Changes of Structure and Morphology of Regenerated Cellulose Caused by Acid and Enzymatic Hydrolysis*

J. LENZ,* H. ESTERBAUER,† W. SATTLER,† J. SCHURZ,‡ and E. WRENTSCHUR,‡ * *Lenzing AG, 4860 Lenzing, Austria,* † *Institute of Biochemistry, University of Graz, 8010 Graz, Austria, and* ‡ *Institute of Physical Chemistry, University of Graz, 8010 Graz, Austria*

Synopsis

The kinetics of the enzymatic hydrolysis of regenerated cellulose fibers before and after acid prehydrolysis has been investigated. This pretreatment changes the kinetics from a monophasic to a biphasic first order reaction. Prolonged acid prehydrolysis increases the proportion of the enzymatically easily hydrolyzable cellulose form. The kinetics were correlated with corresponding changes of the degree of polymerization, crystallinity and crystallite size.

INTRODUCTION

The acid hydrolysis of native and regenerated cellulosic fibers begins with the cleavage of the glycosidic linkages of the cellulose molecules between the crystalline domains. The degree of polymerization (DP) rapidly decreases until it reaches the level-off degree of polymerization (DP_{LO}). The average length of the cellulose crystallites corresponds to the value of DP_{LO} that means an intrinsic viscosity of about 20 mL/g. In the initial stage of hydrolysis of modal and viscose fibers the crystallinity increases significantly from 30 to 45% and remains constant thereafter.[1,2] This is probably the result of a growth, respectively recrystallization of the microcrystals. The crystallite size perpendicular to the (101)-lattice planes (notation according to Meyer and Misch[4]) amounts to 4.2–5.1 nm.[3] After acid hydrolysis values between 7.8 and 8.9 nm were measured.[1]

According to Schurz et al.[5] the enzymatic hydrolysis of viscose fibers causes a decrease of the intrinsic viscosity from 250 to 140 mL/g and an increase of the crystallinity from 29 to 39% after 44 h. Strong changes of the structure, however, are not typical for the enzymatic hydrolysis of cellulosic materials. Neither cotton nor woodpulp show an essential decrease of the DP during enzymatic hydrolysis. The intrinsic viscosity of spruce sulfite pulp decreases only from 760 down to 710 mL/g, whereas the crystallinity increases from 60 to 66%.[6] Bertrand and Buleon also found only a small increase of the crystallinity of mercerized cellulose.[7] This result has been confirmed by Chang et al.[8] From these results Schurz and Esterbauer deduced the following reaction

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mechanism: The cellulase complex diffuses through the pore system to the microfibrils, attacks the cellulose chains and hydrolyzes each chain to the end.[9] Consequently, neither the ratio of crystalline to amorphous material nor the DP of the residue changes significantly.

The "whole or nothing" concept should be considered in view of the kinetics of the enzymatic hydrolysis which can be described by *two* parallel first-order reactions. This function is valid for such different substrates as microcrystalline cellulose, pulps, cereal straw, and steamed poplar wood.[10] Obviously the enzyme complex discriminates between two components which we shall call cellulose a and cellulose b. It has been proposed that the loosely structured amorphous regions are hydrolyzed more rapidly than the crystalline domains as in the case of acid hydrolysis, leading to a progressive increase of the crystallinity of the substrate.[11] Evidently this assumption is not compatible with the small increase of crystallinity which has been observed.

A definition of cellulose a and cellulose b has to take into consideration that the enzymatic hydrolysis of at least two substrates, namely cotton and regenerated cellulose fibers, can be described by a monophasic first-order reaction.[5,12] As morphology and structure of viscose fibers are simpler than those of cotton regenerated cellulose is a promising object to study the correlation between the kinetics of the enzymatic hydrolysis and the structural changes.

Our goal was to evaluate the mechanism of the enzymatic hydrolysis of regenerated cellulose fibers as well as of samples prehydrolyzed by means of hydrochloric acid. This treatment destroys the fiber morphology and results in a fibrillar powder.

