be supposed that their individual activities are dependent on the DP of cellulose, since the number of free non-reducing ends increases as DP decreases. Thus, the role of DP in cellulose hydrolysis appears to depend on both the structure of substrate and the composition of the cellulase complex.

Physico-chemical parameters of cellulose change in the course of enzymatic hydrolysis, and this, in turn, affects the kinetics of the process (Klyosov & Sinitsyn, 1981; Sinitsyn & Klyosov, 1981). In this report, the reasons for the decrease in cellulose reactivity during enzymatic hydrolysis are discussed and the changes in the cellulose structure with regard to crystallinity, specific surface area and degree of polymerization are assessed.

MATERIALS AND METHODS

Substrates and enzymes

The substrates were ball-milled cotton cellulose and hydrocellulose obtained from cotton by HCl treatment (Wong *et al.*, 1977).

Cellulases were from filtrates of *Trichoderma viride* (activity 3 Filter Paper Units (FPU) g^{-1}) and *Trichoderma longibrachiatum* (activity 5 FPU g^{-1}), and 'Rapidase' preparation (Société 'Rapidase', Seclin, France) from *Sporotrichum dimorphosporum* (activity 1·5 FPU g^{-1}). Components of *T. viride* cellulase complex were isolated using a scheme described elsewhere (Klyosov *et al.*, 1985*b*). The enzymes were pre-

cipitated with ammonium sulfate, desalted on an Acrylex P-2 column and chromatographed on a DEAE-Spheron column. The first of two main fractions contained most of the *endo*-1,4-β-glucanase activity (470 International Units (IU) g^{-1}); the second fraction was rich in cellobiohydrolase (8 IU g^{-1}) while the *endo*-1,4-β-glucanase activity was negligibly low. β-Glucosidase was absent in both fractions.

endo-1,4-β-Glucanase activity was assayed viscometrically from the hydrolysis of sodium carboxymethylcellulose (40°C, pH 4·5) (Klyosov *et al.*, 1980). Cellobiohydrolase activity was determined from the initial rate of cellobiose formation from amorphous cellulose, obtained by regeneration from the solution of microcrystalline cellulose in cadoxen (substrate concentration 5 g liter^{-1}, 40°C, pH 4·5) (Sinitsyn & Klyosov, 1981). One unit of cellobiohydrolase activity was defined as the amount of enzyme which leads to formation of one micromole of cellobiose per minute (Sinitsyn *et al.*, 1986).

Cellulose hydrolysis and analysis of products

Cellulose (5 g liter^{-1}) was hydrolyzed at 50°C in glass vessels (0·5–2 liter volume) with magnetic stirring (pH 4·5, 0·1 M acetate buffer). During hydrolysis by tightly-adsorbed enzymes, the loosely-adsorbing enzymes were removed by filtration through a glass filter 30 min after mixing the enzyme preparation solution with cellulose. The non-soluble cellulose residue with tightly-adsorbed enzymes was washed and resuspended in 0·1 M acetate buffer (pH 4·5) of a volume equivalent to the filtrate. To observe the action of loosely-adsorbing enzymes, the filtrate was added to a weighed sample of fresh cellulose.

The soluble hydrolysis products were analyzed by high performance liquid chromatography (HPLC) using a Silasorb-NH_2 column (4·6 mm × 25 cm) with acetonitrile–water (70:30) as the mobile phase and a differential refractometer (Knauer Oberu Sel, Taunus, FRG) for detection. In some cases D-glucose was also analyzed by the D-glucose oxidase–peroxidase method (Klyosov *et al.*, 1980). Reducing sugars were assayed by the modified Nelson–Somogyi method (Klyosov *et al.*, 1980).

