The Complexity and Mode of Action of the Cellulase System of Cellvibrio gilvus*

WALDEMAR O. STORVICK AND KENDALL W. KING

From the Departments of Biochemistry and Nutrition and of Biology, Virginia Polytechnic Institute, Blacksburg, Virginia

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Most of the cellulases studied to date have been of fungal origin. A limited number of investigations of the complexity and mode of action of bacterial cellulase systems from mixed cultures has been reported (1-4). Hulcher and King (5) observed that pure cultures of Cellvibrio gilvus degrade cellulose yielding cellobiose as the only detectable product of hydrolysis.

Miller and Blum (6), Grimes et al. (7), and Hash and King (8) have reported that a multiple enzyme system participates in the hydrolysis of cellulose by cultures of the mold M. verrucaria, but the differences in the specificity and mechanism of action of these components have not been clearly defined. Whitaker (9) has described a highly purified preparation from M. verrucaria which is capable of degrading cellulose to a mixture of cellobiose and glucose.

Several workers studying M. verrucaria have reported the production of intermediate oligosaccharides during the cleavage of cellulose (8, 10-13), indicating that the hydrolysis of glucosidic linkages occurs internally. Random cleavage has also been suggested by Norkrans (14, 15) as the reason for the occurrence of relatively large changes in degree of polymerization (ΔD.P.) of the substrate concurrent with only a small amount of reducing sugar production (ΔR.S.). Whitaker (16), with the use of a purified enzyme, demonstrated a similar phenomenon with both native cellulose and several soluble oligoglucosides. In addition, Whitaker established that the terminal glycosidic linkages are less rapidly hydrolyzed than the internal linkages.

There is little evidence of endwise cleavage of cellulose, although production of a single sugar as end product without detectable intermediates has been interpreted by several workers to indicate an endwise mechanism (5, 17-19). Walseth (17) has observed a low ΔD.P. and high ΔR.S. with cellulase from Aspergillus niger in the breakdown of cellulose, and suggested that these data were probably caused by an endwise attack on the substrate.

The present report concerns the purification and mode of action of cellulases from C. gilvus which appear to remove successive cellobiosyl units from the substrate. The relationship between depolymerization of substrate and release of reducing sugars has been examined with both the random-cleaving enzymes of M. verrucaria and the purified enzymes from C. gilvus.

EXPERIMENTAL

Materials and Methods

Enzyme Preparations—Enzymes were obtained from culture filtrates of C. gilvus grown in 2 liter batches of cellulose broth as described by Hulcher and King (5). The culture filtrates were cleared of cells and residual cellulose by centrifugation at 17,300 \(\times g\) for 15 minutes, concentrated 10-fold under reduced pressure, dialyzed against cold, running tap water for 6 to 8 hours, concentrated an additional 10-fold, dialyzed as before, cleared by centrifugation, and lyophilized. The dry tan colored powder (yield of 2.0 to 2.2 g per liter) was stored in a desiccator over calcium sulfate at -18°C and used as the crude enzyme source.

Assays of Enzymatic Activity—The viscosimetric assay was that of King (2). The reducing sugar assay was that of Hash and King (8).

Purification Procedures—Starch electrophoresis was conducted with an apparatus similar to that described by Raacke (20) with starch (potato, powder, purified grade, Baker). Wicks, four thicknesses of Whatman No. 3MM filter paper, 3.6 \(\times\) 10.0 cm, were held firmly against the ends of the starch bed with glass slides by means of rubber bands. A strip of Parafilm was pressed firmly over the starch bed to minimize evaporation. The electrode vessels consisted of the base of a model R Spinco paper electrophoresis cell. Buffer was recirculated (model T8, Sigmamotor pump) between the electrodes to eliminate pH changes resulting from electrolysis of the buffer.

After 12 hours of equilibration with recirculating buffer, the sample was dissolved in 1.0 ml of buffer and cleared by centrifugation. The centrifuge was made up to a thick slurry with starch and was placed in a 0.5-cm slot across the starch bed near the cathode end. The remainder of the slot was filled with starch which had been removed in making the slot.