EXPERIMENTAL

Fiber Sample

For the investigation 5 samples of viscose fibers of the high wet modulus type were chosen, so-called modal fibers, because this type has a smoother surface and a higher crystallinity than the normal viscose fiber type.

Acid Prehydrolysis

The fiber samples were prehydrolyzed with 2 *N* hydrochloric acid at 60°C during 120 h at most. The prehydrolyzed material was filtered off, washed thoroughly, resuspended in water, sonified and dried at 100°C. After a hydrolysis time of 120 h the fibers turned in to a fast sedimenting fibrillar powder.

Enzymatic Hydrolysis

After preswelling of 1 g of the untreated, respectively prehydrolyzed fiber sample in 0.05 M citrate buffer (pH 4.8) 10 FPU of cellulase (Celluclast CCN, Novo) was added, supplemented with 10 IU β-glucosidase to give a final volume of 100 mL. The flask was sealed and incubated in a rotary shaker (200 rpm) at 50°C to periods up to 120 h. At different times 2.5 mL samples of the mixture were withdrawn and transferred into screw capped tubes, heated for 10 min on

a boiling water bath and centrifuged. The concentrations of glucose were then determined by HPLC using a HPX 87P column (For details see Ref. 10).

Determination of the Intrinsic Viscosity ($GVZ_{Cuen/200}$)

The relative viscosity was measured according to the Zellcheming Merkblatt IV 36/61 in Cuen and converted into the intrinsic viscosity by the Martin equation.

Determination of the Degree of Crystallinity

The degree of crystallinity was determined by means of wide-angle X-ray diffraction (WAXD) using a Kratky camera with pinhole collimation. During the exposition the sample was rotated with constant velocity in order to eliminate the influence of orientation effects, which simulate a higher degree of crystallinity. The diffractograms were evaluated by planimetry, eliminating the background by drawing the basis line visually as a steady curve, linking the minima.

Determination of the Crystallite Dimensions

After a prehydrolysis time of 120 h in 2 *N* HCl at 60°C the suspension was further disintegrated by means of a sonifier. The sample was prepared for transmission electron microscopy (TEM) according to the method of negative staining with phosphotungstic acid. The cellulose crystallites could clearly be distinguished as bright platelets. They were partly connected to beadlike chains. On the micrographs length and width of all clearly visible platelets were measured in certain gridsquares. Each value is the average of 80–90 single measurements.

RESULTS AND DISCUSSION

The Effect of Acid Prehydrolysis on the Subsequent Enzymatic Hydrolysis

Viscose fibers were prehydrolyzed with hydrochlorid acid to different degrees by heating the material at 60°C up to 120 h. The kinetics of the hydrolysis is shown in Figure 1. During the first 30 h, about 8% of the cellulose is rapidly hydrolyzed. Thereafter the hydrolysis proceeds at a more or less constant rate and reaches 16% after 120 h. The residues remaining after a 30-, 50-, 70-, and 120-h acid prehydrolysis were then subjected to enzymatic hydrolysis and compared to the untreated fibers (Fig. 2). In this case the fraction of cellulose hydrolyzed by the enzyme is expressed in g glucan solubilized as glucose per 100 g initial material.

The results show that a short acid pretreatment of 30 or 50 h makes the fibers significantly more resistant to enzymatic hydrolysis compared with the original untreated fibers. In the particular experiment shown in Figure 2 50.6% of the untreated material could be hydrolyzed by the enzyme in 120 h, but only 31, 0, and 40.3% of the fibers pretreated with acid for 30 or 50 h were hydrolyzed

