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The Effect of Cellulase on the Degree of Polymerization of Cellulose and Hydrocellulose

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Abstract

On acid hydrolysis of cotton, the degree of polymerization (DP) of the insoluble residue falls rapidly to a low level (leveling off DP). Continued hydrolysis results in further losses in weight, but the DP, and the micelle dimensions, remain unchanged. Immergut and Rånby [6] suggest that individual micelles at the leveling off DP disappear in entirety; i.e., further action of acid yields soluble fragments only. The present investigation was undertaken to compare the action of enzymes on the micelles with that of acids. The study was broadened by the inclusion of hydrocelluloses from cottons treated in various ways, and further extended to include the parent materials from which the hydrocelluloses were prepared.

Methods

A. Preparation of Samples

All samples were prepared from a single lot of single-ply, low-twist yarn, purchased from a commercial source as grey yarn, i.e., spun from raw cotton without any sort of purification treatment. At the Southern Utilization Research Branch (SURB), the entire lot was given a kier-boil consisting of passing dilute sodium hydroxide solution (approx. 1% NaOH) through the yarn in skein form in a pressure vessel at 15 lb./sq. in. at a temperature of approximately 120° C. The alkali was removed by washing in water, souring with 1% acetic acid, and again washing in water. This treatment removed most of the wax and the water- and alkali-soluble noncellulosic constituents of the cotton fiber. Sample A was drawn directly from this lot (stock lot).

Sample B was prepared by mercerizing the stock lot without tension in 24% sodium hydroxide containing Alkamerse (a wetting agent) at room temperature, washing, souring in dilute acetic acid, and washing free of acid.

Sample C was prepared by immersion of the stock lot in liquid ethylamine for 4 hr. at ice-bath temperature under nitrogen atmosphere, draining off the excess amine, and washing out the remainder of the amine by Soxhlet extraction with chloroform (Segal, et al. [13]). This is the "decrystallization" process.

Sample D was decrystallized in a pilot plant operation by treatment of a portion of the stock lot with liquid ethylamine. The amine was removed by extraction with hexane.

Samples C and D probably contained residual amounts of the organic solvents used to remove the amine. Sample C may have contained 2 to 3% of chloroform; sample D contained about 5 to 6% of hexane. These solvents become trapped or occluded and cannot be removed in the ordinary drying process, but are removed when the sample becomes water-swollen. The subsequent preparation for enzymatic hydrolysis, involving dispersion of the finely ground material in water in a Waring Blendor, probably removed the trapped solvents.

Hydrocelluloses (AH, BH, CH, DH) were prepared from the four samples just described, as follows: Samples A, B, and C were hydrolyzed in 2.5*N* hydrochloric acid at 80° C. for 4 hr., washed in water, neutralized with dilute ammonia, and again

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washed in water, corresponding to the standard conditions for producing a "leveling-off degree of polymerization" (Nelson and Tripp [7]). Sample D was hydrolyzed under more severe conditions (4 *N* hydrochloric acid at 97° C. for 30 min.) in order to preserve as much as possible of the cellulose III lattice type which was present in this material. The cellulose III lattice tends to revert to the original cellulose I lattice in the presence of water or dilute acid, but has been found to be preserved if drastic hydrolytic conditions are used (Segal and Nelson [12]).

Lattice types in the four samples are indicated in Table I. In D, mixed I and III, type III predominates in both the cellulose and the hydrocellulose.

B. Enzymatic Hydrolysis

The yarn samples (A, B, C, D) were ground in a Wiley Mill to 40 mesh. Two grams of each material were stirred with water for 3 min. in a Waring Blendor and then filtered. This wash was employed to remove any solubles remaining from earlier treatment, and to aid in wetting the material. The washed sample was placed in a liter flask, wet with 25 ml. of cellulolytic filtrate, and allowed to stand overnight in the refrigerator. An additional 300 ml. of cellulolytic filtrate were added and the flasks were incubated on a shaker at 40° C. After 24 hr., the contents were filtered and sugar determinations were made on the filtrates. Samples DH, C, and D (Table I) were sufficiently digested (i.e., 20 to 30% solubilized), and hydrolysis of these discontinued. Fresh enzyme was added to the remaining samples and digestion was continued for a second day, except for BH which was digested for a total of 3 days. Sample D-2 was prepared in such a way (more dilute enzyme and a lower temperature) that the extent of the enzyme hydrolysis

was of the same magnitude as that for the other celluloses.

