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# Cellulolytic Activity of *Thermomonospora curvata*: Optimal Assay Conditions, Partial Purification, and Product of the Cellulase

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*Thermomonospora curvata* produces cellulases active against both cotton fibers (designated $C_1$ activity) and carboxymethylcellulose ($C_x$ activity). In reaction systems employing optimal substrate concentration, *p*H, and temperature, hydrolysis rates (measured by the release of soluble reducing sugars) were initially linear and decreased on prolonged incubation, although only a small amount of substrate (1 to 2%) had been hydrolyzed. Persistence of this lower rate, even after addition of fresh enzyme (in the $C_1$ assay system), indicated alteration of cellulose susceptibility to hydrolysis rather than enzyme inactivation. Partial purification by $(NH_4)_2SO_4$ precipitation and exclusion chromatography resolved cellulase activity into two fractions. The sole product of purified cellulase activity on ground cotton fibers appears to be cellobiose.

Cellulases (EC 3.2.1.4) are produced by a variety of microbial forms including protozoa, fungi, bacteria, and actinomycetes. However, only a few of these produce culture filtrates which can rapidly degrade solid cellulosic substances such as cotton fibers (16). This inability is generally attributed to lack of the $C_1$ cellulase factor. This factor, originally postulated by Reese and co-workers (14), appears to be responsible for the activation of native cellulose (such as cotton fibers) to form shorter linear polyanhydroglucose chains. Hydrolysis to soluble sugars such as cellobiose would then be catalyzed by the $C_x$ enzyme (a beta-glucanase) which is active against soluble substituted celluloses such as carboxymethylcellulose (CMC), but has no detectable activity against cotton fibers. Noncellulolytic microorganisms grown on CMC can often attack its beta 1-4 glucosidic linkage and produce culture fluids having $C_x$ activity; therefore, culture fluid activity against CMC is no firm criterion by which to evaluate the cellulolytic ability of a microorganism in nature (R. E. Hungate, *personal communication*). As pointed out by Wood (23), no direct measure of the $C_1$ cellulase component is available because its mode of action is not clearly understood. In the present study on the cellulolytic activity of *Thermomonospora curvata*, cotton fibers have been used as the substrate for measurement of $C_1$ activity, and CMC has been used for $C_x$ activity as recommended by Mandels and Weber (10). The primary purposes of the study were: (i) to develop a standardized assay system allowing maximal activity for both $C_1$ and $C_x$ cellulases of *T. curvata*; (ii) to purify the components of the cellulase complex; and (iii) to determine the product liberated by the action of the purified cellulase on an insoluble cellulosic substrate such as cotton fibers.

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## MATERIALS AND METHODS

**Cellulase assays.** The substrate used in the $C_1$ assay system was long-fiber surgical-grade cotton (Johnson and Johnson Co., New Brunswick, N.J.). This absorbent cotton appeared to be over 99% cellulose as compared with the microcrystalline cellulose, Avicel (FMC Corp., American Viscose Div., Newark, Del.). The absorbent cotton routinely used in these studies allowed a rate of enzymatic hydrolysis which averaged 1.37 times greater than native cotton fibers untreated except for a distilled water wash. The substrate used for the $C_x$ assay was CMC (type 7L; Hercules Inc., Charlotte, N.C.) which had a degree of polymerization of 300 and a degree of substitution of 0.7. The use of these substrates in cellulase assays, the measurement of hydrolysis by the release of soluble reducing sugars, and the expression of enzyme units were as previously described for cell-free culture fluids (18). The effects of substrate concentration, temperature, *p*H, and linearity of reaction rates were measured to determine optimal conditions for both $C_1$ and $C_x$ activities.

