Hydrolysis of Cotton Fibers by Cellulase Enzyme

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Synopsis

Cotton cellulose has been subjected to continuous and repeated enzymatic hydrolysis for different periods. It has been observed that the length of hydrocellulose particles obtained on repeated enzyme action is reduced to about 300–500 Å as compared to 900–3000 Å obtained on prolonged enzyme treatment. Corresponding changes in moisture regain, crystallinity of the hydrolysates, and weight loss brought out by the hydrolysis are also discussed.

INTRODUCTION

It is well known that microcrystalline cellulose, which is widely used in pharmaceutical industries, is obtained by heterogeneous acid hydrolysis and subsequent mechanical disintegration of alkali cellulose, regenerated cellulose or special grade (high α content) wood pulp. The main advantage of acid hydrolysis is rapid decrease in chain length, thus reducing the substrate to hydrocellulose particles.[1]

In the enzymatic hydrolysis of native cellulose, on the other hand, the molecular weight of residual cellulose remains almost invarient. For instance, the hydrolysis of cotton linters by cellulase enzyme of *Aspergillus niger* brought about the reduction in DP from 1385 to 1105 in 144 h, while the DP of the same cellulose material decreased to 105 on acid hydrolysis in 48 h.[2] Similar observations have also been reported by Reese et al.[3] and Nisizawa et al.[4] The cellulases involved were mostly from *Trichoderma* species. Ogiwara and Arai[5] have further reported that up to 50% weight loss was attained without any change in molecular weight distribution of degraded cellulose. It has also been shown that enzymatic hydrolysis of crystalline hydrocellulose leads to fragmentation of longer particles into smaller ones.[6,7] Rautela and King[8] have reported that ground cotton linters, when exposed to enzyme action give crystalline cellulose of length 650 nm. Our recent studies[9–11] have shown that enzymatic hydrolysis of bagasse pulp (BP) and wheat straw pulp (WSP) for 6 h followed by mechanical degradation yields hydrocellulose-like particles. However, this was not the case with cotton cellulose since it is highly crystalline. In a recent paper from this laboratory it has been demonstrated that even the highly crystalline cotton cellulose could be drastically hydrolysed by treating it repeatedly with fresh enzyme solution.[12] The present paper deals mainly with the investigations on repeated enzyme action on cotton cellulose with a view to obtaining hydrocellulose with particle sizes much smaller than reported hitherto. The changes observed in fiber morphology, moisture regain, crystallinity, and crystalline dimensions of the hydrolysates have also been discussed.

Journal of Applied Polymer Science, Vol. 29, 2573–2580 (1984)
 CCC 0021-8995/84/082573-08$04.00

EXPERIMENTAL

The cellulase enzyme filtrate was obtained by growing *Penicillium funiculosum* (isolate F4)[13] in the medium recommended for *Trichoderma viride*.[14] The activity of enzyme filtrate was determined using filter paper assay[15] and expressed in terms of glucose produced in reaction mixture after one hour of inoculation at 50°C. The strength of cellulase enzyme was adjusted to release 1000 μg/mL of reducing sugar in all the experiments. The enzyme hydrolysis of purified cotton sample was carried out using following system: 100 mg cotton; 5 mL enzyme filtrate; 10 mL acetate buffer (pH 4.8); 5 mL of distilled water.

In one set of experiments, the system was incubated at 50°C in water bath with constant shaking for different time of intervals varying from 1 day to 18 days continuously.

In another experiment of enzyme hydrolysis, the enzyme solution was replaced by fresh enzyme solution after 3 days of incubation time. The reason for such repeated treatment after 3 days has been explained in an earlier paper.[12] Considering this 3 days' treatment as one cycle of enzyme hydrolysis the specimen was subjected to different cycles varying from 1 to 6 cycles of enzyme hydrolysis. At the end of the incubation period, the system was filtered through previously weighed sintered glass crucible. From the filtrate the total reducing sugar produced as a result of enzymatic hydrolysis was determined using Somogyi's method.[16] The residue was washed with distilled water till it was free from enzyme. The residue was then dried at 105°C in an oven.