Determination of the physico-chemical parameters of cellulose

To determine the physico-chemical parameters, suspended samples of cellulose were taken from the reaction mixture and centrifuged for 5 min at 5000 RPM. Enzymes and products were removed by multiple washings with distilled water, boiling for 2 min, washing with phosphate buffer

(pH 7·0), distilled water and acetate buffer (pH 4·5). To prevent alteration of the cellulose surface due to crystallization the substrate was washed with ethanol, then with benzene, and dried in air (Knappert *et al.*, 1980). These dried cellulose preparations were used to determine CI, SSA and DP. CI was determined by X-ray diffraction (Sinitsyn & Klyosov, 1981; Focher *et al.*, 1981) and the diffractograms were processed by the Segal method (Tripp, 1971). SSA was assayed from the maximum adsorption of chymotrypsin, and DP from the viscosity of cellulose in cadoxen (Sinitsyn & Klyosov, 1981).

The reactivity of cellulose in the course of hydrolysis was expressed as a percentage of the initial reactivity with the same enzyme preparation. To this end, after washing and drying, the partially-hydrolyzed substrate was further hydrolyzed under the same conditions as the fresh substrate.

RESULTS

Changes in cellulose reactivity

Ball-milled cotton cellulose and hydrocellulose were chosen as the substrates because they differ in their physico-chemical parameters. The CI of the ball-milled cotton cellulose was 66% and the DP was 1600. Hydrocellulose had a high CI of 86% and a low DP of 150. The SSA of both substrates was 0·27 $m^2 g^{-1}$.

Figure 1 shows that the reactivities of the two substrates decreased in different ways. For instance, after 80% hydrolysis of cotton cellulose by the *T. viride* cellulase complex, the reactivity decreased three-fold, whereas hydrocellulose reactivity decreased only 1·3-fold (Fig. 1A, curves 3 and 1). The susceptibility of hydrocellulose to *T. longibrachiatum* cellulases did not change at all until it had undergone 50% hydrolysis, while that of the ball-milled cotton cellulose decreased 1·3-fold (Fig. 1A, Curves 2 and 4).

The reactivity of the ball-milled cotton cellulose decreased during hydrolysis with both the *T. viride* complex (Fig. 1A, Curve 3) and its *endo*-1,4-β-glucanase and cellobiohydrolase fractions (Fig. 1B). In the latter case, the decrease in reactivity was similar for both fractions, that is, 1·8-fold and 2-fold, respectively, at 20% hydrolysis.

It is known that there are two types of cellulolytic enzymes which differ in adsorption characteristics (Rabinowitch *et al.*, 1982; 1983; Klyosov *et al.*, 1985*a*). Some cellulases are almost completely adsorbed on the cellulose surface at the optimum pH for enzyme activity. When, after enzyme adsorption, the water phase is replaced by a fresh portion