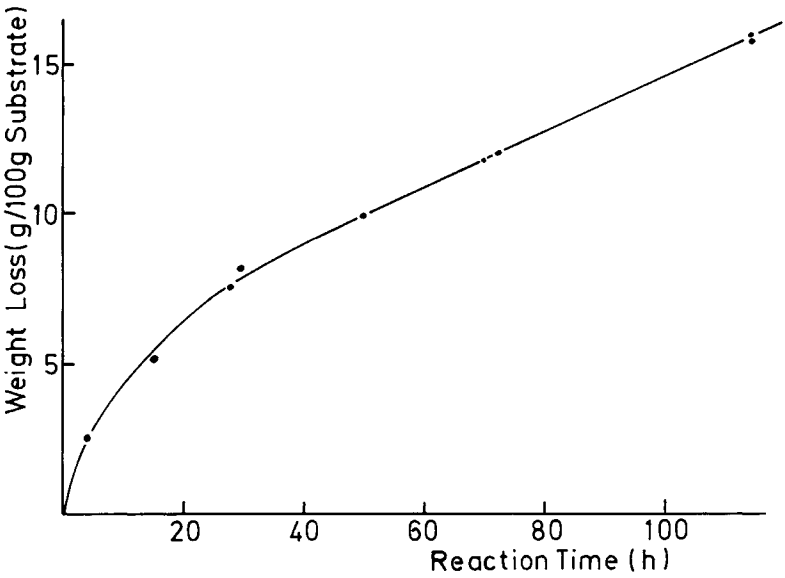

Fig. 1. Weight loss of modal fibers during acid prehydrolysis.

(Table I, Series 2a–2e). This effect was reproducible in a second set of experiments (Table I, Series 1a–1d). Again the acid pretreated fibers (50, 70, 120 h) were considerably more resistant to enzymatic hydrolysis compared with the untreated material.

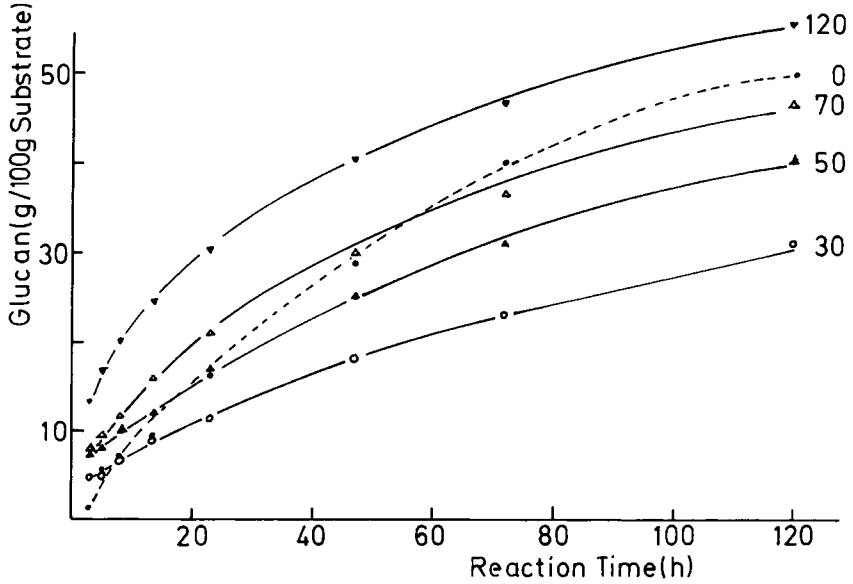

Fig. 2. Solubilization of native and acid prehydrolyzed modal fibers by enzymatic hydrolysis expressed in g glucan solubilized as glucose per 100 g initial material. Indication of the curves: time of acid prehydrolysis in hours.

