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Fungal Cellulases and the Microbial Decomposition of Cellulosic Fabric

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The biological degradation of cotton fabric involves more than the enzymatic hydrolysis of the glycosidic linkages of the cellulose chain. Cell-free enzyme preparations from active cotton-degrading organisms often have extremely limited action on native cellulose (cotton), although they readily degrade soluble cellulose derivatives or swollen celluloses. Cultures of *Trichoderma viride* produce an extracellular enzyme complex which is active on cotton fiber causing 30% digestion in a week, 70-80% upon extended treatment. Frequent changes of the enzyme solutions are not required. Under similar conditions, preparations from other cellulolytic fungi – including the widely used *Myrothecium verrucaria* – are much less effective.

Our recent attempts at separation of the hydrolytic (Cx) and solubilizing (C-1) factors of the cellulase complex have given partial separation of the two factors by zone electrophoresis and good separation by chromatography on DEAE-dextran. Recombination of the two components is markedly synergistic for action on cotton (Cx + C-1), but not for action on carboxymethyl cellulose (Cx alone).

The activity of the *Trichoderma* enzymes on cotton is relatively unaffected by dialysis or acetone precipitation. A slight inhibition results from the addition of cellobiose (1%), urea (1.5%), glycerol (2%), or ethanol (2%). Over 50% inhibition is caused by addition of Na keryl sulfonate (0.01%), methyl cellulose (0.005%), and the persimmon cellulase inhibitor. The activity is markedly reduced by treating at pH 2.0 or pH 8.0 for 1 hour at 30 C and is completely destroyed by heating to 80° for 20 minutes at pH 4.4. These responses of the C-1 component are similar to those of the Cx factor.

INTRODUCTION

Practical control of microbial deterioration of cellulosic fabrics is obtained by the uniform distribution of a suitable fungicide in the item to be protected. Suitable fungicides are available. Current problem areas arise because of other requirements imposed by the user. Thus, in garments which contact the skin, the fungicide must not irritate or poison the wearer. In multifunctional fabrics, the fungicide must not interract with the other additives in such a way as to destroy their usefulness.

While the search for new fungicides continues, other approaches are also being investigated:

(1) Modifications of cellulose for crease resistance and other purposes have indicated that many such treatments confer resistance to decay. However, when decay resistance is achieved, the item may lack consumer acceptance because of excessive 'add on' weight, inferior 'hand,' or decreased moisture or dye uptake. While this method gives better decay resistance than fungicides, its use must be limited by its compatibility with the other demands on the fabric.

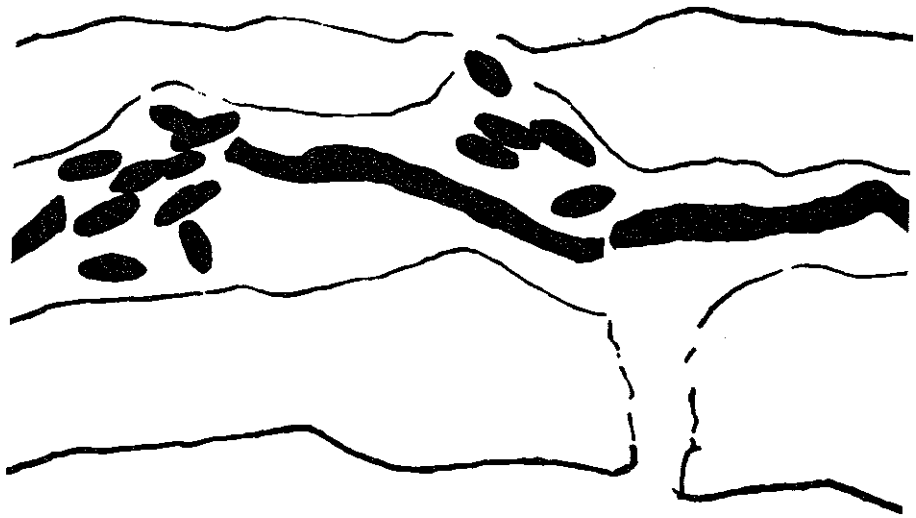

FIG. 1. Hyphae and spores of *Myrothecium verrucaria* within the lumen of jute. (Drawn from photomicrograph). After Basu and Ghose (1962a) used by permission, *Textile Research Journal.*

(2) Synthetics may be substituted for cotton in special situations. Thus, nylon thread in shoes avoids the requirement for uniform distribution of fungicide in the cotton thread previously used. This, of course, is avoiding the problem.

(3) Our understanding of decay is dependent upon a clearer picture of the fine structure of cellulosic materials. Much of the work at the Southern Utilization Research and Development Division of the U.S. Department of Agriculture (Rollins and Tripp, 1961) and the Empire State Paper Research Institute (Sumi, Hale, and Ränby, 1963) is aimed at broader usage of cellulose through improved methods of treatment based on a better understanding of the morphology of the fiber. The work of Stamm and Wagner (1961) at the Forest Products Laboratory on the porosity of wood may be helpful in determining whether or not enzymes can penetrate cell walls to an appreciable distance from the living hyphae (Cowling, 1963).

(4) Finally, there has been a renewed attack on understanding the nature of the enzymes responsible for microbial damage of cellulose fibers. Approximately half of all fungi that have been tested are able to destroy cellulosic fabrics. The attack usually involves penetration of the fiber wall, growth of the hyphae within the lumen, and digestion from within (Basu and Ghose, 1962b; Heyn, 1954; Marsh, 1957; Reese, 1959; Taylor and Marsh, 1963; Fig. 1). Under favorable conditions, the attack is rapid, and the fabric loses its strength, pulls apart, or is punctured easily. Loss of functionality precedes extensive digestion.