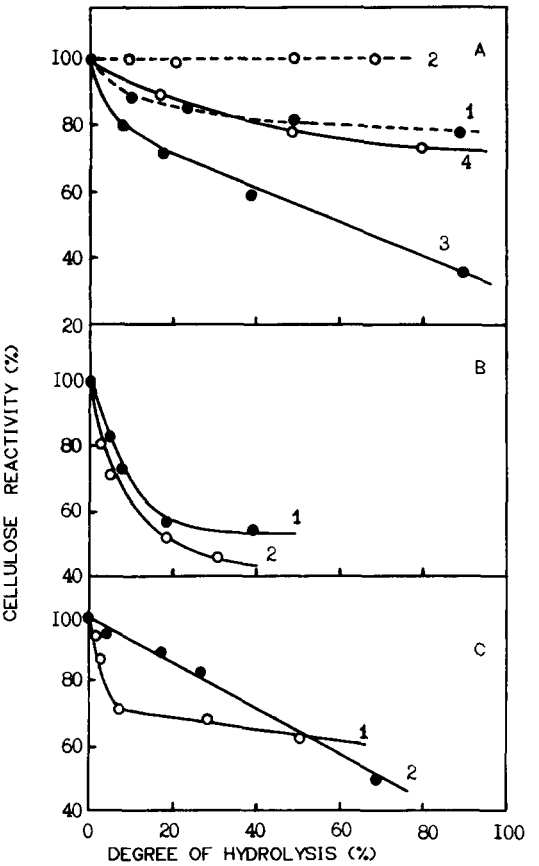

Fig. 1. Dynamics of reactivity change of hydrocellulose (- - - -) and ball-milled cotton cellulose (——) in the course of enzymatic hydrolysis. A: Curves 1 and 3, cellulase complex preparation from *T. viride*; curves 2 and 4, cellulase complex preparation from *T. longibrachiatum*. B: Curve 1, *endo*-1,4-β-glucanase fraction from *T. viride*; curve 2, cellobiohydrolase fraction from *T. viride*. C: Curve 1, tightly-adsorbed enzymes from *T. viride*; curve 2, loosely-adsorbing enzymes from *T. viride*.

of buffer, these tightly-adsorbed enzymes are not desorbed from the cellulose surface. Loosely-adsorbed enzymes, on the other hand, do not link tightly with the cellulose surface and most remain in the water phase on removal of cellulose. Figure 1C shows that in the case of *T. viride* cellulase complex, where hydrolysis does not exceed 10%, cellulose reactivity decreases 30% with tightly-adsorbed cellulases, but only 7% with loosely-adsorbing enzymes. After further hydrolysis the reactivity of cellulose under the action of tightly-adsorbed enzymes did not change, but with loosely-adsorbing enzymes reactivity continued to decrease at a constant rate (Fig. 1C).

Changes in crystallinity index

It is generally believed that the reactivity of cellulose declines because of an increase in CI, since enzymes tend to hydrolyze the more reactive amorphous regions of cellulose (Sasaki *et al.*, 1979; Shewale & Sadana, 1979; Tanaka *et al.*, 1979; Ryu *et al.*, 1982; Charpuray *et al.*, 1983). Indeed, in each case after 10–20% hydrolysis an increase in CI was observed (Fig. 2A and B). For example, in the case of hydrolysis of ball-milled cotton cellulose by the *T. viride* complex preparation, the CI increased from 66 to 79% after 8% hydrolysis (Fig. 2A). For more crystalline hydrocellulose, this parameter varied less (Fig. 2A).

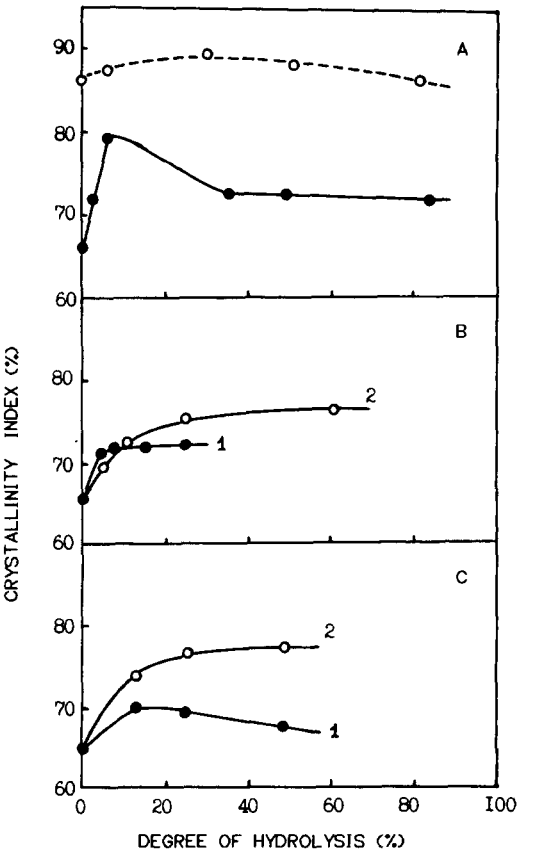

Fig. 2. Change in crystallinity index of hydrocellulose (- - - -) and ball-milled cotton cellulose (——) in the course of enzymatic hydrolysis by *T. viride* cellulases. A: Cellulase complex preparation. B: Curve 1, loosely-adsorbing enzymes; curve 2, tightly-adsorbed enzymes. C: Curve 1, *endo*-1,4-β-glucanase fraction; curve 2, cellobiohydrolase fraction.