The enzyme solution in the above was a filtrate obtained by growing *Trichoderma viride* QM 6a on Solka Floc (a wood cellulose) for 16 days. The cell-free solution was diluted with *M/20* citrate, pH 4.8, to a cellulase activity of 10 Cx μ /ml. (Reese and Gilligan [11]).

The residues were washed on the filter with water, dried at 70° C., and weighed.

C. Chemical Analysis

Weight loss during hydrolysis is based on dry weights before and after acid hydrolysis (Table I).

Degree of polymerization was determined viscometrically by methods in use at the SURB [2]. The viscosity of cuprammonium solutions was determined in burette-type viscometers (concentration of dispersion, 0.5 g./dl.) by the method of Conrad and Tripp [5]. Intrinsic viscosities were calculated from viscometric data by means of the formula (Tripp, et al. [14]):

$$[\eta] = 1.70 (\eta_{sp.})^{0.694} - 0.160$$

Multiplication of $[\eta]$ by Kraemer's factor of 260 gave the degree of polymerization. Where necessary, viscosities were adjusted for rate of shear.

Results

The celluloses (A, B, C, D) and hydrocelluloses (AH, BH, CH, DH) were digested by the cellulase of *Trichoderma viride*. The relative susceptibilities of the eight samples were determined under two sets of conditions (Table II), and a value of one given to the most resistant material—the hydrocellulose of the slack mercerized yarn (BH). The differences are greater (for the most part) in the test involving the

TABLE I. Samples

Yarn sample	Cellulose			Hydrocellulose		
	Lattice	SURB No.	Crystallinity,* %	Sample	SURB No.	Weight loss during acid hydrolysis, %
A Kiered cotton	I	Co 4189	90	AH	Co 5768	4
B Slack mercerized	II	Co 5678	70	BH	Co 5769	6.5
C Decrystallized	I	Co 5507	40-50	CH	Co 5770	7
D Amine-decryst.	III, I	Co 5358	—	DH	Co 5793	8.9

* These are estimates based on data on previous samples similarly treated (acid hydrolysis method). Attempts to determine crystallinity of cellulose III lattice by this method have been unsuccessful.

least decomposition (Experiment 1), but the order of susceptibility is essentially the same in both tests. Our experience has been that hydrocellulose is always less susceptible than the cellulose from which it is prepared, but in Experiment 2 an exception occurs which we are at a loss to explain.

Of all the celluloses, the sample D (amine-swollen, decrystallized, lattice III and I) is by far the most susceptible to enzyme hydrolysis. It is known that such material also hydrolyzes very rapidly with acid. A factor tending to cause high reactivity of this sample is the presence of occluded nonpolar solvent, giving an aerogel or expanded structure in the amorphous regions. The original amine-swollen structure is prevented from collapsing (by the trapped hexane) to the extent that it would if water were the solvent from which it was dried. Judging from the high reactivity found in the hydrocellulose prepared from this cellulose, it would appear that the expanded structure extends even into the micelles.

The enzyme hydrolyses were continued for different time periods aimed at obtaining equivalent amounts of decomposition (20 to 30%) for all samples (Table III). Calculation of decomposition from reducing sugar values always gave lower values than those obtained by direct weight determinations. The reason for this lies in our calculation of the reducing sugar values as glucose, while in reality cellobiose (and perhaps other sugars) are also present.

In all celluloses, the DP of the residue (after enzyme hydrolysis) was lower than that of the parent material (Table III). The change in DP was least for the native cotton and greatest for the amine-decrystallized cellulose. In other words, the substrate least susceptible to enzyme hydrolysis showed the least change in DP; the substrate most susceptible, the greatest change in DP.

In the hydrocelluloses, comparatively little change in DP accompanied degradation.