**Partial purification of the cellulase complex.** The cellulase of 6-day cell-free culture fluids of *T. curvata* was precipitated at *p*H 4.8 to 5.0 by 40% saturation with $(NH_4)_2SO_4$ in an ice bath. After storage overnight, the precipitate was sedimented at 40,000 × *g* at 2 C for 1 hr. The supernatant fluid was discarded, and the precipitate was redissolved to one-tenth the original volume with 0.01 M acetate buffer, *p*H 6.0. Five-milliliter samples of this preparation were then fractionated at room temperature by exclusion chromatography during passage through a Pharmacia K-15/90 column (1.5 by 90 cm; Pharmacia Fine Chemicals Inc., Piscataway, N.J.) packed with Corning CPG-10-240 controlled-pore glass beads with a pore diameter of 24.2 nm and a particle diameter of 75 to 125 μm (Corning Glass Works, Corning, N.Y.). The sample was eluted with a solution of 0.1 M NaCl in 0.01 M sodium acetate buffer at a flow rate of 0.6 ml/min. Five-milliliter fractions were collected on an LKB 7007 fraction collector (LKB Products, Bromma, Sweden) and assayed for $C_1$, $C_x$, and protein.

**Identification of products from enzymatic hydrolysis of cellulose.** Reducing sugar was allowed to accumulate in stirred mixtures of 200 mg of cotton (ground to pass through a 20-mesh screen) and 4 ml of purified cellulase. After stirring the mixtures for 14 to 16 hr at 60 to 63 C on a Mark I magnetic stirring unit with controlled heating block (Cole-Parmer Instrument Co., Chicago), residual cellulose was removed by centrifugation at room temperature. One-milliliter samples of the supernatant fluid were desalted by passage at room temperature through a column (1.7 by 32 cm) packed with Corning CPG-10-75 controlled-pore glass beads (pore diameter of 8.6 nm, particle diameter of 120 to 200 μm). The sample was eluted with an aqueous solution of 2% ethanol at a flow rate of 0.24 ml/min. Fractions of 2 ml were collected, and those fractions containing the reducing sugar peak were chromatographed in the following manner. Samples (150 to 300 μliters) were spotted onto sheets (23 by 57 cm) of Whatman 3MM medium flow rate chromatography paper (W. & R. Balston Ltd., England) by intermittent application with Drummond Microcap micropipettes (Drummond Scientific Co., South Broomall, Pa.) and dried with a stream of unheated air. Standards consisted of glucose and cellobiose applied in 50- to 100-μg amounts. The chromatograms were developed for 16 hr with a descending solvent system of isopentanol-pyridine-$H_2O$ (7:7:6) as recommended by Heftmann (4) for the separation of oligosaccharides. Chromatography of similar samples was also performed on Baker-flex silica gel, type IB, thin-layer, flexible sheets (20 by 20 cm) Baker Chemical Co., Phillipsburg, N.J.) by using a solvent system consisting of n-butanol-isopropanol-$H_2O$ (5:3:1). The sheets were placed with their lower ends directly in the solvents to a depth of 5 mm. After development, for 3 hr at room temperature, and drying the location of carbohydrates on both paper and thin-layer chromatograms was determined with a carbohydrate detection spray consisting of 0.2% acidified aniline-diphenylamine (Sigma Chemical Co., St. Louis, Mo.).

**Other methods.** Reducing sugar concentrations were determined by the method of Nelson (11). Protein determinations were by the method of Lowry et al. (6).

## RESULTS

**Optimal substrate concentration for cellulase activity.** The determination of optimal substrate concentrations for both $C_1$ and $C_x$ activities of culture fluids was necessary to attain maximal initial reaction rates. Figure 1 illustrates the effect which the cotton-cellulase ratio had on the $C_1$ reaction rate. Maximal activity occurred when the reaction system contained about 28 mg of cotton per ml of cell-free culture fluid. This ratio was maintained for further routine assays. The effect of CMC concentration on $C_x$ activity was determined over the range of 1 to 40 mg/ml. The $K_m$ (substrate concentration at one-half of maximal velocity) was calculated (Fig. 2) by using the linear transformation suggested by Hofstee (5), considered the most statistically sound (and the most critical of experimental error) of the three linear transformations used for $K_m$ and $V_m$ determinations. The $K_m$ was calculated to be 3.5 mg of CMC per ml from the slope fitted to the data by the least squares method. In additional assays, the CMC concentration was maintained at 10 times the $K_m$ (35.0 mg/ml).