It is important to note that in some cases an early increase in CI was followed by a decrease. For example, at 40–80% hydrolysis of cotton cellulose, the CI returned almost to its initial value; for hydrocellulose it became even lower than the initial value (Fig. 2A). This may be explained by the fact that as amorphous cellulose content decreases in the substrate, the enzymes start to hydrolyze the crystalline regions, making them somewhat amorphous.

The CI of cellulose with cellobiohydrolase increased initially and then remained constant, but with *endo*-1,4-β-glucanase after 10–40% hydrolysis the degree of crystallinity returned to its initial value (Fig. 2C).

Changes in specific surface area

The SSA of cellulose is another important parameter affecting its reactivity. Since the average pore size of cellulose microcapillaries is estimated to be 25 Å and the minimum diameter of cellulase molecules (as a minor diameter of a corresponding ellipse) is usually 20–40 Å (Knappert *et al.*, 1980), the enzymes can often interact with cellulose only at its 'outer' surface. So, in enzymatic hydrolysis, it is not the 'fine' structure of cellulose that is important (for example, the surface area determined by gas adsorption), but rather the SSA determined from protein adsorption (Sinitsyn & Klyosov, 1981).

Figure 3 shows SSA changes during hydrolysis. In cotton cellulose, after only 5% hydrolysis by any of the three cellulase preparations, the SSA decreased 1·5–2·5-fold. Changes in the SSA of hydrocellulase early in hydrolysis can also be seen in Fig. 3. At later stages of hydrolysis the SSA sometimes increased. For example, the SSA of cellulose increased by factors of 1·4 and 2 under the action of *T. longibrachiatum* cellulases (Fig. 3B) and tightly-adsorbed enzymes of *T. viride* (Figure 3A, upper curve), respectively, compared to the minimal value in hydrolysis.

Changes in degree of polymerization

The changes in DP of cellulose under various conditions are shown in Fig. 4. For ball-milled cotton cellulose the DP decreased markedly even at 5–10% hydrolysis, and continued to decrease at further hydrolysis (Fig. 4A). The DP of hydrocellulose also decreased early then remained approximately the same.

The changes in DP were related to the properties of the substrate. Also important were the strength of cellulase adsorption and the mode of enzyme action, such as the presence of *endo*-1,4-β-glucanases and cellobiohydrolases in cellulase preparation.

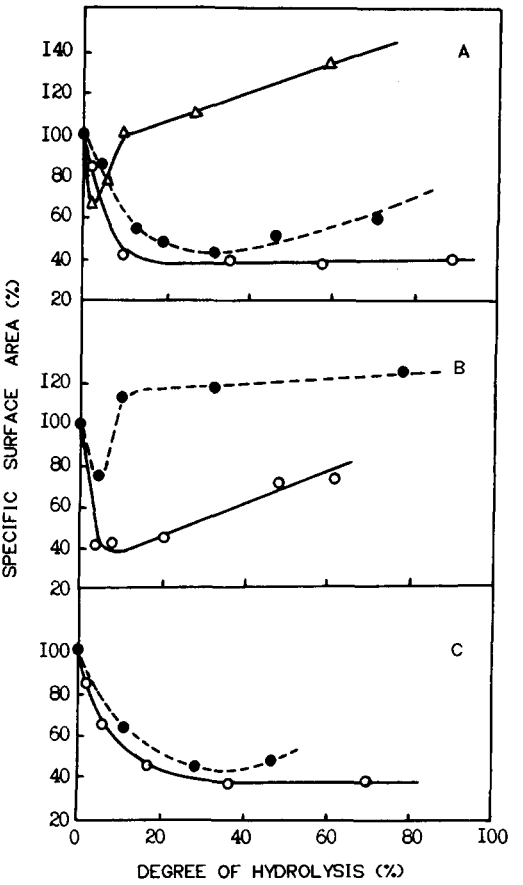

Fig. 3. Change in specific surface area of hydrocellulose (- - - -) and ball-milled cotton cellulose (——) in the course of enzymatic hydrolysis by cellulase complex preparations. A: *T. viride* (the upper curve shows the action of only tightly-adsorbed enzymes); B: *T. longibrachiatum*; C: *S. dimorphosporum*.