The action of the organism on the cellulose is by means of digestive enzymes secreted by the fungus. The action of these enzymes results in several changes:

a. Alkali swelling provides the most rapid and sensitive measure of the activity of filtrates of cellulolytic fungi on native cellulose. Cotton fibers treated with culture filtrates diluted 1 : 100 for as little as 30 minutes at 50 C show increased swelling in 18% sodium hydroxide (Reese and Gilligan, 1954). Under these conditions, no changes are

detectable by any other method. The action is probably on the cellulose network of the primary wall and may be related to increased staining of damaged fibers with Congo red (Marsh et al., 1953).

b. Transverse cracking (Marsh, 1957) occurs on short exposure (2 hr) and spiral fissures on more prolonged incubation (5 days) of cotton fibers in cellulolytic filtrates (Blum and Stahl, 1952). Spiral fissures appear much earlier (in 1 hr) if undried fibers, fresh from the boll, are used (Marsh, 1957). Similar fissures and cracks appear in cotton (Heyn, 1954) and jute (Basu and Ghose, 1962a) attacked by the living fungus (Fig. 2).

c. Loss in tensile strength (Blum and Stahl, 1952) is the next detectable change in cotton fibers treated with enzyme. It may be related to the spiral and transverse cracking. For many enzyme preparations, loss in tensile strength reaches a maximum of about 30% unless several changes of fresh enzyme are applied (Selby, 1963).

d. The degree of polymerization is lowered (Reese, 1959).

e. An increase in alkali (10% NaOH) solubility occurs apparently due to formation of water-insoluble intermediates (Abrams, 1950) of fairly short chain length (18).

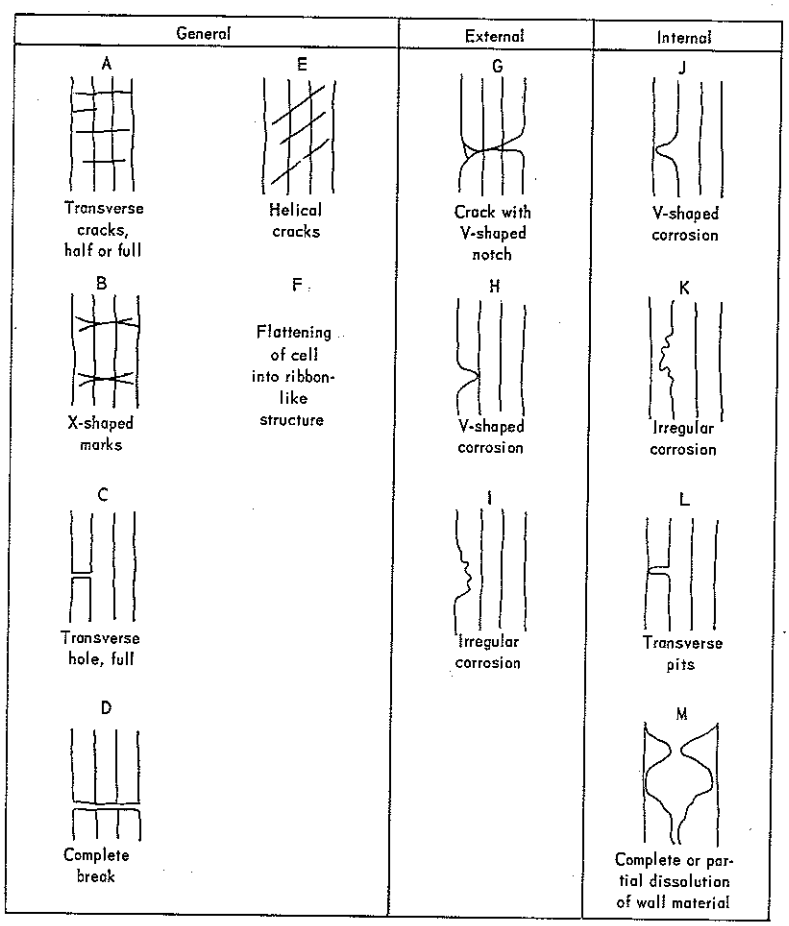

FIG. 2. Types of structural damage to jute fibers by living fungi. After Basu and Ghose (1962b), used by permission, *Textile Research Journal.*

f. Loss of weight and production of reducing sugars occur much more slowly than the preceding changes. For many enzyme preparations, the loss of weight of native cellulose (cotton) is restricted to about 3%, and the resulting residue is highly resistant to further degradation (Reese, 1959; Walseth, 1952). The residue shows a decrease in moisture regain. This is generally considered evidence for the removal of the more amorphous, readily accessible portion of the cellulose.

The C_1 and C_x Concept

The enzymatic changes above, although limited, are sufficient to destroy the functional usefulness of cellulosic fibers. Attack by the living organism leads to more rapid and complete solubilization of the cellulose, and absorption by the fungus, of the sugars produced. The isolated enzymes, despite their restricted attack on native cellulose such as cotton, extensively degrade soluble derivatives and swollen or ball-milled celluloses. During the past 10 years, investigations have shown that cellulase is a complex of enzymes. Rapid attack on the cotton fiber requires a member or members of the complex which are absent in many cellulase preparations (Fig. 3).