**Optimal temperature for cellulase ac-**

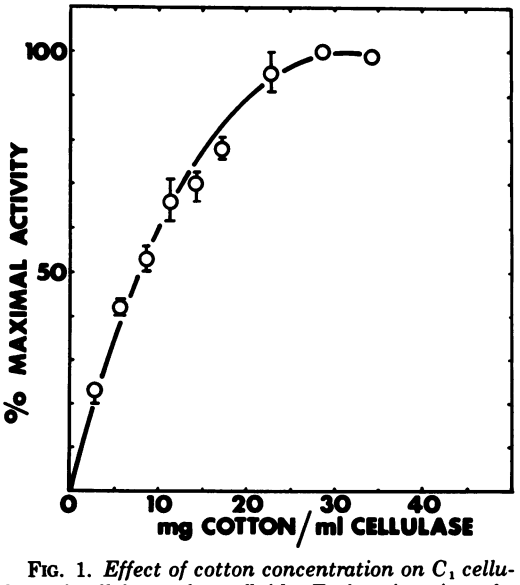


FIG. 1. *Effect of cotton concentration on $C_1$ cellulase of cell-free culture fluids. Each point gives the average and range of three determinations. The maximal activity in each experiment was assigned a value of 100%.*

**tivity.** In a previous study (17) on the ability of *T. curvata* culture fluids to hydrolyze a CMC with a degree of substitution of 1.2, the optimal temperature was 65 C. Figure 3 contains the results of a comparative study of activity against cotton fibers. The $Q_{10}$ values were calculated to be 1.99 for the 35 to 45 C range and 2.03 for the 45 to 55 C range. The peak activity against cotton fibers (as against CMC) was 65 C; an increase in temperature from 65 to 75 C resulted in nearly a 50% reduction in activity.

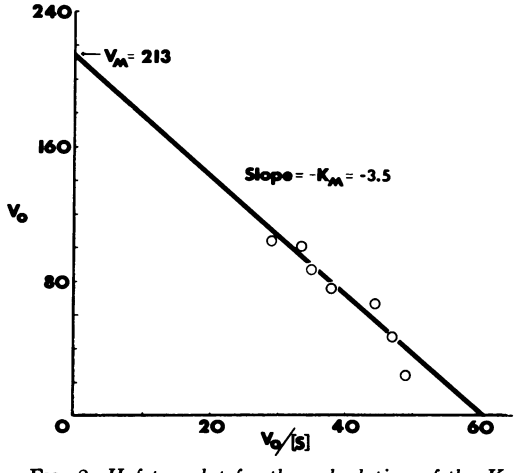


FIG. 2. *Hofstee plot for the calculation of the $K_m$ for carboxymethylcellulose (CMC) in the $C_x$ cellulase assay. The line is fitted by the least squares method to points which are the averages of three determinations. The $K_m$ is expressed in mg of CMC per ml of reaction mixture. The V is simply expressed as the accumulation of μg of reducing sugar (compared to glucose standard) during a 10-min reaction period.*

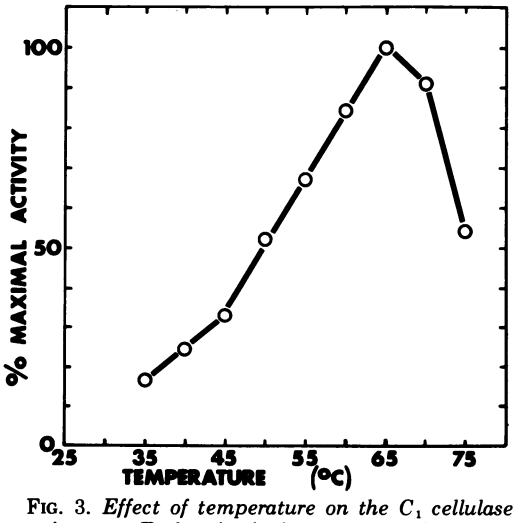


FIG. 3. *Effect of temperature on the $C_1$ cellulase reaction rate. Each point is the average of six to nine determinations.*

In a preliminary calculation, the Arrhenius experimental activation energy ($E_a$) for the hydrolysis of cotton fiber cellulose was estimated in the manner described by Dixon and Webb (1). Figure 4 is an Arrhenius plot of the data given in Fig. 3; the slope of the linear portion of the curve was fitted to the points by the least squares method and yielded an $E_a$ of 11.98 kcal/mole of susceptible glycosidic bonds.