TABLE I
The Dependance of the Glucan Yield after an Enzymatic Hydrolysis of 120 h of Modal Fibers on the Duration of the Acid Prehydrolysis

Experiment	Acid prehydrolysis reaction time (h)	Enzymatic hydrolysis glucan yield after 120 h (%)
1 a	0	66.6
1 b	50	31.5
1 c	70	36.9
1 d	120	46.8
2 a	0	50.6
2 b	30	31.0
2 c	50	40.3
2 d	70	47.3
2 e	120	55.5
3 a	0	36.0
3 b	120	82.8
4 a	0	27.0
4 b	120	82.8
5 a	0	34.2
5 b	120	94.0

This resistance to enzymatic hydrolysis is obviously progressively lost as hydrolysis time increases. Fibers pretreated for 120 h with HCl were in three experiments (Table I, Series 3–5) highly susceptible to the enzyme and about 80–90% could be hydrolyzed, which is highly significant compared with the 30–35% hydrolysis of the untreated sample. In one experiment, this effect was much less pronounced and the 120 h pretreated material showed only a slight increase in hydrolyzability (Table I, Series 2a–e). In one case, the fibers treated 120 h were still more resistant to enzymatic hydrolysis than the untreated material (Table I, Series 1a–d), but the increase in enzymatic hydrolyzability with increasing acid prehydrolysis was found also in this case.

It has been shown for various types of cellulosics including microcrystalline cellulose that the kinetics of the enzymatic hydrolysis can be described phenomenologically by the summation of two parallel first order reactions according to:

$$v = v_a + v_b = k_a \cdot [\text{cell a}] + k_b \cdot [\text{cell b}]$$

where v is the rate of the formation of soluble sugars (g/L h), [cell a] and [cell b] are the concentrations of the two kinetically different forms of cellulose (g/L), and k_a and k_b are the rate constants associated with the hydrolysis of the two cellulose components in h^{-1}. In this case k is not the absolute rate constant but rather a parameter which includes also accessibility, adsorption phenomena, enzyme loading and other so far unknown effects.[5] Nevertheless, this parameter k has been proved by several authors as a useful index for the characterization of different substrates. Details are described elsewhere.[10]

With the experimental hydrolysis data shown in Figure 2, we have computed by means of a nonlinear regression statistic program package (BMDP) on a VAX 750 for each hydrolysis curve the associated values for [cell a] and [cell b], k_a and k_b. The results of this calculation are summarized in Table II. For the untreated modal fiber, the convergence criteria in the calculation were only reached with the assumption that its hydrolysis proceeds by only one first-order reaction, which means that for the cellulase enzyme the untreated fiber behaves like an uniform substrate. This finding is fully supported by other independent methods.[5] From the data in Table II it is also evident that the acid pretreatment leads to the following changes of the regenerated cellulose:

(a) The original fiber is converted by acid pretreatment into two kinetically different forms. One of which is about two- to threefold less accessible for the cellulase enzyme (i.e., rate constant k_b drops from 0.0068 to 0.0036 h^{-1}), and another one which is about 20 times more accessible for the enzyme, i.e., rate constant increases from 0.0068 to 0.140 h^{-1}.

(b) The proportion of the easily hydrolyzable cellulose (cell a) increases with increasing time of acid pretreatment from 0 to 24.3% after 120 h acid pretreatment.

(c) The rate constants k_a and k_b slightly increase with increasing duration of acid pretreatment.

The peculiar finding that the extent of enzymatic hydrolysis reached after 120 h (Table I, Fig. 2) is either lower or higher—depending on the duration of the acid pretreatment—than that of untreated fibers results then from the increase of the proportion of the easily hydrolyzable cellulose form with increasing duration of acid pretreatment on the expense of the heavily hydrolyzable form.

After a pretreatment time of 30–70 h the abundant fraction (about 90%) of the substrate still exists in a form less easily hydrolyzable than the native fibers. The small fraction (about 10%) of the easily hydrolyzable fraction generated by the acid pretreatment does not essentially contribute to the hydrolysis yield at 120 h. If the acid pretreatment becomes more severe, however, the

TABLE II
Percentage of Easily (Cell a) and Heavily (Cell b) Enzymatically Hydrolyzable Cellulose in Untreated and Acid Pretreated Modal Fibers and Associated Rate Constants k_a and k_b (Series 2a–e)