On the molecular level, hydrolysis of cellulose is a simple reaction; addition of water cleaving the glucosidic bonds resulting in depolymerization of the chain leading to loss in viscosity and strength and, finally, in the production of glucose and cellobiose as end products. However, in the attack on cotton cellulose, one must consider intermolecular binding, aggregation of chains, insolubility, degree of hydration, accessibility of the linkage to the enzyme, variations in chain length, and presence of noncellulosic substances, which make the story more complicated. Reese and co-workers postulated some years ago that cellulose digestion occurs by a series of steps (Reese, 1959). The first step, carried out by a factor C_1, converts native cellulose into a reactive cellulose. The nature of this action and even the question as to whether it is enzymatic was not known. The C_1 factor has not been studied because there is no direct measure of its activity. Until very recently, no one had claimed more than 3% degradation of cotton cellulose by cell-free enzymes. We have assumed that if a preparation acts on cotton it contains C_1, and we have pictured C_1 acting in such a way as to permit an increased moisture uptake, hydrating the cellulose and pushing apart the closely packed chains. The resultant reactive cellulose (including also swollen celluloses and soluble derivatives) is acted on by C_x, a group of enzymes which hydrolyze the glucosidic bonds producing reducing sugars. The C_x enzymes have been studied in a number of laboratories, usu-

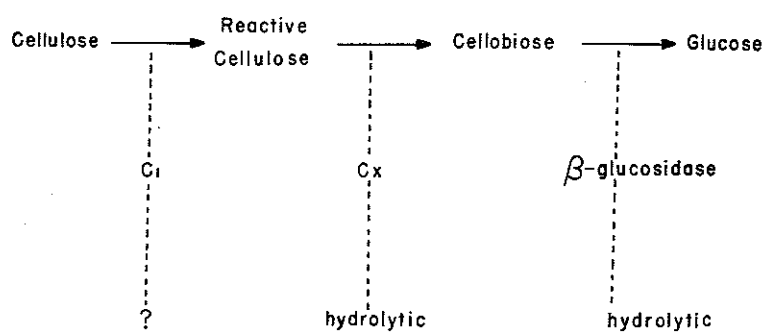

FIG. 3. Degradation of cellulose, the C_1, C_x concept.

ally using carboxymethyl cellulose as a substrate. Most workers have found that a single organism produces a series of C_x enzymes which can be separated by physical procedures such as electrophoresis and adsorption (Gilligan and Reese, 1954; Hash and King, 1958; Miller and Birzgalis, 1961; Grimes, Duncan, and Hoppert, 1947). The β-glucosidases which hydrolyze cellobiose to glucose are well known and are readily distinguished from cellulases.

Production of a Cellulase Having High Activity on Cotton

Selby (1963; Selby, Maitland, and Thompson, 1963) recently obtained losses of 30% in the weight of cotton yarn treated with 30 daily changes of *Myrothecium verrucaria* cellulase. Under comparable conditions, we were able to achieve a weight loss of only 13% and a loss of only 2% in 35 days with four changes of enzyme (Table 1). How-

TABLE 1. *Production of C_1 and C_x by various fungi*

QM No.	Preparation	C_1 units	C_x units	Hydrolysis, percent	
				Sugar	Wt. loss
	Buffer (0.05 M acetate)	0	0	0	1
6a	*Trichoderma viride*	50.0	50.0	58	53
826	*Sporotrichum pruinosum*	30.0	70.0	11	19
137g	*Penicillium pusillum*	27.0	110.0	22	23
1224	*Fusarium moniliforme*	NT	3.5	39	39
72f	*Aspergillus terreus*	5.0	36.0	28	24
806	*Basidiomycete*	5.0	75.0	15	23
94d	*Stachybotrys atra*	1.0	8.0	5	6
B814	*Streptomyces sp.*	0.7	40.0	9	12
38g	*Fusarium roseum*	0.7	10.0	9	10
381	*Pestalotiopsis westerdijkii*	0.7	60.0	4	8
460	*Myrothecium verrucaria*	0.4	28.0	2	2
459	*Chaetomium globosum*	0.2	0.5	NT	NT

Cultures grown on Solka Floc, except *Penicillium pusillum* 137g grown on cotton duck.
C_x units (Reese and Mandels, 1963): action on carboxymethyl cellulose (0.5%) for 1 hr 50°.
C_1 units (text): action on cotton sliver (4.0%) for 24 hr at 40 C.
Hydrolysis of 1.0% cotton sliver at 29 C, pH 4.5, for 35 days (4 changes of enzyme).

ever, when dewaxed cotton sliver was treated with 30 changes of the cellulase of *Trichoderma viride,* weight losses of 80% were obtained (Fig. 4). Unlike Selby's findings, frequent changes of enzyme solution were not required for extensive weight losses. In an experiment lasting 45 days, the losses were 71% for 30 enzyme changes, 73% for 18 changes, 69% for 7 changes, and 59% when the enzyme solution was unchanged.

The cellulase of *T. viride* is much more effective in hydrolyzing native cellulose than are the cellulases of other organisms (Table 1). It is rich in the C_1 factor. We have made a study of this cellulase, comparing its action on carboxymethylcellulose (C_x) with its action on cotton (C_1). C_x activity is maximal at pH 4.8, 60 C; C_1 activity is greatest

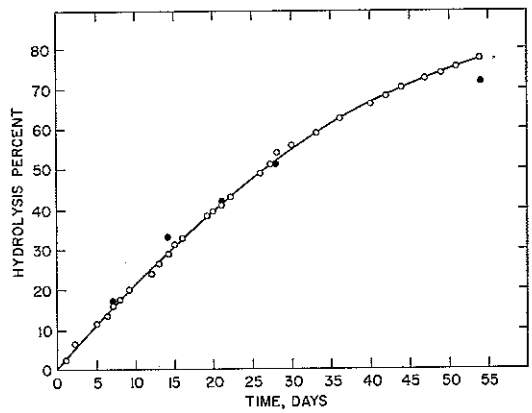

FIG. 4. Hydrolysis of cotton by *Trichoderma viride* QM 6a cellulase. 10-ml culture filtrate, 100-mg cotton sliver, 0.05 M acetate buffer, pH 4.8, 0.005% merthiolate, 29 C.
o —— o reducing sugar production
● weight loss of cotton, 30 changes of enzyme solution.