Trichoderma viride and *T. longibrachiatum* cellulases contain both tightly- and loosely-adsorbing enzymes. Under the action of the *S. dimorphosporum* cellulase preparation, containing mostly the loosely-adsorbing enzymes (Rabinowitch *et al.*, 1982), the DP of cellulose decreased far less (Fig. 4A and B). The DP of cotton cellulose after 50–80% hydrolysis by *T. longibrachiatum* cellulases decreased 2·5 times, while with *S. dimorphosporum* cellulases DP decreased only 1·3 times (Fig. 4A). The DP of hydrocellulose hydrolyzed by the *S. dimorphosporum* preparation did not decrease at all, at least up to 40–50% hydrolysis (Fig. 4B), despite the fact that the *endo*-1,4-β-glucanase (viscometric) activity in the reaction mixture with the *S*

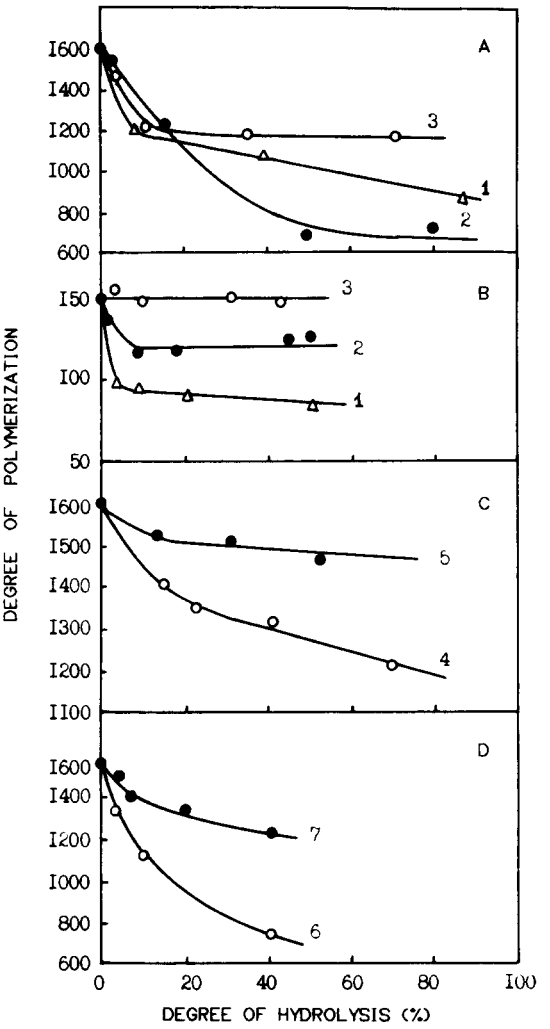

Fig. 4. Change in degree of polymerization of cellulose in the course of enzymatic hydrolysis. A, C, D: Ball-milled cotton cellulose; B: Hydrocellulose. 1, *T. viride* cellulase complex preparation; 2, *T. longibrachiatum* cellulase complex preparation; 3, *S. dimorphosporum* cellulase complex preparation; 4, *T. viride* loosely-adsorbing enzymes; 5, *T. viride* tightly-adsorbed enzymes; 6, *T. viride endo*-1,4-β-glucanase fraction; 7, *T. viride* cellobiohydrolase fraction.

dimorphosporum preparation was twice as high as for *T. longibrachiatum*, as *endo*-1,4-β-glucanase should cause the greatest DP shifts due to its specificity. A comparison of Fig. 4A and C reveals that the DP of cotton cellulose decreased about half as much with tightly- or loosely-adsorbing *T. viride* cellulases as with the whole complex. *Trichoderma*

viride endo-1,4-β-glucanase and cellobiohydrolase were adsorbed on cellulose to about the same extent (57 and 50%, respectively), so the ratio of *endo*-1,4-β-glucanase to cellobiohydrolase in tightly- and loosely-adsorbing fractions was similar to that in the initial cellulase preparation. This eliminated possible effects of variations in the component composition of tightly- and loosely-adsorbing fractions on cellulose hydrolysis.