The power source for electrophoresis was a Spinco model B Duostat adjusted to a constant voltage of 11 volts per cm. Barbiturate buffer, pH 8.5, \(\frac{1}{2} = 0.06\) or acetate buffer pH 5.1, \(\frac{1}{2} = 0.08\) was used in all electrophoresis experiments. At constant voltage the current increase during the separation ranged from 0 to 10%. Electrometer readings ranged from 2 to 3 cm towards the cathode when measured with 1,3,5-trinitrobenzene as a marker.

After separation at 3°C the starch bed was cut into 1 cm sections and each section was eluted with 2.0 ml of 0.05 M phosphate buffer, pH 7.0. The eluate of each section was assayed for protein (21) and assayed for enzyme activity.

Intrinsic Viscosity Determination—Intrinsic viscosities, [\(\eta\)], were determined with 2- to 20-fold dilutions of a 2.0% solution of carboxymethyl cellulose (CMC-70-L of degree of polymerization = 125). From the specific viscosities, \(\eta_0 = (\eta_{rel} - 1)\)

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of each dilution, the log \( \log (v_{j}/v) \) was plotted against \( e \ (e = g \ per \ 100 \ ml) \). The intrinsic viscosity, \([\eta]\), was obtained by extrapolating the straight line plot to zero concentration. Reference was made to this graph for determining the \([\eta]\) of residual substrates during kinetic studies.

**Small Scale Reactions**—Reaction vessels of 300 \( \mu \)l volume were employed in enzymatic hydrolysis of the lower oligosaccharides. Pyrex glass tubing of 8 mm (outside diameter) was drawn out to capillaries 1 mm (outside diameter) on each end of a 1 cm section. Fifty microliters of toluene-saturated enzyme solution, and then 100 \( \mu \)l of substrate were drawn into the bulb. The solutions were mixed by gently sucking air through the mixture. Both ends of the capillaries were then sealed in a flame. After incubation the tips were broken off and the entire sample was applied to the Whatman No. 3MM chromatography papers in small ends of the capillaries were then sealed in a flame. After incubation, the mixture were mixed by gently sucking air through the mixture. Both ends of the capillaries were then sealed in a flame. After incubation the tips were broken off and the entire sample was applied to the Whatman No. 3MM chromatography papers in small aliquots.

The paper chromatographic procedures and indicator sprays used were described by Hash and King (8).

Reagent grade chemicals were used throughout except that chromatographically pure cellotrioce, cellotetraose, cellopentaose, and cellohexaose were prepared from an acid hydrolysate of cellulose (8). Identification of the oligosaccharides was based on \( R_f \) values \( \left[ \log \left( \frac{1}{R_f} - 1 \right) \right] \), with a pyridine-water-\( \beta \)-butanol solvent (1:1:1 volume per volume), total conversion to glucose after acid hydrolysis, and on the color reaction specific for \( \beta \)-1,4-glucosides when paper chromatograms were sprayed with aniline-diphenylamine-phosphoric acid reagent (8).

**RESULTS**

**Characteristics of Crude Filtrates**—With the reducing sugar assay the crude enzyme was found to be stable for 1 hour over a pH range of 5.0 to 8.5. A pH optimum at 5.9 to 6.0 was observed as shown in Fig. 1. Temperature stability data, obtained after 1-hour tests, indicated that the crude enzyme was stable at 37°, approximately 30% labile at 47°, and inactivated at 55°.

A specific incubation of the enzyme with cellulose powder (Solka Floe, Brown Company) for 14 days at pH 7.0 resulted in the production of only cellobiose as indicated by paper chromatographic analysis of the supernatant. The increased activity of the enzyme as it was diluted in the enzyme-time study shown in Fig. 2 demonstrates the presence of an inhibitor in the crude enzyme preparation.

**Purification of Enzymes**—Several methods of purification were evaluated. Ethyl alcohol fractionations (22) resulted in losses of up to 50% of the activity. Fractionations were carried out at alcohol concentrations of 0 to 70% (volume per volume) at pH levels of 5.0 and 6.0 and at 3° except during manipulation of the tubes. Whether the loss was caused by brief exposure to room temperatures, denaturation by the alcohol, or some other factor was not determined.