Moisture regain of the samples, continuously or repeatedly treated with cellulase enzyme was determined at 65% RH by following standard procedures.[17]

Radial X-ray diffractograms of both continously and repeatedly enzyme-treated samples were obtained using Ni-filtered CuK_α radiation with a Philips stabilized X-ray generator fitted with diffractometer attachment. The intensity data were read out from the strip-chart recorder and then normalized so that the areas under the intensity curves between the limiting ordinates $2\theta = 10°$ and $2\theta = 40°$ were the same for all samples. The percent crystallinity was estimated using the method described by Patil et. al.[18] The crystallite width perpendicular to the 002 plane was calculated from the width at half maximum intensity of 002 profile after substracting the background and using the Scherrer line broadening relationship.[19]

Transmission electron microscope (TEM) observations were made to study the cell wall morphology and determine the length of particles obtained after enzymatic hydrolysis of cotton cellulose. The cellulase enzyme-treated samples were suspended in distilled water and put in an ultrasonicator for 5 min to form a uniform collidal suspension. The suspension was further diluted by distilled water. A drop of suspension was put on carbon-coated grids and dried at room temperature. The specimen were then coated with Au/Pd at an angle of 27° to increase the contrast. The specimens were examined in Hitachi Hu11E TEM which was operated at 75 kV accelerating voltage. The length of the isolated particles were directly measured on the enlarged micrographs. These data were used to construct histograms to

obtain the distribution of particle lengths in both continuously and repeatedly enzyme-treated samples.

RESULTS AND DISCUSSION

Table I gives the results of various parameters measured for samples subjected to continuous and repeated enzymeatic hydrolysis.

The reducing sugar produced as a result of continuous enzymatic hydrolysis increased from 400 μg/mL for 3 days hydrolysis to 900 μg/mL for 18 days hydrolysis with weight loss of 6.7% and 14.2%, respectively. In the case of repeated cellulase treatment the reducing sugar produced increased progressively with the number of cycles of treatment. The total reducing sugar produced at the end of the sixth cycle of enzyme treatment (i.e., total 18 days hydrolysis) was 2885 μg/mL with the weight loss of 62%, which was much higher compared to that of 18-day-continuous enzyme treatment.

The hydrolyzed cellulosic materials showed marked fall in moisture regain after enzymatic hydrolysis. The moisture regain value decreased from 6.5% for the control sample to 3.7% at the end of the first cycle of enzymatic hydrolysis. The moisture regain decreased to only 3.5% for the 6-day-continuous enzyme-treated sample; however, it came down to 2.3% for the sample treated continuously for 18 days. The repeated cellulase enzyme action for the second and third cycles of treatment brought about only a marginal decrease in moisture regain, although a large amount of reducing sugar with significant weight loss was observed for these samples. Further, repeated cycles of enzyme treatment after the third cycle caused progressive decrease in moisture regain from 3.5% to 2.0%.

The normalized X-ray diffractograms of control and samples of first, fourth, and sixth cycles of cellulase enzyme treatment are given in Figure 1. The crystallinity slightly increased from 70% for the control to 73% at the end of 3 days enzymatic hydrolysis without any concomitant change in crystallite width perpendicular to (002) planes. The crystallinity remained constant at 73% after second and third cycles of cellulase treatment which was in line with the observed marginal fall in moisture regain values for these two samples. For subsequent cycles, however, the crystallinity increased and reached 81% at the end of the sixth cycle with an increase in crystallite width perpendicular to (002) planes. The lack of effect of repeated enzyme action on percent crystallinity and also on moisture regain in the case of second and third cycle of cellulase treatment particularly when considerable weight loss was observed (see Table I) needs some further detailed studies. The commonly accepted mode of action is that, in the initial stage of hydrolysis, easily accessible regions in cellulose are attacked by the large enzyme molecules. When all the readily accessible regions are dissolved, further cellulase action during the subsequent period or cycles of enzyme treatment must take place on other less accessible surfaces including surface of crystallites.[2] This simple two-phase model of fiber structure seems to be inadequate and the role of mesomorphous regions needs to be critically assessed in view of the observed results.

The transmission electron microscopy (TEM) of cellulase-treated samples revealed secondary cell wall with formation of open spaces in a fibrillar

TABLE I
Changes in Various Parameters of Cotton Cellulose after Continuous and Repeated Enzyme Treatments

Parameters	Control	Continuous enzyme treatment			Repeated enzyme treatment					
		3 days	6 days	18 days	1st cycle	2nd cycle	3rd cycle	4th cycle	5th cycle	6th cycle
Reducing sugar (μg/mL)	—	400	350	900	400	750	1390	1805	2365	2855
Weight loss (%)	—	6.7	5.4	14.2	7.0	13.2	26.6	35.5	45.2	62.0
Moisture regain (%)	6.5	3.7	3.5	2.3	3.7	3.6	3.5	2.7	2.2	2.0
Crystallinity %)	70	73	73	81	73	73	73	80	80	81
Crystallite width ⊥ to (002) plane (Å)	58	56	56	62	56	56	—	53	56	62