Pretreatment time (h)	Cell a		Cell b	
	%	k_a (h^{-1})	%	k_b (h^{-1})
0	0	—	(100)	(0.0068 ± 0.0003)
30	8.2 ± 1.0	0.109 ± 0.031	91.8	0.0024 ± 0.0001
50	11.4 ± 1.6	0.145 ± 0.049	88.6	0.0033 ± 0.0002
70	14.4 ± 1.0	0.141 ± 0.023	85.6	0.0041 ± 0.0001
120	24.3 ± 2.1	0.165 ± 0.036	75.7	0.0046 ± 0.0004
	Mean	0.140 ± 0.023	Mean	0.0036 ± 0.0009

TABLE III
Change of the Intrinsic Viscosity of Modal Fibers After Acid Respectively Enzymatic Hydrolysis of 120 h

Experiment	Prehydrolysis (120 h)	Enzymatic hydrolysis (120 h) intrinsic viscosity (mL/g)	
		Before	After
4a	No	259	231
4b	Yes	17.5	21.3
5a	No	259	224
5b	Yes	14.7	21.6

TABLE IV
Change of Crystallinity of Modal Fibers after Acid Respectively Enzymatic Hydrolysis of 120 h (Elimination of Orientation Effects)

Experiment	Prehydrolysis (120 h)	Enzymatic hydrolysis (120 h) crystallinity (%)	
		Before	After
3a	No	29.5	29.3
3b	Yes	47.2	47.8
4a	No	34.4	32.9
4b	Yes	46.2	49.1
5a	No	31.0	31.0
5b	Yes	47.5	51.1

TABLE V
Change of Crystallinity of Modal Fibers after Acid Respectively Enzymatic Hydrolysis (Orientation Effects are Present)

Experiment	Prehydrolysis reaction time (h)	Enzymatic hydrolysis (120 h) crystallinity (%)	
		Before	After
2a	0	43.2	36.5
2b	30	43.9	47.9
2c	50	45.7	51.0
2d	70	51.4	50.4
2e	120	50.8	54.6