The pattern and values of DP changes depended on the mode of action of cellulolytic enzymes. *endo*-1,4-β-Glucanase from *T. viride* hydrolyzed the ball-milled cotton cellulose randomly, destroying mostly the inner glucoside linkages in the polymer cellulose molecule, which resulted in a considerable decrease in cellulose DP even at a low extent of hydrolysis (1·4-fold in 10% hydrolysis, Fig. 4D). *Trichoderma viride* cellobiohydrolase hydrolyzed cellulose by the terminal mechanism, decreasing the DP of cellulose to a lesser extent (1·1-fold in 10% hydrolysis and 1·3-fold in 40% hydrolysis, Fig. 4D).

DISCUSSION

The effect of the physico-chemical and structural features of cellulose on the efficiency of enzymatic hydrolysis has been much studied (Klyosov, 1984). Many recent papers on the subject report dissimilar results, and improved research methods seem only to enlarge the controversy. However, most of the authors have worked with only one or two cellulosic materials and a single cellulase preparation, or preparations from one microbial source. Often the study has been performed to elucidate the role of just one or two factors, such as degree of polymerization, crystallinity index, specific surface area, particle dimensions, or moisture content, and then generalizations have been made about the hydrolysis of all cellulosic materials.

It is probably impossible to create a consistent picture of the relationship of the physico-chemical and structural features of cellulose to the efficiency of enzymatic hydrolysis without taking into account a number of factors. These must include the composition of multienzyme complexes, especially the relative content of *endo*- and *exo*-enzymes; the properties of cellulases (in particular, their adsorption ability); the size and shape of the molecules and the ability of enzymes to penetrate into the pores and capillaries of cellulosic materials, and the composition of cellulosic material itself. In addition, parameters that affect the course of the process, such as thermostability of cellulases, the changes in their conformations on the cellulose surface leading to subsequent inactivation, and so on, should be considered.

The change in cellulose reactivity has been studied, as well as changes in its physico-chemical parameters during enzymatic hydrolysis, as a function of the degree of cellulose conversion, rather than of the reaction time, as is usually done. This allowed a somewhat unusual description of regularities in hydrolysis of crystalline cellulose (CI = 86%) and partially-amorphized (CI = 66%) cellulose by three cellulase preparations (*T. viride, T. longibrachiatum* and *S. dimorphosporum*), the *endo*- and *exo*-enzymes and the tightly- and loosely-adsorbing cellulases from *T. viride.*

The results allow two new inferences on regularities of the enzymatic cellulose hydrolysis and provide insight into the course of enzymatic hydrolysis of pure cellulose under the action of cellulase preparations differing in composition and sorption.

The first inference is that tightly- and loosely-adsorbing cellulases have different effects on the structural parameters of cellulose. The reactivity of amorphized cellulose decreases rapidly at the initial stage of hydrolysis by tightly-adsorbed cellulases, then levels off but remains relatively high (Fig. 1C). Tightly-adsorbing enzymes enlarge the specific surface area, probably due to dispersion of cellulose, whereas the action of the total *T. viride* preparation does not produce this effect (Fig. 3A). On the other hand, the loosely-adsorbing cellulases depolymerize cotton cellulose more than tightly-adsorbing enzymes (Fig. 4C).

The second inference is that changes in the physicochemical parameters of cellulose (CI, SSA, DP) occur early in hydrolysis. At 5–10% hydrolysis the crystallinity index of cellulose increases (relatively more for amorphized than crystalline cellulose, and more for cellobiohydrolase than *endo*-1,4-β-glucanase; Fig. 2); the specific surface area decreases (somewhat more in the case of amorphized cellulose or the use of tightly-adsorbed cellulases; Fig. 3) and depolymerization takes place (Fig. 4). These changes lead to a lower cellulose susceptibility for enzyme attack during the initial phase of the reaction (Fig. 1). The decrease in reactivity is not so noticeable, however, in hydrolysis of crystalline cellulose or in the case of amorphous cellulose hydrolyzed by loosely-adsorbing enzymes.