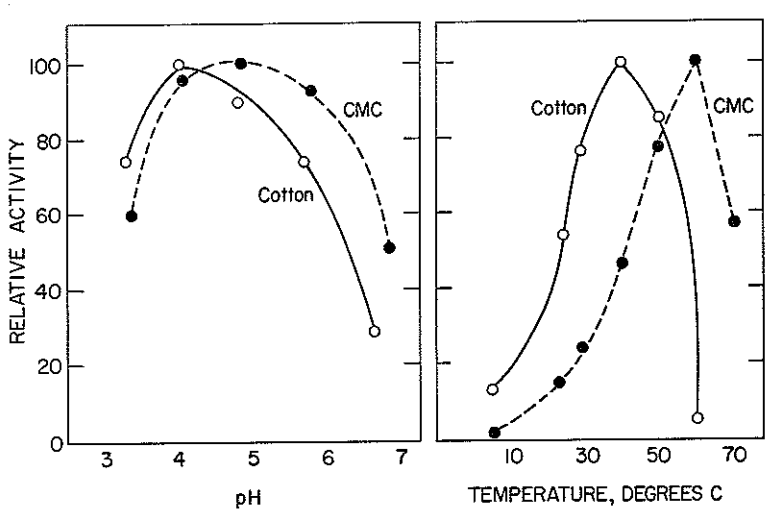

FIG. 5. Effect of pH and temperature on activity of *Trichoderma viride* 6a, cellulase.
o —— o activity on 1% cotton sliver (by sugar production). pH curve: 50 C_x units/ml, 29 C, 25 days, 3 changes; temperature curve: 20 C_x units/ ml, pH 4.5, 22 days, 3 changes.
● —— ● activity on 0.5% carboxy methyl cellulose. pH curve: 0.2 C_x units/ml, 50 C, 1 hr; temperature curve: 0.2 C_x units/ml, pH 5.0, 1 hr.

at pH 4.0, 40 C (Fig. 5). Since CMC was digested for only 1 hour by a highly diluted enzyme, and the cotton was treated for 25 days with an undiluted enzyme, the differences in the two curves may not be of much significance. The action on cotton is proportional to enzyme concentration, and to concentration of substrate (Fig. 6). Similar relationships were reported earlier for C_x (Mandels and Reese, 1963).

The C_1 activity of *T. viride* was greatly reduced by heating to 80 C for 20 minutes or by treating at pH 2 or 10 at 30° for 1 hour. The addition of most chemicals had little effect on C_1 (Table 2). Anionic detergents were markedly inhibitory, cationics were not. Methyl cellulose and cellobiose were inhibitory, glucose was not. The natural inhibitors (Mandels and Reese, 1963) were active. No marked differences were noted in the response of C_1 and C_x to chemicals. Dialysis, addition of versene, or acetone precipitation did not affect the activity of *T. viride* cellulase on cotton (not shown).

In our initial studies with C_1, long incubation periods were used (Fig. 4, Table 1). Use of optimal conditions, particularly the increased cotton concentration, permits a much shorter assay period (24 hr). As a C_1 activity unit, we now use that amount of enzyme which in 1 ml acts on 40 mg of cotton sliver at pH 4.0 for 24 hours at 40 C to produce 0.40 mg of reducing sugar measured as glucose. This is not wholly satisfactory since the production of reducing sugar is dependent not only on C_1 but also on the subsequent action of C_x. However, the unit works well for preparations rich in C_x and C_1 activity can be demonstrated in other preparations by addition of C_x (free of C_1).

Many fungi rapidly degrade cellulose in shaker flasks, but the extra-cellular fluids vary greatly in their content of the various cellulolytic enzymes. In active *Chaetomium globosum* cultures, very little C_1 or C_x has ever been detectable, in *Pestalotiopsis westerdijkii* cultures, large amounts of C_x and little C_1 are found, while in *Trichoderma viride* relatively large amounts of both factors are present (Table 1). No instance of high C_1, low C_x has been observed.

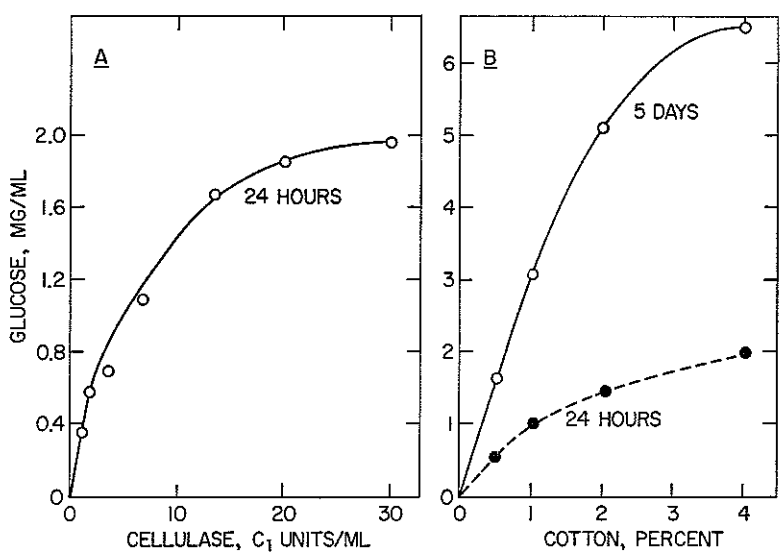

FIG. 6. Effect of enzyme and substrate concentrations on the hydrolysis of cotton by *T. viride* cellulase. Conditions: 0.05 M acetate, pH 4.0, 40 C. A 4% cotton sliver–24 hr, B 30 C_1 units per ml; 24 hr, and 5 days.