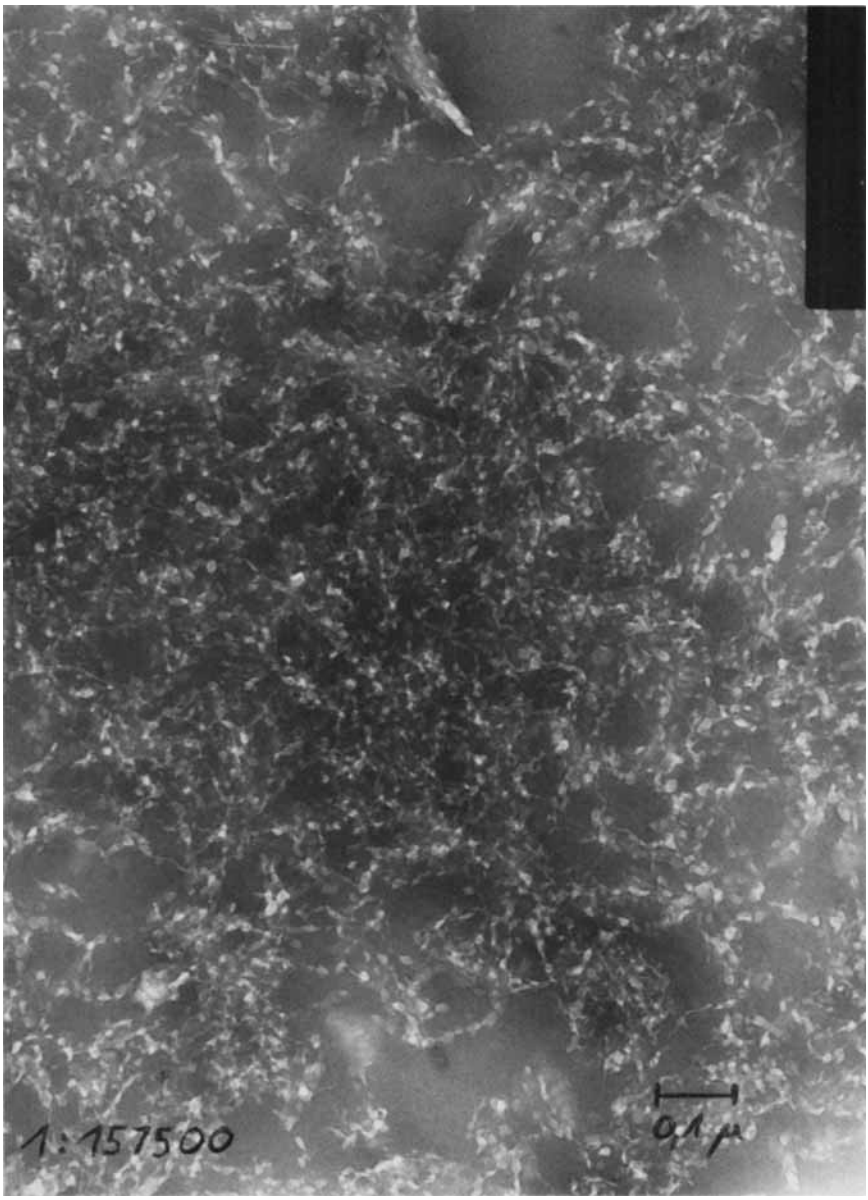

Fig. 3. Transmission electron micrograph of acid prehydrolyzed modal fibers. Magnification 1 : 157.500.

easily hydrolyzable fraction substantially increases, which in turn results in high hydrolysis yields at 120 h enzymatic hydrolysis. As a consequence, the slow hydrolysis rate ($v_b = k_b \cdot [\text{cell b}]$) is progressively compensated and finally (120 h pretreatment) overcompensated by the fast hydrolysis rate ($v_a = k_a \cdot [\text{cell a}]$).

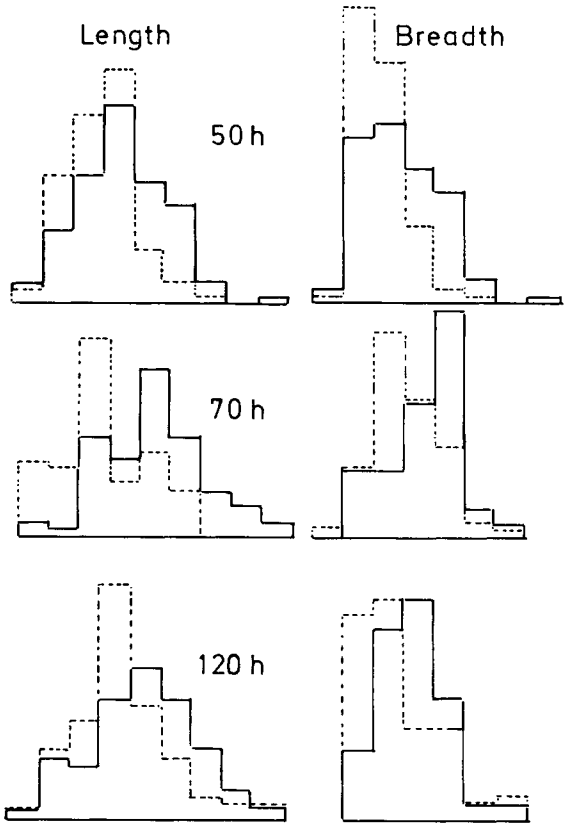

Fig. 4. Distribution of the size of microcrystals of acid prehydrolyzed modal fibers before (——) and after (---) enzymatic hydrolysis of 120 h. Indication of the curves: time of acid prehydrolysis in hours.