Fractional precipitation with ammonium sulfate (22) indicated at least two active protein components in the crude filtrates. The fractions were carried out at pH levels of 4.0, 5.0, 6.0, 7.0, and 8.0 and at salt concentrations of 10, 20, 30, 40, 50, 60, and 70% saturation at 3°. At pH 8.0 there appeared to be three distinct components and a 6-fold increase in specific activity of one component.

Purification by selective adsorption on cellulose (Whatman Cellulose Powder, standard grade) and calcium phosphate gel (23) was attempted. In the cellulose adsorption experiments 1.0 ml of a 1% solution of crude cellulase at pH levels of 3.0, 5.0, 6.0, 7.0, and 8.0 in 0.05 M phosphate buffer was shaken for 15 minutes with 5, 50, and 100 mg of adsorbent. The tubes were cleared by centrifugation and the supernatant was assayed viscosimetrically and analyzed for protein. Approximately 90% of the protein in the crude filtrate was recovered in the supernatants. The results are recorded in Fig. 3. Calcium phosphate adsorption with a 1 to 1 ratio of protein to calcium phosphate gave evidence at pH 6.0 that the same activation of enzyme or removal of inhibitor was taking place as observed in the cellulose adsorption experiment.

Starch zone electrophoresis resulted in recoveries of 75 to 100% after 8 hours of separation. A typical distribution pattern is shown in Fig. 4. The relative amounts of each component varied between filtrates from different cultures but were relatively constant for a given filtrate. Each of the components demonstrated reproducible mobility when resubjected to electrophoresis with the exception of Component III in which case a small

![Fig. 1. Effect of pH on the activity of crude and purified cellulases. With approximately 30 units of enzyme per ml of substrate and incubating for 1 hour at 37° at the pH levels indicated, the production of reducing sugars was measured. I, II, III, and IV represent the electrophoretically resolved components in order of increasing mobility.](http://www.jbc.org/)

![Fig. 2. Enzyme x time relationship of crude enzyme. Product of enzyme x time was 2 mg minutes. R.S. = reducing sugar.](http://www.jbc.org/)
component migrated as it had in the original separation and a major peak appeared with a mobility slightly greater than that of Component II.

Evidence that an inhibitor was present in the crude filtrates limits the significance of recovery data throughout these experiments.

Characteristics of Purified Components—The pH optima of the individual components are recorded in Fig. 1. In an attempt to determine if the difference between the pH optima of the purified components and that of the crude enzyme were a result of synergism between the components or of the inhibitor, the following experiment was conducted. A mixture of the purified components reconstituted to resemble their relative concentrations in the crude filtrate was assayed to determine the pH optimum. In parallel, similar mixtures were also assayed in the presence of 0.08 ml of a 1.0% solution of heat-inactivated crude enzyme from which the precipitated protein had been removed by centrifugation.

The results of this experiment are recorded in Fig. 5.

Native cellulose was hydrolyzed at barely detectable rates by either the crude filtrates or the purified fractions, but hydrolysis of alkali-swollen cellulose by the purified components yielded large amounts of cellobiose identified by paper chromatography. Fraction III produced a trace of glucose in addition to the cellobiose. No detectable cellobiase activity was associated with any section of the entire electrophoresis bed.

Action of Purified Components on Lower Oligoglucosides—Each of the purified components (I, 250 units; II, 250 units; III, 25 units; and IV, 25 units) was incubated aseptically with cellobiose (2.7 μmoles), cellotetraose (5.7 μmoles), cellopentaose (4.9 μmoles), and cellohexaose (7.2 μmoles) for 25 hours. No apparent hydrolysis of cellobiose or cellotetraose resulted. Cellobiose and glucose in a mole ratio of approximately 2 to 1 were chromatographically identified as degradation products from cellopentaose. Only cellobiose was produced from cellohexaose through the action of each of the components.

Kinetics of Cellulases—The kinetics of $\Delta$ [η] and AR.S. with each of the four purified components of the cellulase system were investigated with the results shown in Fig. 6. With these preparations log (AR.S.) was proportional to log (ΔR.S.).