TABLE 2. *Effect of various additions on the C_1 and C_x of* Trichoderma viride

Additive	Concentration mg/ml	Change %	
		Action on 1% cotton (C_1)*	Action on CMC (C_x)†
Glucose	10.0	+ 8	+13
Cellobiose	10.0	−54	−34
Giberellin	0.1	+ 8	−10
Protease (R&H, PF)	3.3	−15	0
Peptone	1.0	+ 9	0
Gelatin	1.0	+16	+ 5
Persimmon inhibitor	0.01	−50	−97
Merthiolate	3.3	−36	0
NaCl	2.9	+ 1	0
$CaCl_2$	0.5	+ 9	− 4
$MgCl_2$	0.5	+ 5	+16
Na keryl benzene sulfonate	0.05	−48%	−30
Methocel (methyl cellulose)	0.05	−50%	−25
Alkyl dimethyl benzyl ammonium chloride	0.5	+ 2%	−15
Dioctyl sodium sulfosuccinate	0.5	−40%	−10
Urea	15.0	−33%	0
Glycerol	20.0	−36%	0
Ethanol	20.0	−45%	−20%
Brassic acid	0.1	−65%	−28%
Lignin mixture	0.02	−33%	−34%

*Cotton—20 days at 29 C; †CMC—1 hr at 50 C.

In a similar vein, the correlation between the short-term C_1 assay and the long-term digestion of cotton is not always a good one (Table 1). *T. viride* cellulase is rich in C_1 measured either as units or as extent of hydrolysis of cotton in 35 days (Table 1). *Sporotrichum pruinosum* and *Penicillium pusillum* filtrates also have high unit values, but they are not nearly as active as the *T. viride* enzyme on long-term hydrolysis. Conversely, the *Fusarium moniliforme* solution with low unit activity had high activity in the long term experiment. Differences in stability, or in adsorbability on cellulose, of the enzymes possibly account for the variations observed. The C_1 factor is produced by most *T. viride* cultures. Of 71 *Trichoderma* culture filtrates tested, 58 produced 10-44% hydrolysis of cotton in 18 days. The most active organism on the C_1 unit basis was *T. viride* QM 6a. Most active in long-term hydrolysis was *T. viride* QM 317 which produced almost 5% sugar from 6% wood cellulose (Solka Floc).

Next in importance to the organism itself for the production of these enzymes are the conditions of growth (Table 3). The growth substrate affects both the amount and the

TABLE 3. *Effect of growth substrate on production of C_1 and C_x by* Trichoderma viride *QM 6a*

Substrate (1%)	C_1 units	C_x units	R C_1/C_x
	μ/ml	μ/ml	
Cotton sliver	88.0	43.0	2.0
Cotton sliver (ground)	98.0	54.0	1.8
Cotton duck (ground)	100.0	83.0	1.2
Solka Floc (ground)	88.0	100.0	0.9
Filter paper (ground)	70.0	85.0	0.8
Cellobiose	25.0	38.0	0.7
Glucose	11.0	25.0	0.4
Lactose	10.0	48.0	0.2
Starch	0.3	0.4	—
Glycerol	0.2	0.3	—

relative levels of C_1 and C_x. Cultures grown on glycerol or starch contain no C_x and do not act on cotton. Cultures grown on cellulose have more C_1 and more long term activity on cotton than those produced on cellobiose, glucose, or lactose. It should be emphasized, however, that *T. viride* cultures have more C_1 activity even when grown on sugars than do cultures of most other organisms grown on cellulose.

Most cellulosic substrates (Table 4) are hydrolyzed by the cellulase of *T. viride* QM 6a. The rate of the reaction depends on the form of the cellulose. Materials like filter paper or newspaper show considerable breakdown in 1 day. Cotton is the most resistant of the pure celluloses. Woody materials are resistant unless thoroughly ground by ball milling (Pew and Weyna, 1962). As an example of the possible conversion of cellulosic wastes to sugar by the use of cellulase, 50 g of newspaper were digested, producing 12.8 g of sugar. The product was predominantly glucose, but cellobiose and xylose were also present.

The length of incubation, or age of the culture, affects the relative amounts of the various cellulase components present in the medium. This has been shown for *M. verrucaria,* with CMC (C_x), and with swollen cellulose (Gilligan and Reese, 1954) or cotton (Selby, 1963; Fig. 7), as substrates. In both cases, the activity against solid cellulose appeared before C_x. In our present tests, using cotton hydrolysis as a measure of C_1, we have not been able to show great differences in the C_1/C_x ratio during growth (Fig. 8). We have also been unable to elute either C_1 or C_x from culture solids during the early stages of incubation before their appearance in the medium. Selby (1963), on the other hand, has eluted *M. verrucaria* cellulase from the culture solids using borate at pH 9 or soluble cellulose derivatives at pH 5.5.

In T. viride QM 6a, we have an excellent organism for the production of C_1 and C_x. The use of different organisms and of different assay techniques explain why our results do not always agree with those of others. The high C_1 level of *T. viride* cellulase has led to its commercial application in the Japanese food industry (Toyama, 1963).

Separation of C_1 and C_x

Both similarities and differences between C_1 and C_x are shown above. If C_1 and C_x are different enzymes, one should be able to separate them. In early experiments (Gilligan

TABLE 4. *Activity of* Trichoderma viride *cellulase on a number of cellulosic substrates*

	Hydrolysis—percent		
	Reducing Sugar		Wt. Loss 14 days
	1 day	14 days	
*Substrate—unground**			
Cotton sliver	9	45	48
Cotton duck	5	36	33
Filter paper	33	97	96
Newspaper unprinted	30	40	41
Newspaper printed	30	37	35
Esparto cellulose	33	85	79
Bran flakes	16	38	29
*Substrate—ground**			
Cotton sliver	7	38	34
Cotton duck	11	48	41
Cellulose powder (Whatman)	6	36	29
Solka Floc (SW 40A)	23	77	83
Spruce wood (Wiley Mill)	7	11	8
Spruce wood (ball milled)	39	60	41
Pine twigs	5	11	12
Pine lumber	3	4	3
Bayberry twigs	2	5	0

*Substrate (1%) in cellulase solution (20 C_x μ/ml), pH 4.0; 29 C.

and Reese, 1954) with phosphate gel columns, we successfully separated cellulase components differing in their relative activities on CMC (C_x), swollen cellulose, and cotton (swelling factor). Selby (1963) differentiated a C_x component from a second factor responsible for loss in strength of cotton fiber (his component A). Toyama and Shibata (1961) electrophoretically separated factors differing in their activities on CMC and on filter paper.