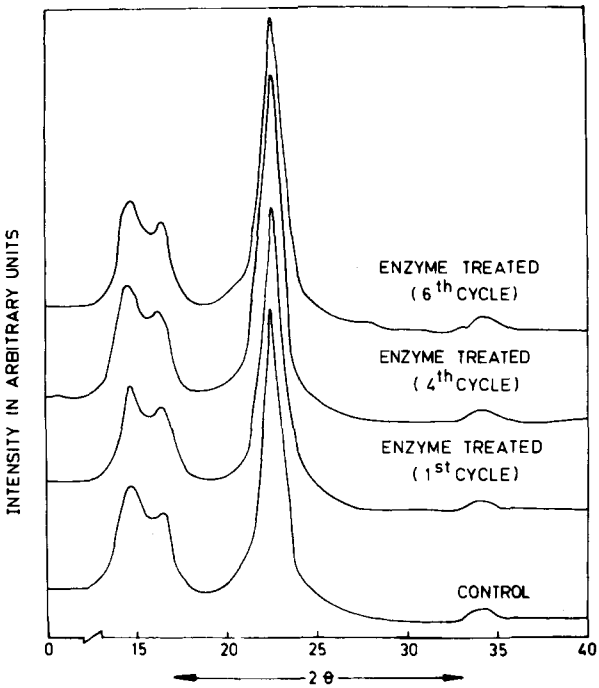

Fig. 1. Normalized radial X-ray diffraction profiles of enzyme-treated cotton samples: (a) control, (b) first, (c) fourth and (d) sixth cycles of enzyme treatment.

sheet and erosion of fibrillar surface at the end of the first cycle of enzyme hydrolysis (Fig. 2). After the continuous enzyme treatment for 18 days, considerable fragmentation of cell wall and dissolution of fibrillar structure was observed [Fig. 3(a)]. Also the continuous enzyme treatment for 18 days caused eventual formation of discrete needle shaped particles akin to hydrocellulose like particles [Fig. 3(b)]. The length of these particles varied between 900 and 1100 Å with an exception of some particles of length 3000 Å. The interesting feature observed is that the hydrocellulose like particles

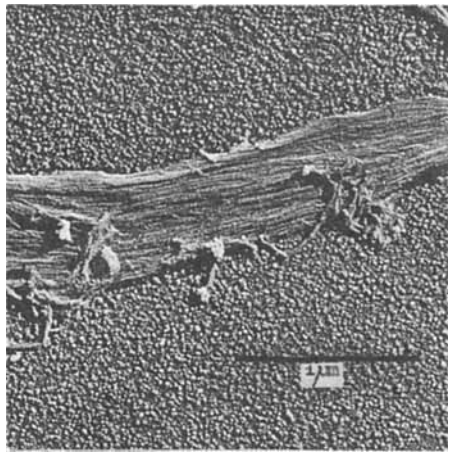

Fig. 2. Electron micrograph of 3-day enzyme-treated cotton sample. Note secondary cell wall with some open spaces.

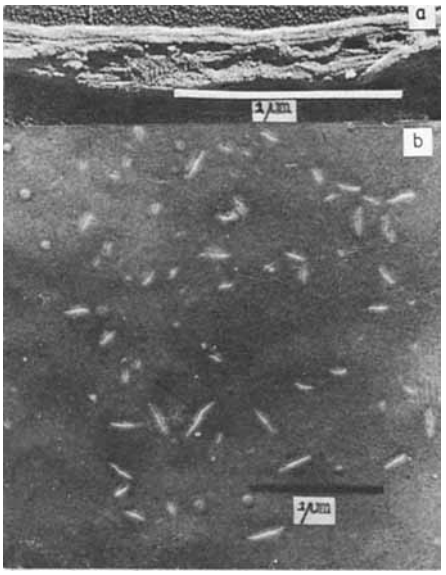

Fig. 3. Electron micrograph of 18-day continuously enzyme-treated cotton: (a) secondary cell wall fragment; (b) hydrocellulose-like particles.

with length around 3000 Å are having kink similar to that observed by Fernando[20] [Fig. 3(b)]. These particles have kink angle in the range of 100–150°. Such kinking can occur in the hydrolyzed particles due to weakening of the structure because of partial hydrolysis caused by continuous enzyme treatment.