**Determination of pH optimum for cellulose activity.** The *p*H optimum for $C_x$ activity in cell-free culture fluids of *T. curvata* had previously been observed at 6.0 (17). To ascertain whether this optimum held for $C_1$ activity also, culture fluids were diluted twofold with 0.2 M acetate or phosphate buffers at various *p*H values and assayed for $C_1$ activity. The results (Fig. 5) indicated a *p*H optimum in the range of 6.0 to 6.5 with a decrease to about one-half the maximal activity at *p*H 5 or 8.

**Linearity of cellulase reaction rates under optimal conditions.** When maintained under optimal conditions, as just described, the release of reducing sugars with time was linear, which allowed ample product accumulation for measurement of initial rates. Fig. 6 is a plot of reducing sugar accumulation with time in the $C_x$ reaction system. The release was linear up to 20 min, followed by a decreased rate until termination of the reactions at 30 min with a

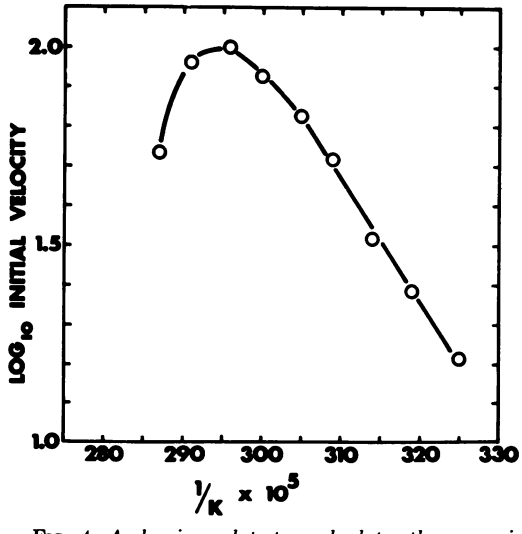


FIG. 4. *Arrhenius plot to calculate the experimental activation energy for the hydrolysis of cotton fiber cellulose by cell-free culture fluids. The linear portion of the curve was fitted by the least squares method.*

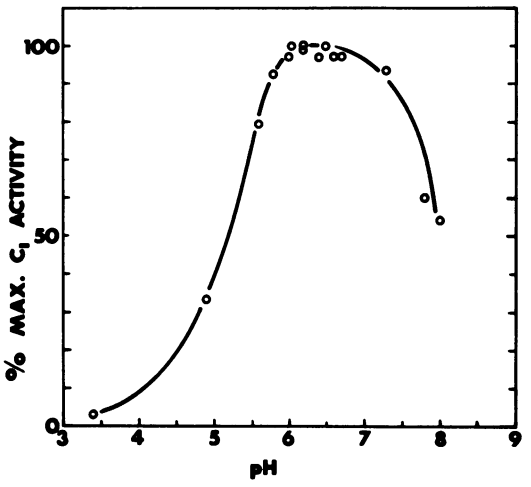


FIG. 5. *Influence of pH on the $C_1$ cellulase activity of culture fluids. The points are cumulative data of duplicate experiments. Maximal activity observed in each experiment was assigned a value of 100%.*

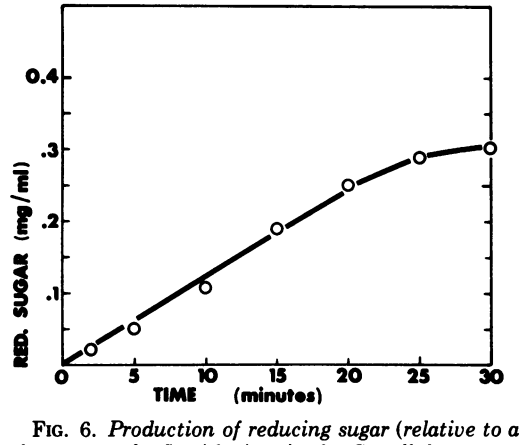


FIG. 6. *Production of reducing sugar (relative to a glucose standard) with time in the $C_x$ cellulase assay system. Each point is the mean of two determinations.*

reducing sugar accumulation equivalent to about 0.3 mg of glucose per ml.