Similar kinetic studies were made with a purified, cellobiase-free, cellulase from M. verrucaria (Fraction No. 12 of the chromatographic preparation of Hash and King (8)) which showed a linear relationship between log (ΔR.S.) and Δ [η] as seen in Fig. 7.

Data of other random-cleaving systems demonstrated a similar log-linear relationship between ΔR.S. and ΔD.P. as shown in Fig. 8 for enzymic hydrolysis and in Fig. 9 for hydrolysis by phosphoric acid. It is generally assumed that [η] and the degree of polymerization are proportional, although the proportionality constants are not established.

DISCUSSION

There appear to be no major differences in the function of the four resolved components. In each case the optimum pH was between 7.0 and 7.5. Each of the components possessed similar hydrolytic properties. The unstable mobility of Component III suggests that it may be converted to Component II by polymerization.

The fact that cellobiose was the only detectable product of the action of the crude enzyme and each of the purified compo-

![Fig. 3. Adsorption of crude cellulase on cellulose. Enzyme levels are indicated in the text.](http://www.jbc.org/)

![Fig. 4. Starch zone electrophoresis of crude cellulase. Active components are referred to as I, II, III, IV in order of increasing mobility.](http://www.jbc.org/)

![Fig. 5. Effect of pH on activity of recombined purified components (O—O) and of the addition of heat-inactivated, crude enzyme (Δ—Δ). R.S. = reducing sugar.](http://www.jbc.org/)
The differences in the slopes of the curves in Fig. 6 may be indicative of the mode of endwise attack (25). The slope of these curves would depend upon the number of hydrolytic events associated with formation of a single enzyme-substrate complex. If a single substrate molecule were completely hydrolyzed after formation of each enzyme-substrate complex, a slope of zero would result. Of the four components, IV shows the greatest tendency in this direction, and II the least. The data do not allow calculation of the specific number of cleavages per enzyme attachment because of uncertainties regarding the size distribution of the substrate molecules.

Cellotetraose was not detectably hydrolyzed and cellohexaose appeared to be hydrolyzed more rapidly than cellopentaose by the purified components. This suggests that the enzymes have greatest affinity for the higher oligosaccharides. It is not clear, however, why cellotetraose failed to be hydrolyzed. Whitaker (26) observed that the turnover number of a purified M. verru-
Culina cellulase increased with the degree of polymerization of oligoglucosides containing from 2 to 6 glucosyl units.

The limited rate of hydrolysis of native cellulose is indicative that some factor other than the four components described here participates in the utilization of cellulose by growing cultures.

The adsorption data, Fig. 3, and the enzyme-time relationship, Fig. 2, demonstrate the presence of an inhibitor in the crude enzyme. The inhibitor appears to be heat labile from the data in Fig. 5. The increased activity after the addition of the heat-treated crude enzyme in Fig. 5 and the bimodal curve seen in Fig. 2 possibly reflect the effect of a heat-stable cofactor or activator.

Synergism does not appear to offer an explanation of the difference between the pH optima of the crude enzyme and the purified components. The possibility that an inhibitor may cause this shift in pH optimum has not been investigated.

SUMMARY

Data from ammonium sulfate fractionation and from starch zone electrophoresis yielded evidence of the multiplicity of the cellulase system of Cellulibrio gilus. Four distinct cellulase components have been isolated and their mechanisms of action have been studied.

Several lines of evidence indicated that the cellulase system removed terminal cellobiosyl units. The production of cellobiose as the only detectable degradation product of cellulose by the crude filtrates and the production of a 2 to 1 mole ratio of cellobiose and glucose from cellopentaose and of only cellobiose from cellohexaose through the action of each of the purified components support the endwise theory. Furthermore, the relationship of the change in the production of reducing sugars to the change in the degree of polymerization during the action of the Cellulibrio cellulases is distinctly different from that of systems known to cleave the substrate internally.

The presence of an unidentified inhibitor in the crude enzyme preparation has been demonstrated.

REFERENCES

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Waldemar O. Storvick and Kendall W. King


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