The extent to which each of the substrates can be hydrolyzed is not shown in the tables. When the hydrocelluloses, AH, BH, and CH, are enzyme hydrolyzed more than 20%, the rate of hydrolysis is very low. DH hydrolysis, on the other hand, continues at a fair rate even after a 40% loss in weight.

The extent to which the celluloses can be hydrolyzed also varies. Leveling off in the rates for the four substrates occurs at approximately the following per cent decomposition: A 25, B 35, C 35, D 65%.

Alkali solubility data are empirical, the values depending upon temperature, concentration of alkali, particle size, and other details of procedure. Only fairly large differences may be considered significant. Within limits, such data are correlated with chain length (Table IV). The celluloses (DP 5000) are less soluble than the hydrocelluloses (DP 200), and within each of these groups there is also some correlation between DP and alkali solubility. Deviations may be interpreted as being due to differences in degree of heterogeneity.

Enzyme digestion of the celluloses gave products of greater alkali solubility (and of shorter chain lengths). But enzyme digestion of the hydrocelluloses had little effect on either solubility or DP, with the possible exception of mercerized cotton hydrocellulose (BH) where the DP increased a little and the solubility decreased.

Another correlation can be pointed out. In the cellulose series, the order of susceptibility to enzyme hydrolysis is the same as the order of increasing alkali solubility. (This does not hold for the hydrocelluloses.) Yet, the enzyme-hydrolyzed products having *greater* alkali solubility (than the parent ma-

TABLE II. Relative Rates of Enzyme Hydrolysis of Cellulose Samples

Yarn sample	Experiment 1*		Experiment 2*	
	Cellulose	Hydrocellulose	Cellulose	Hydrocellulose
A Kiered cotton	1.7	1.1	1.2	1.5
B Slack mercerized	2.6	1.0	1.6	1.0
C Decrystallized	3.5	1.3	2.3	1.4
D Amine-decryst.	7.4	3.2	6.0	2.7

* Under conditions of Experiment 1 (pH 4.8; 50° C.; 24 hr. unshaken; substrate 1%), there was a 2% loss in B hydrocellulose.

Under conditions of Experiment 2 (as above but shaken; substrate conc. 0.65%), there was a 9.4% loss in B hydrocellulose.

terials) are *less* susceptible to further enzyme attack. This discrepancy may, in part, be due to the introduction of another factor—accessibility—the action of the enzyme being limited to a large degree to the available surfaces.

Electron micrographs (Figure 1) were made of hydrocelluloses before and after enzyme hydrolysis. In none of the samples was dispersion of the hydrocellulose down to "ultimate" particles achieved. Nevertheless, careful inspection of the micrographs indicates differences in particle size and in manner of aggregation between samples. The ultimate particles from hydrolyzed native cellulose (AH) appear to be 1,000 to 1,500 Å long, and approximately 150 Å wide. The hydrocellulose particles from mercerized cotton (BH), cellulose III (DH), and decrystallized cotton (CH) are 500 to 800 Å long and slightly narrower than those from native cellulose. The particles appear to be 50 Å or less in thickness in all of the samples. No difference in size could be demonstrated between the particles which had been exposed to enzyme attack and those which had not.

In the native hydrocellulose (AH), the particles are aggregated in relatively long clumps, in which most of the individual particles are parallel to the length of the clumps. The other samples showed shorter, usually broader, clumps and the arrangement of the particles seemed to be less parallelized than in the native hydrocellulose. The clumps in cellulose II (BH) and cellulose III (DH) suggest arrangements brought about by the surface tension forces operative during drying of the specimen.

In so far as observation permitted, the ultimate

TABLE IV. Effect of Enzyme Hydrolysis on Alkali Solubility of Celluloses and Hydrocelluloses

Substrates	Solubility in alkali,* %	
	Before enzyme hydrolysis	After enzyme hydrolysis
Celluloses		
A Kiered	4.9	7.6
B Mercerized	6.8	12.3
C Decrystallized	7.9	15.7
D Amine decrystallized	11.2	35.8
Hydrocelluloses		
AH	55.1	52.3
BH	64.9	52.3
CH	94.2	91.3
DH	90.4	90.5

* One per cent of substrate in 10% NaOH at 5° C. for 30 min. Results are averages of two values.

particles of each specimen did not appear to be uniform in length. If their apparent lengths are an indication of their degree of polymerization, it is clear that a distribution of chain lengths exists in each of the specimens.