In similar studies on the $C_1$ reaction rate (Fig. 7), release of reducing sugar with time was linear for periods of about 10 to 15 min, followed by a prolonged period of decreased rate for up to 30 hr. The use of the insoluble cotton fibers in the $C_1$ assay system allowed an additional experiment: it was reasoned that if the decrease in $C_1$ reaction rate was due to enzyme denaturation at the relatively high incubation temperature (65 C), addition of fresh enzyme at 10-min intervals, while retaining the same substrate, would allow extension of the linear portion of the reaction rate. If, however, exhaustion of susceptible sites for hydrolysis on the cotton fiber cellulose polymer was the basis for a decreased reaction rate with prolonged incubation, the reaction rate would decrease in spite of enzyme addition. The experiment to test this hypothesis was carried out in the following manner. A 4-ml amount of culture fluid (*p*H 6.2) was added to 110 mg of cotton and incubated at 65 C for 10 min after a zero time sample was taken. After incubation, another sample of fluid was taken, and the rest was drawn off from the cotton fibers. After washing the cotton once with 4 ml of distilled water and removing the wash fluid, a fresh 4-ml sample of identical culture fluid was added and the incubation process was repeated. After six cycles of incubation, using the same cotton fibers with six successive samples of fresh enzyme, all samples were analyzed for reducing sugar and the rates for each of the six incubation periods were calculated. Table 1 contains the averages of results from three replicates of the experiment just described. These data indicated diminution of susceptible sites on the cellulose polymer, or production of cellulose residues for which the enzyme has low specificity, rather than denaturation of enzyme which was responsible for decreased $C_1$ reaction rates during prolonged incubation.

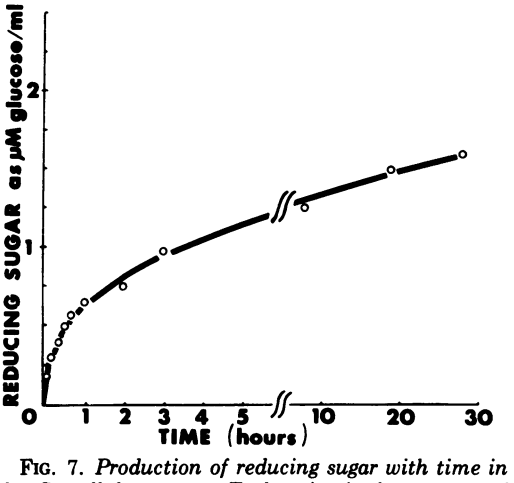


FIG. 7. *Production of reducing sugar with time in the $C_1$ cellulase assay. Each point is the average of two to three determinations.*

**Cellulase purification.** Initial attempts at precipitation of the cellulase complex from cell-free culture fluids of *T. curvata* by *p*H, alcohol, or acetone were unsuccessful. During

precipitation studies employing $(NH_4)_2SO_4$, it was found that *p*H of the preparation was important for recovery of $C_1$ activity. At *p*H 7.3, an average of only 28% of the $C_1$ activity could be recovered in the precipitate at $(NH_4)_2SO_4$ saturations of 40 to 80%. Reducing the *p*H to 4.8 allowed about 75% recovery of $C_1$ activity in the precipitate. A further reduction to *p*H 4.0 decreased the recovery to an average of 60%. $C_x$ activity did not appear sensitive in this regard; an average of 92% of the original $C_x$ activity could be precipitated at *p*H values of 7 to 4 by 40% saturation with $(NH_4)_2SO_4$. Although recovery of total $C_1$ and $C_x$ activity in the precipitate (75 and 92% respectively) was acceptable, little increase in specific activity was gained. The average increase amounted to 1.5-fold for $C_1$ and 1.9-fold for $C_x$. However, precipitate formation allowed concentration of activity prior to fractionation by exclusion chromatography. Figure 8 gives profiles of $C_1$, $C_x$, and protein on passage through the column packed with Corning controlled-pore glass beads, type CPG-10-240. Peak $C_1$ activity occurred in fractions 28 and 29, peak $C_x$ activity was found in fractions 25 and 26, and the protein peak occurred between the $C_1$ and $C_x$ peaks in fractions 27 and 28. $C_1$ activity appeared to consist of a major peak followed by a smaller peak at fraction 42. $C_x$ activity consisted of a single peak with a trailing shoulder.