For the understanding of these results one has to realize that during the acid prehydrolysis the morphology of the substrate changes fundamentally (i.e., the fibers are converted into a fine fibrillar powder similar to microcrystalline cellulose). This could be confirmed by the determination of the intrinsic viscosity. Table III shows the changes of the intrinsic viscosity during the acid and the enzymatic hydrolysis. As expected the hydrochloric acid decomposes the cellulose until it reaches a viscosity level, which corresponds to the DP_{LO}, whereas the enzymatic hydrolysis does not change the intrinsic viscosity neither of the untreated nor of the prehydrolyzed fibers significantly.

In the case of the intact, original fiber we found a decrease of the fiber cross section during the enzymatic hydrolysis. After 120 h reaction time it decreased from 11.6 ± 1.8 to 8.2 ± 2.0 μm corresponding to an estimated volume decline of 50%. As the cross-section of these fibers is not circular, a complete conformity with the unhydrolyzed residue of 64% cannot be expected. Consequently, the cellulase complex attacks the fiber only at its surface. It does not penetrate into the fiber through the pore system. This seems to be impossible, because the elementary fibrils are aggregated to nonswelling clusters to an extent of

TABLE VI
Change of the Crystallite Size of Modal Fibers Acid Prehydrolyzed for 50, 70, and 120 h After an Enzymatic Hydrolysis of 120 h (Series 2c–e)

Acid prehydrolysis			Enzymatic hydrolysis (120 h) crystallite	
	Crystallite			
Reaction time (h)	Length (nm)	Breadth (nm)	Length (nm)	Breadth (nm)
50	9.7 ± 2.9	6.5 ± 2.0	8.4 ± 2.0	5.2 ± 1.7
70	13.3 ± 3.3	8.0 ± 2.1	10.0 ± 2.9	6.8 ± 2.2
120	11.4 ± 3.2	7.1 ± 2.0	10.2 ± 3.0	6.4 ± 2.4

more than 50%.[13] The existing pores have an average diameter of 5–6 nm in the swollen state (i.e., rather small for the enzyme having a diameter of 4–5 nm). Thus, the enzyme "sees" only one kind of surface that of the entire fiber. This result harmonizes with the kinetic of the enzymatic hydrolysis of the native fiber which shows only one first-order reaction.

In the case of the pretreated fibers one has to consider another kind of surface, which has developed during the acid prehydrolysis. As the elementary fibrils consist essentially of microcrystals the further investigations concentrated upon the changes of crystallinity and the size of crystallites.

Crystallinity Changes During the Enzymatic Hydrolysis of Untreated and Acid Prehydrolyzed Modal Fibers

As mentioned earlier some cellulosic materials show a slight increase of crystallinity during enzymatic hydrolysis. Therefore the degree of crystallinity of the native and prehydrolyzed fibers was determined before and after an enzymatic hydrolysis of 120 h. The results are listed in Table IV. From these values the following conclusions can be drawn:

(a) As expected the acid prehydrolysis causes a significant increase of crystallinity.

(b) Enzymatic hydrolysis does not change the crystallinity of the native fibers. This can be expected assuming that the enzyme complex peels off the cellulose molecules from the fiber surface according to the "whole or nothing" concept.[9]

(c) With prehydrolyzed fibers, a slight increase of the crystallinity was found in the same range as reported previously.[6–8]

The possibility that the slight difference of crystallinity before and after the enzymatic hydrolysis is only casual cannot be excluded. Therefore, the crystallinity changes of series 2a–e were determined too. In this experiment the orientation effect was not eliminated by rotating the fiber sample in the X-ray