Discussion

A. Enzyme Action on Hydrocelluloses

Enzymes act on the particles of hydrocellulose of cotton in somewhat the same way as do acids. The loss in weight which results is not accompanied by a change in degree of polymerization, nor by an observable change in particle dimensions. Thus far,

TABLE III. Effect of Enzyme Hydrolysis on Degree of Polymerization

Substrates	Solubilization, %		Degree of polymerization	
	Red. sugar	Wt. loss	Before enz. hydrolysis	After enz. hydrolysis
Celluloses				
A	17.3	20.1	4970	4200
B	26.6	31.0	5040	3040
C	20.3	24.7	4670	3100
D	57.5	68.6	3920	1630
D-2	25.2	32.6	3920	1050
Hydrocelluloses				
AH	20.8	23.4	225	227
BH	15.6	18.0	138	145
CH	18.6	23.1	133	128
DH	23.7	27.4	112	104

the enzyme results and the acid data are in complete agreement.

Acid hydrolysis of hydrocellulose is essentially complete (Philipp, et al. [9]) and the rate, based on the amount of solids present, is nearly constant for each successive time period. In contrast, the rate at which the substrate is solubilized by enzyme decreases rapidly; i.e., the residual particles are more resistant than those previously attacked. For example, hydrocellulose BH was hydrolyzed at the following rates: first day 8.7, second 4.9, third 2.9%. After a 20% loss in weight, the enzyme hydrolysis rates approach zero.

Enzyme attack is due to a relatively few molecules too large ($200 \times 30 \text{ \AA}$, Whitaker, et al. [16]) to penetrate the particles of the hydrocelluloses (they are almost as large as the particles themselves!). Action must, therefore, be at the surface. Of equal importance is the fact that the chains to be attacked must be hydrated. In terms of the particle, there must be soluble chains attached to an insoluble center. Visualizing the particle as a taut bundle of cellulose chains, we see the small, numerous acid molecules successively splitting linkages to liberate chain ends which can become completely hydrated. When enough chains are so liberated, solubilization of the entire particle occurs. Hydrocelluloses—the products of incomplete acid action—are composed of such particles. Enzymes appear to be capable of hydrolyzing the hydrated chains only. When these have been removed, the particle is again a taut bundle, but now without dangling chains. As such, it is highly resistant to enzyme action.

Based on this interpretation, we should not expect enzymatic action to have an appreciable effect on DP. It doesn't. Since the dangling chains are not portions of the longer chains of the particles, their removal should actually give a slight increase in DP.

As to particle dimensions, the 20% decrease in weight obtained by enzyme hydrolysis would amount to a diameter decrease of about 10%. It is unlikely that a change of this magnitude would be detectable.

We conclude that enzyme hydrolysis of most hydrocelluloses is limited to the removal of the loose chains from the particle surface, and that further attack is at an extremely low rate. There is no indication, then, that particles disappear in entirety as indicated from data on acid hydrolysis by Immergut and Rånby [6]. In the hydrocellulose from decrystallized cellulose III (DH), the more expanded particles may permit hydration to the extent required for enzyme attack. In this particular instance, leveling off in the rate of hydrolysis was not reached even at a 40% loss in weight.

B. Enzyme Action on Cellulose

The cellulose samples are so diverse that one hesitates to ascribe the susceptibility to enzyme attack to any one particular factor. If enzymatic hydrolysis follows the same pattern as acid hydrolysis, it would be expected that extent of hydrolysis under given conditions would show a positive correlation with accessibility. The results confirm the conclusions of others that susceptibility to acid (Table I) and to enzyme hydrolysis (Table II) is inversely related to the degree of crystallinity (Walseth [15]).

Moisture uptake is related to crystallinity; the higher the crystallinity, the lower the moisture regain. Ease of hydration is probably the fundamental reason why cellulose of low crystallinity is so susceptible to enzyme hydrolysis. Any factor which reduces the hydratability of the cellulose will increase its resistance (and *vice versa*).