**Identification of products from enzymatic hydrolysis of cellulose.** After incubation of 200 mg of ground cotton with 4 ml of purified cellulase from the $C_1$ peak, the soluble sugar resulting from the reaction was desalted by passage through a column of Corning glass beads, type CPG-10-75. Reducing sugar analysis of the fractions resulted in a profile having a single peak (Fig. 9). Samples taken from the peak fractions were subjected to descending paper chromatography. Figure 10 illustrates the chromatographic pattern of the reducing sugar produced by the action of cellulase on cotton fibers compared to the glucose and cellobiose standards. The $R_F$ value for glucose was 0.40. The $R_F$ values for the product and for cellobiose were identical ($R_F$ of 0.31). The similarity of $R_F$ values was confirmed by using thin-layer chromatography with a solvent of *n*-butanol-isopropanol-$H_2O$ (5:3:1). The $R_F$ values for glucose, cellobiose, and the product were 0.62, 0.43, and 0.42, respectively.

TABLE 1. *Relative reaction rates during successive additions of fresh $C_1$ enzyme to the same cotton fibers*

| Incubation period | Relative reaction rate (% max) |
|---|---|
| First 10 min | 100 |
| Second 10 min | 83 |
| Third 10 min | 63 |
| Fourth 10 min | 42 |
| Fifth 10 min | 43 |
| Sixth 10 min | 41 |

## DISCUSSION

A confusing variety of substrates and analytical methods has been employed for the measurement of cellulase activity (3). Some of the more commonly employed substrates are: soluble cellulose derivatives such as CMC; regenerated celluloses, prepared by dissolving cellulose in phosphoric acid and reprecipitating it as a powder from water; celluloses swollen by either acids or alkali; cellophane; wood-cellulose; ball-milled cotton; filter paper powder or strips; and occasionally, relatively undergraded cotton fibers. Estimates of enzymatic activity on these substrates have been based on loss of viscosity (in the case of soluble cellulose derivatives), production of reducing sugars (from both soluble and insoluble celluloses), or weight losses (used with insoluble celluloses). The role of the $C_1$ cellulase component becomes more important as the resistance of the substrate increases from that of soluble cellulose derivatives (the most susceptible) to that of cotton fibers (the most resistant). Cotton is considered the best indicator of $C_1$ activity and measures the combined action of $C_1$ and beta-glucanase, whereas CMC, as a substrate, allows measurement of the beta-glucanase independently of $C_1$ (10).

The ability to degrade cotton fibers by cellulase contained in cell-free culture filtrates is restricted to relatively few microorganisms (3). This ability, demonstrated by *T. curvata* in the present study, provides an opportunity to investigate the thermophilic degradation of cellulose which is important in the high-temperature composting of municipal solid wastes. The *p*H and temperature optima (*p*H 6.0 to 6.5 and 65 C) for activity against cotton fibers by *T. curvata* are more compatible with existing conditions in the open windrow composting of municipal solid waste (19) than are the optima (*p*H 4.0 to 5.0 and about 50 C) for the beta-glucanases of most fungi (13). Furthermore, thermophilic actinomycetes of the genera *Thermomonospora* and *Thermopolyspora* dom-