Enzymatic hydrolysis of pure cellulose can be presented by the following simplified scheme. Cellulose, consisting of amorphous (susceptible) and crystalline (resistant) regions, is attacked by loosely- and tightly-adsorbing cellulases containing *endo*- and *exo*-enzymes (*endo*-1,4-β-glucanases and cellobiohydrolases). Amorphous regions are preferentially attacked by cellulases, since, in addition to their higher reactivity, they make up an essential portion of cellulose SSA and, therefore, enzymes have a higher probability of being absorbed on the amorphous regions. This leads to the initial rapid decline in DP and SSA

as well as to the increase in CI, the effects being more evident for the amorphous substrate.

As cellulose amorphous regions are exhausted, enzyme attacks on crystalline regions increase, which leads to a decline in the overall reactivity of cellulose (Fig. 1). At this stage the tightly-adsorbed cellulases that remain at the sites of primary attachment cannot move along the cellulose surface and, after a series of attacks, sink inside the cellulose matrix reaching new reactive glucoside linkages of the substrate. Loosely-adsorbing enzymes can desorb and pass to new sites on the surface. However, since the loosely-adsorbing enzymes hydrolyze crystalline cellulose poorly (Klyosov *et al.*, 1982), they appear to mostly attack amorphous regions and thereby expose crystallites. Tightly-adsorbed cellulases, on the other hand, attack both amorphous and crystalline regions. This leads to a constant decrease of substrate susceptibility to the action of loosely-adsorbing cellulases, while reactivity in relation to the tightly-adsorbed enzymes stabilizes after a certain initial decrease. The present experimental results totally support these ideas (Fig. 1C). It also becomes evident why the loosely-adsorbing enzymes depolymerize cellulose more rapidly than the tightly-adsorbing enzymes (Fig. 4C). The fact is that the DP of crystallites is relatively low (about 150) and their hydrolysis by tightly-adsorbing enzymes does not especially affect the average DP of cotton cellulose. On the other hand, the loosely-adsorbing cellulases, attacking mostly amorphous regions with a high DP, markedly depolymerize the substrate.

The same ideas help the understanding of why crystalline hydrocellulose retains its reactivity with increasing hydrolysis by *Trichoderma* cellulases, while the reactivity of more amorphous ball-milled cotton cellulose decreases under the same conditions (Fig. 1A).

The attack of cellulases, mostly tightly-adsorbed, on exposed crystalline regions, leads to their dispersion and an increase in the SSA of the substrate after its initial decrease. This shows the typical dependence of SSA on the extent of hydrolysis, which has a shape of a curve with a minimum (Fig. 3). As expected, the curve with the most apparent minimum was observed in the case of the action of the tightly-adsorbing enzymes on ball-milled cotton cellulose, where after the initial decrease SSA increased more than 2-fold (Fig. 3A). Such dependence was also seen in the hydrolysis of more crystalline hydrocellulose (Fig. 3A and B). This process appears to be accompanied by some amorphization of cellulose crystallites, since the decrease in the CI occurs at the same degree of hydrolysis (Fig. 2).

These data show that the pattern for changes of some physicochemical and structural features of pure cellulose, as well as for changes

in its reactivity in the course of hydrolysis, cannot be reliably predicted without the knowledge of not only initial characteristics of substrate but also the composition of the cellulase complex and adsorption parameters of the constituent enzymes. It should be emphasized that in previous work concerned with cellulose structure on the efficiency of enzymatic hydrolysis, neither the composition of the cellulase complex nor the adsorption characteristics of the enzymes were determined. Taking these parameters into account, it is possible to describe the dynamics of changes in reactivity and basic structural features that occur during enzymatic hydrolysis of cellulose.

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