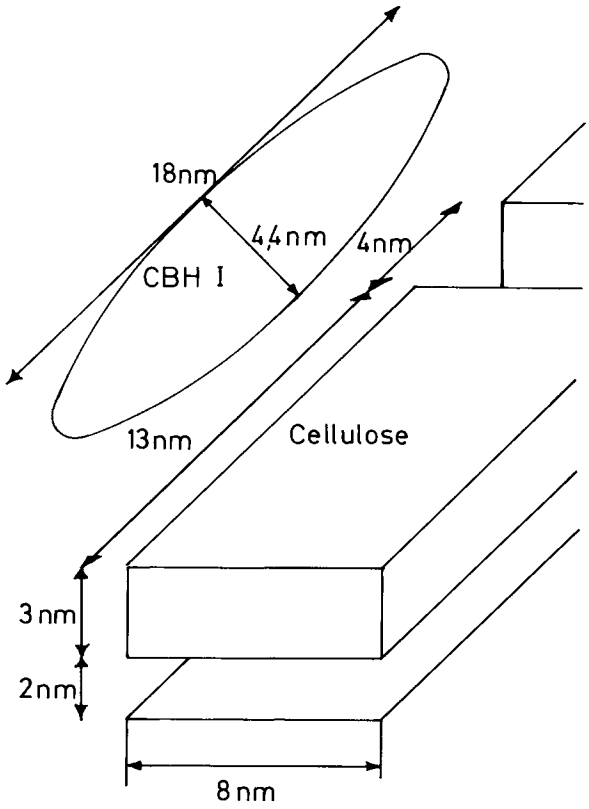

Fig. 5. Schematic drawing of a cellobiohydrolase I molecule and a cellulose II microcrystal of acid prehydrolyzed modal fibers.

beam. The results are shown in Table V. Apart from experiment 2d a slight increase of crystallinity could be observed here too.

Additionally the crystallite dimensions of the prehydrolyzed fibers of series 2c–e before and after enzymatic hydrolysis were determined. The crystallite dimensions of unhydrolyzed fibers cannot be measured, because the ultrasonic disintegration of unhydrolyzed material to single microcrystals is impossible. Figure 3 shows an example of an electron micrograph of an acid prehydrolyzed fiber which was evaluated. The results are given in Table VI. The differences between the mean values are statistically significant in every case. The mean values coincide with the maximum frequency of the distribution curves, as demonstrated in Figure 4. The results show that the broad face of the crystallites become 10–20% smaller and shorter during the enzymatic hydrolysis.

CONCLUSION

The results of the determinations of the crystallinity show that the crystalline and amorphous regions of the untreated and acid prehydrolyzed fibers are hydrolyzed by the enzyme complex essentially at the same rate. Consequently,

the occurrence of a biphasic reaction after prehydrolysis cannot be interpreted by a preferred hydrolysis of the disordered domains.

In the interpretation of the reaction kinetic one has to consider that acid prehydrolysis makes the cellulose more resistant to enzymatic hydrolysis but also gives rise, on the other hand, to the occurrence of cellulose a, the proportion of which increases with increasing time of prehydrolysis. Chanzy et al.[14] have shown by electron microscopy that cellobiohydrolase I (CBH I) adsorbs preferably at certain edges of Valonia microcrystals and to a less extent at their faces. As mentioned earlier, acid hydrolysis broadens the lamellalike microcrystals by recrystallization[1,3] and produces new edges by cleavage of the macromolecules interconnecting the crystallites. So it could be assumed that by the acid pretreatment new sites for the adsorption of the cellulase emerge corresponding to cellulose a, whereas the recrystallization reduces the adsorption by the growth of the broad crystal faces corresponding to cellulose b.

This assumption explains the decrease of the crystallite size during the enzymatic hydrolysis of acid prehydrolyzed modal fibers. Supposing that cellulase adsorbs preferably at the edges of the microcrystals the broad faces of the platelets should become smaller. According to Table VI, this reduction amounts to 10–20%, which corresponds to the percentage of cellulose a as given in Table II.

Figure 5 shows a schematic drawing of a CBH I-molecule, which is the major enzyme in cellulase, and a cellulose II microcrystal. The dimensions of CBH I were taken from Schmuck et al.,[15] giving the molecule the general shape of an ellipsoid. The figure shows that sterically the crystallites can be attacked by the enzyme at their edges.

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