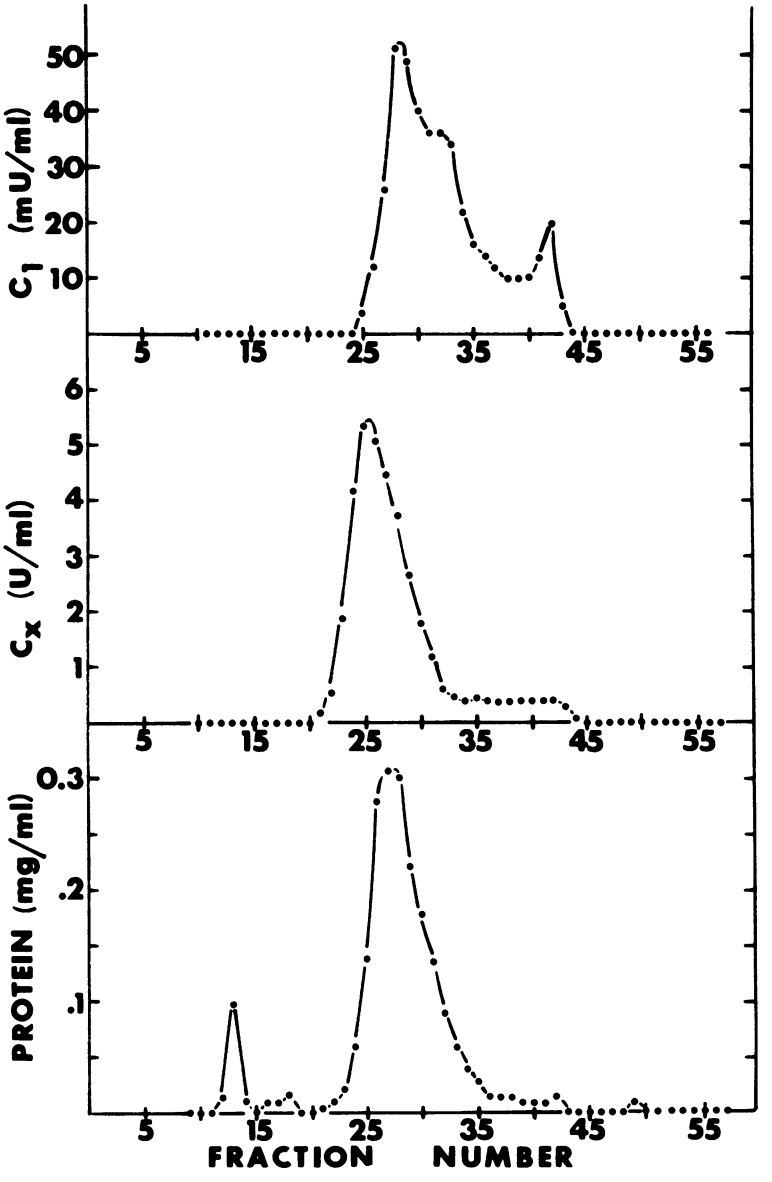


FIG. 8. *Elution profile of $C_1$, $C_x$, and protein after passage through a column of controlled-porosity glass beads. The void volume ($V_o$) of the column was 58 to 60 ml; fraction volume was 5 ml. The percent of recovery (total activity in fractions versus activity in original sample) averaged 63% for $C_1$ and 61% for $C_x$.*

inate in cellulose decomposition during high-temperature composting of straw, manure, and plant residues under conditions where fungi no longer occur (20, 21).

In these studies on the linearity of cellulase rates, accumulation of soluble reducing sugars was proportional to time for periods of 15 to 20 min under optimal conditions in both $C_1$ and $C_x$ assay systems. On further incubation, rate of reducing sugar production decreased, even though reducing sugar accumulation could account for only about 1% of the substrate present. This is characteristic of cellulase assay systems. When cellulase acts on insoluble cellulose such as cotton fiber (as in the $C_1$ assay used here), the most susceptible portions are rapidly digested and the residue becomes increasingly resistant to enzyme attack (10). In the $C_x$ assay, where CMC is the substrate, the degree of substitution has a great influence on

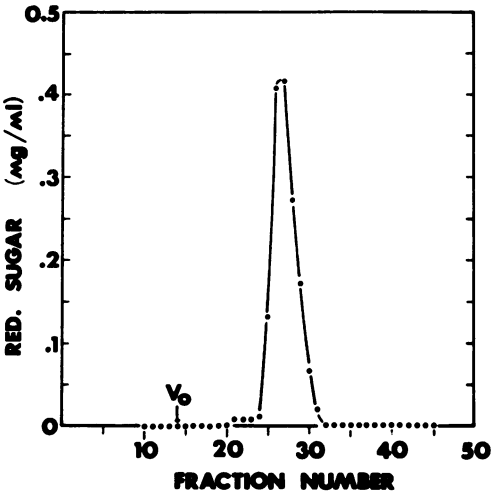


FIG. 9. *Elution profile of reducing sugar (produced by purified cellulase activity on cotton fibers) after passage through a glass-bead column. Reducing sugar in each 2-ml fraction was assayed by using glucose as the reducing sugar standard.*

the susceptibility of the polymer to cellulase action. It appears that the requirement for cellulase activity on a CMC polymer is two unsubstituted glucose residues joined by the beta 1-4 linkage (12). As the degree of susceptibility of CMC approaches 1.0 (one carboxymethyl group per glucose residue), the number of susceptible linkages approaches zero, assuming that each glucose residue is equally substituted. In this study, by using a CMC with a degree of substitution of 0.7, the plateau of reducing sugar accumulation is probably a reflection of the number of susceptible linkages in the polymer.