In the case of the second cycle of enzyme treatment, lateral cracks in the fibrillar sheet were observed. The fibrillar sheets were eventually reduced to hydrocellulose-like particles after repeated cellulase action. This shows that, after the second cycle of enzyme treatment, the microfibrillar structure is so weakened that the enzyme penetrates within the body of microfibrils and causes scissioning of a sufficient number of chains to rupture the fibrils.[21] The typical electron micrograph of hydrolysed particles obtained at the end of the fifth cycle of enzyme treatment is shown in Figure 4.

The distribution of particle lengths obtained after the fourth and successive cycles of cellulase treatment is illustrated in Figure 5. At the end of the fourth cycle of cellulase treatment, a large majority of hydrocellulose particles have the length in the range of 700–900 Å. The particle length progressively reduces with repeated cycles of enzyme treatment giving rise to maximum number of particles of length 500–700 Å after the fifth cycle and 300–500 Å after the sixth cycle of cellulase enzyme treatment. It may be mentioned here that the maximum number of particles obtained after the sixth cycle of enzymatic hydrolysis have length of about 350 Å, which is of the same order as that of the length of crystallites reported for cotton cellulose.[22,23] It may further be mentioned, that the length of the particles obtained at the end of the sixth cycle of enzymatic hydrolysis is quite comparable to the size of 100–300 Å obtained by mechanical degradation of acid hydrolyzed cellulosic material from various sources as reported by

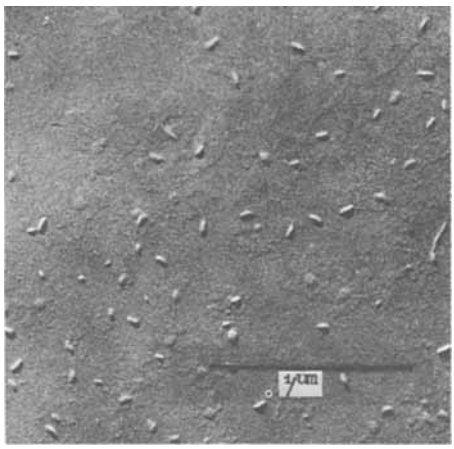

Fig. 4. Electron micrograph of discrete hydrocellulose like particles of cotton obtained after 5th cycle of enzyme treatment.

Battista.[1] It appears from the above that the residue obtained after repeated enzymatic attack or acid hydrolysis followed by mechanical degradation consists of almost perfect crystallites of cellulose having the same length. Similar values for the length of crystallites have also been reported based on the X-ray method.[18]

From the above results it may be concluded that prolonged enzyme action on native cotton cellulose yields hydrocellulose particles of length 900–3000

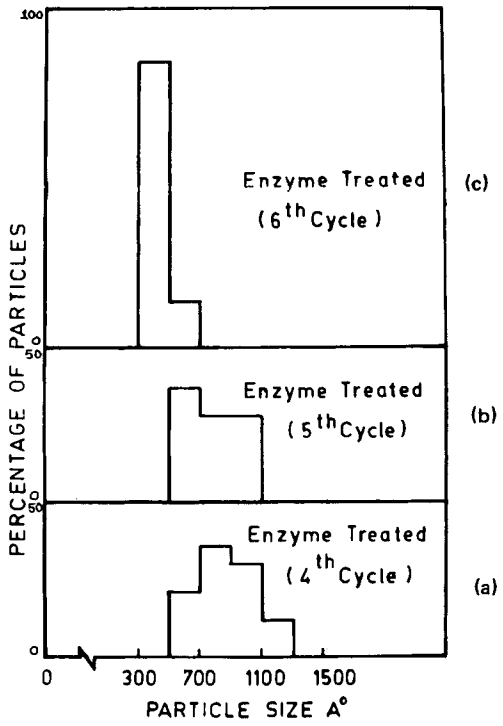

Fig. 5. Histograms of length measurement of particles obtained after repeated enzyme treatment: (a) fourth cycle; (b) fifth cycle; (c) sixth cycle.

Å. However, repeated action of the enzyme on cotton fibers leads to hydrocellulose particles of length of about 300 Å, which may be associated with perfect crystals of cellulose.

Thanks are due to Dr. V. Sundaram, Director, for the permission to publish this paper.

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Received November 1, 1983
Accepted December 27, 1983