In the current work, cotton fiber hydrolysis is used as a measure of the pre-C_x enzyme, C_1. Its resistance to hydrolysis by C_x (free of C_1) makes it well suited for this purpose. Other variously degraded celluloses may be, in part, susceptible to both C_x and C_1 and thus confuse the interpretation of the data. Even CMC is not entirely without fault in this respect!

Attempts have been made to separate cellulase components by means of the molecular sieve principle. Four enzymatically active fractions were found in *Polyporus versicolor* (Petterson and Porath, 1963), one of which had a molecular weight of about 11,000, another a weight of about 50,000, but no attempt was made to correlate these with C_1 and C_x. Our efforts at fractionating *Trichoderma viride* cellulase on Sephadex gels G25, G50, and G75 were unsuccessful.

Attempts to fractionate cellulase on DEAE-cellulose were abandoned because rapid degradation of the column occurred (10% digestion in 8 hrs). DEAE-dextran (Sephadex

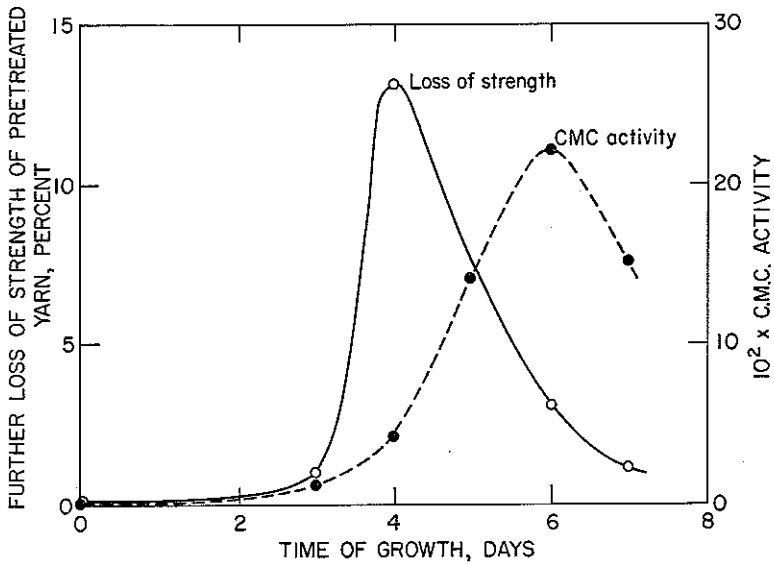

FIG. 7. The rate of development of activity towards cotton yarn and towards carboxymethylcellulose in cultures of *Myrothecium verrucaria*. After Selby (1963), used by permission of the author.

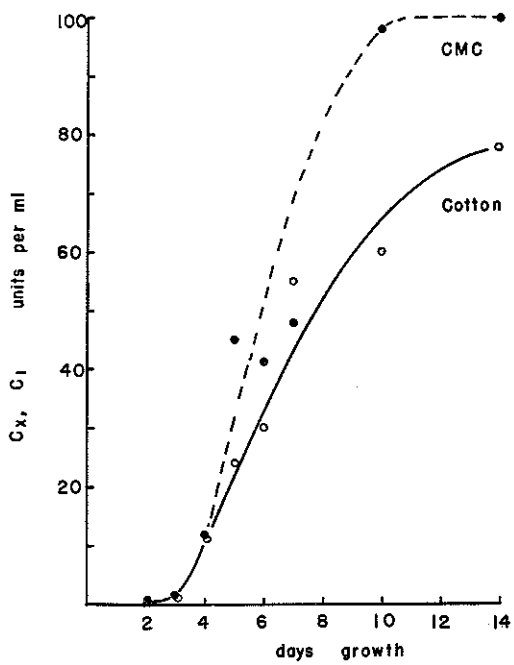

FIG. 8. Appearance of C_x and C_1 during growth of *Trichoderma viride* on cellulose. Mineral salts medium (Reese and Mandels, 1963) + 1% Solka Floc; shake culture at 29 C. o —— o C_x units; ● ---- ● C_1 units

A25 and A50), however, gave good separations when Trichoderma cellulase was introduced at pH 7 and eluted either with a pH gradient (M/50 PO_4, pH 7 $\rightarrow$ 3) or with a salt gradient (O — 0.5 M NaCl in M/50 PO_4 at pH 7.0.) β-Glucosidase passed rapidly through the column while C_x came off as two major peaks, one very early, the other at about pH 5.5 or at about 0.1 M NaCl. Traces of C_x activity were found in almost every fraction. The first C_x peak usually overlapped the β-glucosidase, but, in one experiment, complete separation of C_x and β-glucosidase was achieved (Table 5). The C_x

TABLE 5. *Synergistic effects between fractions of* T. viride *cellulase separated on Sephadex A50*

Fraction	β-glucosidase mg glucose/ml	C_x units/ml	C_1 + water mg glucose/ml	C_1 + Fraction D mg glucose/ml
A	0.84	0	0	0.16
B	0	1.8	0.08	0.16
C	0	0	0.12	0.23
D	0	14.2	0.16	0.17
E	0.05	10.3	0.52	0.54
F	0	0.3	0.48	1.28
G	0	0	0.25	0.48
A B C D E F G				
Found		3.9	0.87	1.28
Calculated		3.5	0.23	