Separation of cotton-hydrolyzing activity from CMC-hydrolyzing activity by partial purification of the cellulase complex of *T. curvata* was not unexpected in view of previous reports on other cellulase systems. Wood (23) separated the $C_1$ from the $C_x$ component of *Trichoderma koningii*, although the $C_1$ fractions were not completely free of $C_x$ activity. Eriksson and Rzedowski (2) separated the extracellular cellulolytic activity of *Chrysosporium lignorum* into three peaks on diethylaminoethyl-Sephadex, with cotton-hydrolyzing activity concentrated in peak III. Although a study on the cellulase of *Myrothecium verrucaria* indicated that it was homogeneous during purification by a variety of techniques (22), Mandels and co-workers (8) were able to demonstrate by starch block zone electrophoresis a wide heterogeneity of molecules in the cellulase systems of several fungi, including *Myrothecium*. A much more extensive study of the purification of the *T. curvata* cellulase system is necessary for evaluation of its heterogeneity.

In the analysis of the reducing sugar produced from ground cotton fibers by the partially purified cellulase of *T. curvata*, cello-

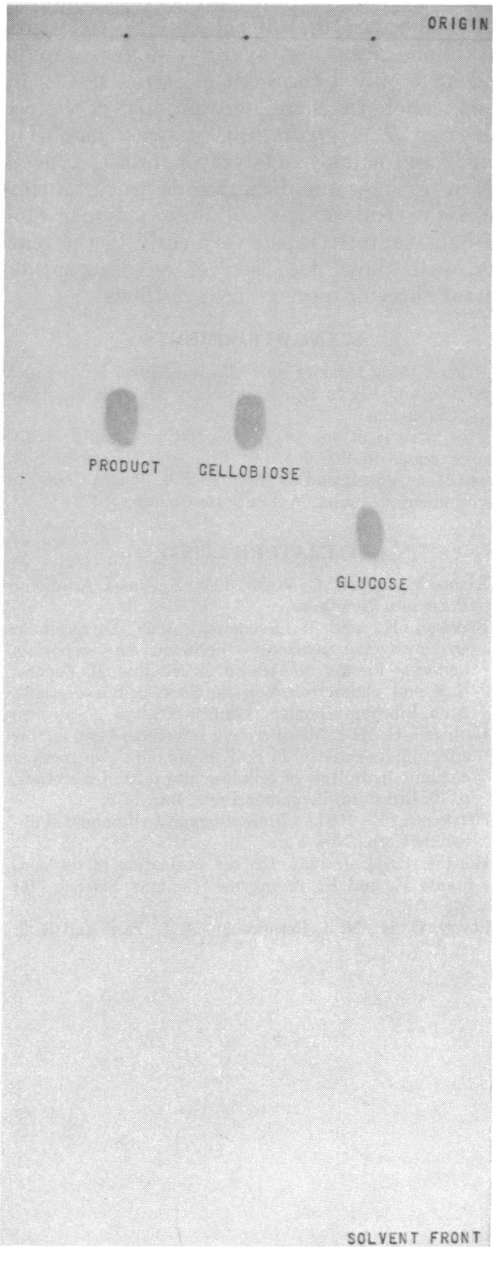


FIG. 10. *Paper chromatogram of glucose, cellobiose, and the product of the purified cellulase reaction on cotton fibers.*

biose appeared as the only detectable product. In a study (15) on the products of cellulose degradation by the cellulase of a *Streptomyces* species, cellobiose appeared as the dominant reducing sugar, although cellotriose was also produced; the ratio of cellobiose to cellotriose was about 3.2 to 1. Usually, cellobiose is the first detectable soluble product of cellulase action, because the oligoglucosides, cellotriose to cellohexaose, are hydrolyzed very rapidly and have only a transient existence (9). If, indeed, cellobiose is the only product of the cellulase of *T. curvata*, the enzyme should be considered as an exopolysaccharidase, type B, which removes one disaccharide unit at a time from the end of the cellulose polymer (15). Perhaps samples taken very early in the reaction will allow detection of oligosaccharides having three or more glucose residues.

## ACKNOWLEDGMENTS

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