The major difference between enzyme and acid hydrolysis is due to the size and the concentration of

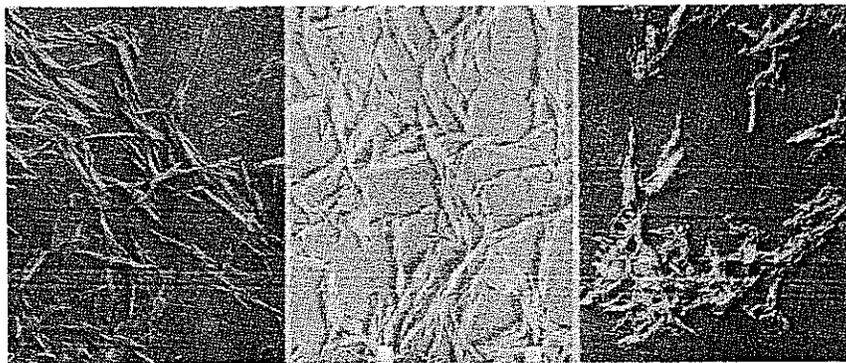


Fig. 1. Electron micrographs of hydrocelluloses from cotton 15,000 X. *Left:* Hydrocellulose from native cotton (cellulose I). *Center:* Hydrocellulose from native cotton after enzyme hydrolysis. *Right:* Hydrocellulose from cotton (cellulose III).

the active molecules. Small, numerous acid molecules diffuse into the cellulose and a rapid depolymerization occurs before extensive solubilization of substrate. The relatively few large enzyme molecules—unable to diffuse into the cellulose—act locally to effect complete solubilization of a relatively few cellulose chains. While it has been shown here that the residue has a somewhat lower DP, it has been shown elsewhere (Reese [10]) that the lower value is due to the presence of a small per cent of alkali-soluble hydrolysis products. The residue, after removal of these, has a higher DP than the original cellulose. This we interpret as a preference of the enzyme for the shorter, more accessible, chains of the amorphous regions.

Enzyme hydrolysis of cellulose reaches a leveling off value which is a function of three factors: (1) particle size, (2) hydratability, and (3) porosity. Even where cellulose sols have been used (Norkrans and Rånby [8]), the reaction reaches a limit, defined by the authors at DP 50 and particle size 300×150 Å. This leveling off in the enzyme hydrolysis rate has been frequently described and commented upon (Walseth [15], Blum and Stahl [4]).

The 40-mesh material used in our tests was hydrolyzed to varying leveling off values. Ball-milling is required to reduce the particles to a size where complete hydrolysis can be reached (Reese [10]). Less drastic reduction of particle size is required when the material is readily hydrated, or when pores of such magnitude that the enzyme can diffuse into the particle are present.

This discussion would not be complete without reference to the complete hydrolysis of cellulosic materials by microorganisms. The importance of particle size and of crystallinity as limiting factors in enzyme hydrolysis has been stressed, but the organism itself seems to be able to overcome these. Both the organism (Abrams [1]) and its cellulolytic filtrates act to produce a water-insoluble, but alkali-soluble, product. The nature of this intermediate is such that it resists enzymatic hydrolysis (note its accumulation in the enzyme hydrolyzed celluloses, Table IV). The fact that the organism solubilizes it leads us to wonder whether some labile factor may be involved, a factor which is rapidly destroyed in the cellulolytic filtrates. In any case, it would appear that further investigation of the alkali-soluble intermediate is required.

Summary

1. Enzymatic hydrolysis of cellulose leaves a residue having a lower degree of polymerization (DP) and a greater alkali solubility than the original material.

2. Enzymatic hydrolysis of hydrocelluloses does not alter the DP, or affect the alkali solubility, or change the particle size as seen in the electron microscope. Particles do not seem to disappear in entirety as reported for acid hydrolysis.

3. Enzyme hydrolysis of both cellulose and hydrocellulose is affected by particle size. The rate of hydrolysis decreases rapidly as some constituents, presumably hydrated cellulosic fragments, are split off, leaving a residue that is quite resistant to further attack.

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