NaCl gradient at pH 7.0.
β-glucosidase—glucose from 0.2% salicin, 2 hrs 50°; C_x units—C_1 eluate ($\times$ ½) on 4% cotton 24 hrs 40° + 1 ml water or 1 ml Fraction D.
Recoveries: β-glucosidase 66%, C_x 66%, C_1 11%.
On recombined fractions C_x 73%, C_1 40% (or 59% with Fraction D added).

components had almost no activity on cotton. C_1 came off much later than C_x, *e.g.*, at about pH 4.5 or at about 0.2 M NaCl. It was associated with the major protein peak and had a trace of activity on CMC (Fig. 9). The C_1 component acted on cotton, but this activity was greatly increased when a small quantity of the separated C_x (from either peak) was added (Fig. 9). The addition of *Pestalotiopsis westerdijkii* C_x (Table 1) also increased the action of the *T. viride* C_1 fraction on cotton. The addition of C_1 to C_x did not stimulate the activity on CMC. When all fractions from a column separation were recombined, the action on CMC was close to the theoretical average, but the action on cotton was increased four- to fivefold (Table 5). Similar synergistic effects were previously reported by Gilligan and Reese (1954) for fractions separated on phosphate gel columns.

When the separated C_1 peak was again chromatographed on the DEAE-Sephadex, the trace of C_x remained associated with it. However, when the separated C_1 peak was subjected to starch block zone electrophoresis, the C_x and C_1 components migrated at different rates (Fig. 9).

Another technique that has given a partial separation of cellulase fractions is differential adsorption on the substrate, cellulose. The C_1 fraction was more strongly adsorbed than the C_x fractions (Reese, 1959; Selby, 1963), but the separation was not

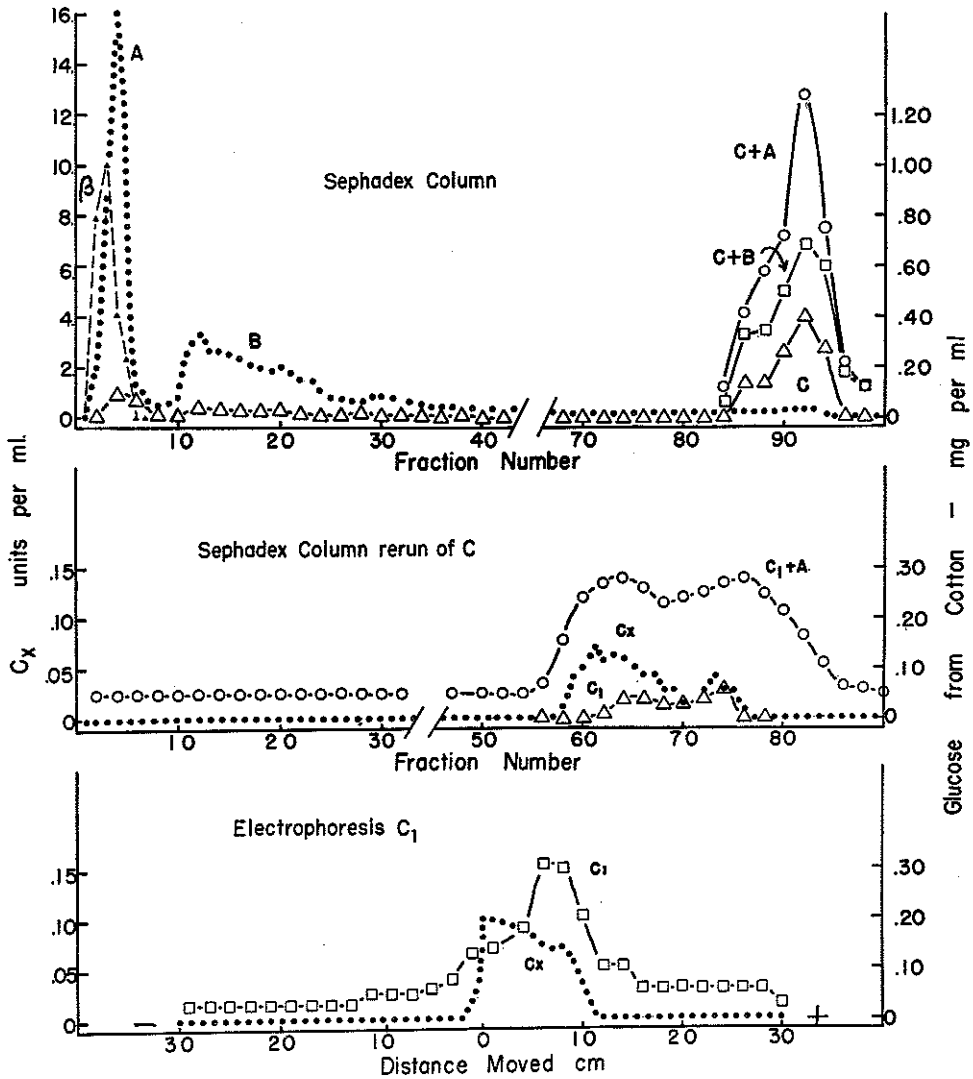

FIG. 9. Separation of components of *Trichoderma viride* QM 6a cellulase. 1. Initial separation on Sephadex A50. 20-ml dialyzed and concentrated culture filtrate applied to 5 g Sephadex A50 (1″ × 7.5″ col.). Eluted with pH gradient (M/50 PO_4, pH 7.2 → 3.5). 20-ml samples collected. Fractions combined:
C_{x_A} = #2-5; C_{x_B} = #9-25; C_1 = #84-98.
C_1 peak dialyzed and concentrated to 25 ml for reseparation. 2. Rechromatogram of C_1 on Sephadex A50. 10-ml C_1 peak from 1 on Sephadex A50, pH gradient 6.5 → 3.5. Note change in ordinate. 3. Electrophoretic separation of C_1. C_1 peak (from 1) separated by starch block zone electrophoresis (Mandels, Miller, and Slater, 1961) at pH 7. 8v/cm, 20 hr. β-glucosidase ▲ —— ▲ action on 0.2% salicin. C_x — units o —— o scale for 2 and 3 = 40 × 1 C_1 — action on 4% cotton 24 hrs 40°, ½ ml acetate buffer, pH 4.0, 3-ml eluate, + 1-ml water △ —— △; or + 1 ml C_{x_B} □ —— □; or + 1 ml C_{x_A} o —— o. Scale for 2 and 3 = 2 × 1.

complete, and elution of the adsorbed enzymes was difficult. Selby attributed the need for daily replenishing his enzyme solutions to the adsorption of "A" enzyme on the cellulose being digested. However, our C_1 factor from *T. viride* was not significantly depleted during the long term incubation with cotton.

The multiplicity of cellulase components is well documented. Not only do the components separate on columns and in electrophoresis, but they differ in their activity on various types of cellulose. Further evidences of difference are: the synergistic effects of recombined fractions acting on solid cellulose, the different slopes – fluidity vs. reducing sugar of the several components (Gilligan and Reese, 1954; Nisizawa, 1961), and the differences in inhibitory power of cellobiose and of methocel (Reese, 1959).

DISCUSSION

C_1 is an enzyme. It is a large molecule, retained in a dialysis sack, precipitated by solvents, and excluded by Sephadex G 75. It is an induced enzyme, pH and temperature dependent, inactivated by heat and by chemical inhibitors. Except in *T. viride,* C_1 is conspicuous by its absence in the culture medium, yet it must be present in all actively cellulolytic fungi. It may be located on the surface of the mycelium or adsorbed on the culture solids. Efforts to remove it have been unsuccessful except for Selby's work with *M. verrucaria.* In his cultures it appears before C_x (Fig. 7) and diminishes on longer incubation. If it had been adsorbed in the culture solids, its concentration in the medium should increase with time as the cellulose is consumed.

The methods used to evaluate the components of cellulase have varied widely, but there has been general agreement that at least two types of activity are being measured. C_x activity is the hydrolytic phase, measured by loss of viscosity, or production of reducing sugars from carboxymethyl cellulose. This is a suitable measure of C_x action since it seems increasingly likely that C_1 has no action on CMC. The best substrate for measurement of C_1 is cotton fiber since it is acted on by C_1 but not by C_x. But not all methods involving cotton fiber are measures of C_1.

(a) Our 'loss in weight' (sugar production) procedure is perhaps the best measure of C_1 but depends on the presence of C_x. A *direct* measure of the prehydrolytic reaction is needed.

(b) Alkali 'swelling factor' is found to be associated more closely with the C_x components isolated from columns than with C_1 but synergistic effects when components are recombined occur here as well as in (a) (not shown).

(c) Loss in tensile strength of cotton may occur with very little weight loss by a factor which has been separated from the C_x component (Selby, 1963) in *Myrothecium verrucaria.* No data are available as to synergistic effects of C_x on this factor, or on its relation to C_1 as defined in (a).

(d) Cotton, filter paper, cellulose sol (Petterson and Porath, 1963), and swollen cellulose (Walseth, 1952) have been used as substrates for C_1. These substrates are acted on to some extent by C_x itself, with a synergistic effect from C_x plus C_1. It appears that the swelling or pulping process has partially simulated the action of C_1.

If all these methods do evaluate C_1, there should be a correlation between the action of various enzyme samples tested by them. This is not the case. Action on filter paper and on swollen cellulose agree fairly well but differ markedly from our measure of C_1 and from swelling factor. The latter, in turn, do not agree well with each other. This supports a previous report (Gilligan and Reese, 1954) that action on swollen cellulose did not correlate with swelling factor (ratios 0.06-10.0). It is time now to compare the

action of isolated components on cotton fibers using as many procedures as are available.

Is C_1 hydrolytic? One possibility is that it is an extremely random acting member of the C_x group, producing maximum fluidity for minimum change in reducing groups. The possibility that it is a result of high local concentrations of C_x, as might occur at a hyphal tip, is ruled out by our data and by that of Selby (1963). C_1 might also be a very small C_x molecule able to penetrate the cellulose lattice. However, our data on gel separation indicates that C_1 does not differ appreciably from C_x in size, at least in *T. viride*. Petterson and Porath (1963) found that for *Polyporus versicolor*, the molecule acting on a cellulose sol was larger than that acting on CMC. Although a large enzyme molecule might be hindered by the CMC substituents, it is unlikely that a small C_x molecule would fail to act on CMC. Another possibility is that C_1 may be hydrolytic, acting on a bond or substrate present in minute amounts in the cotton fiber. 'Weak' linkages have been proposed, and noncellulosic polysaccharides have been found in cotton. The hydrolysis of these could open up the structure for the subsequent action of C_x. However, in our search for enzymes capable of such action, (31 fungal preparations having a wide variety of carbohydrases), none was found.

If C_1 is not hydrolytic, how might it act? It must carry out some action that solubilizes the cellulose and allows C_x to reach the sites where it is active. How? The hydrogen bondase of Siu would be a possible explanation if one could believe in such an enzyme. Possibly C_1 may bind to cellulose relaxing the intramolecular bonds of the cellulose and allowing C_x to act. This could also be an explanation of the usual absence of C_1 in filtrates. In most organisms, C_1 might remain bound to the cellulose or complexed with residual carbohydrate in an inactive form.

In conclusion, we may sum up the current status of the C_1 problem. First, there is ever stronger evidence of a pre-C_x factor both in the results reported here and in those of Selby, Toyama, and others. Second, is the development of a cellulase system (*T. viride*) with high C_1 and with the ability to extensively degrade native cellulose. Third is the successful separation of the C_1 factor from C_x on DEAE dextran.

What we need now is cooperation from those able to detect small changes in cellulosic fibers. We can effect the change. Who can detect it? That is the question.

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