Enzymatic hydrolysis of cellulose: Visual characterization of the process

(Trichoderma reesei/Acetobacter xylinum/high-resolution electron microscopy/endoglucanase/cellobiohydrolase)

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ABSTRACT Cellulose from the Gram-negative bacterium Acetobacter xylinum has been used as a model substrate for visualizing the action of cellulase enzymes from the fungus Trichoderma reesei. High-resolution electron microscopy reveals that A. xylinum normally produces a ribbon of cellulose that is a composite of bundles of crystalline microfibrils. Visual patterns of the process of cellulose degradation have been established. Enzymes are initially observed bound to the cellulose ribbon. Within 10 min, the ribbon is split along its long axis into bundles of microfibrils which are subsequently thinned until they are completely dissolved within 30 min. Incubations with purified components of the cellulase enzyme system produced less dramatic changes in ribbon structure. Purified 1,4-β-D-glucan cellobiohydrolase I (D) (EC 3.2.1.91) produced no visible change in cellulose structure. Purified endo-1,4-β-D-glucanase IV (EC 3.2.1.4) produced some spaying of ribbons into microfibril bundles. In both cases, whole ribbons were present even after 60 min of incubation, visually confirming the synergistic mode of action of these enzymes.

Considering the abundance of cellulose, the decomposition of cellulose is perhaps one of the most common natural degradative processes. The importance of understanding this reaction becomes apparent when one considers the potential exploitation of cellulose as a renewable energy resource through its conversion to ethanol (1), the importance of cellulose degradation in nutrient cycling, and the possible role of cellulase action in the fundamentals of plant cell growth and development (2). Although the action of cellulases has been extensively studied in biochemical terms, little is known about the interconnection of cellulase enzymes with its cellulose substrate at the macromolecular level. This is probably because of the previous lack of a suitable system for visualizing cellulase action and the failure to exploit high-resolution electron microscopic techniques. Cellulase studies to date have characterized the chemical composition of cellulase enzymes, the specificities of their reactions, and certain aspects of their kinetics (3–10); however, biochemical studies are not able to monitor the morphological changes that occur in the cellulose substrate during the process of hydrolysis.

The trivial name cellulase actually refers to a system of three different enzymes whose combined actions lead to the efficient degradation of cellulose. In a currently accepted scheme of cellulase action (4, 11, 12), endo-1,4-β-D-glucanase (EC 3.2.1.4) (endoglucanase) randomly cleaves internal glucosidic bonds within an unbroken glucan chain. The newly created nonreducing chain end then becomes the substrate for 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91) (cellobiohydrolase), which cleaves cellulobiose dimers from the glucan chain and releases them into solution. The hydrolysis of cellulose into the glucose end product is completed by β-glucosidase (EC 3.2.1.21), which splits cellulobiose into glucose monomers. The creation by endoglucanase of nonreducing glucan chain ends that are sites of catalytic action for cellobiohydrolase leads to a synergism in the overall rate of cellulose degradation.

In this paper we examine the progressive degradation of bacterial cellulose with high-resolution electron microscopy and establish the visual patterns of change in cellulose structure that occur during degradation. The cellulose produced by the bacterium Acetobacter xylinum has proved to be a nearly ideal substrate because of its purity, its small size with dimensions approaching those of the cellulase enzymes themselves, and the disperse nature of this cellulose, which is not consolidated into a thick cell wall (13–15). We have visualized cellulases free in solution, their initial binding to the substrate, and their ensuing action upon the substrate.

MATERIALS AND METHODS

Enzymes. Purified samples of cellobiohydrolase I (D), endoglucanase IV, and the complete cellulase enzyme system from Trichoderma reesei QM9414 were gifts from Ross D. Brown, Jr. (Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, FL). Enzyme preparations were purified as described (6, 8, 12). Release of glucose was determined enzymatically with Worthington Statzyme glucose reagent. For protein digestions, cellulases were incubated in Sigma protease type VI (Pronase P) at pH 7.5 and 37°C for 30 min. The pH was lowered to 4.8, and the mixture was then incubated with cellulase for 30 min. Control cellulases were incubated at pH 7.5 and 37°C in the absence of Pronase and returned to pH 4.8 for incubation with cellulose.

Growth Conditions. A. xylinum strain ATCC 23769 from the American Type Culture Collection was grown in 100 ml of the glucose medium of Hestrin and Schramm (16) at 30°C for 24 hr. Pellicles were removed from the air/liquid interface of the medium and soaked in three 20-min changes of cold 50 mM phosphate buffer (pH 7.0) (150 ml per pellicle). Cells and buffer were released from several cubic centimeters of the washed pellicles by twisting a pellicle around a wooden applicator stick. Cell suspensions were kept at 0°C until needed and used without dilution. Cells were transferred to microtomed electron microscope grids supporting thin carbon films by touching the surface of the grid to a drop of the washed cell suspension.

Abbreviations: endoglucanase, endo-1,4-β-D-glucanase; cellobiohydrolase, 1,4-β-D-glucan cellobiohydrolase.

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Cellulose was generated by floating grids with adhering cells face down on a drop of glucose incubation medium in a spot plate for 7-10 min at room temperature.

**Micronets and Thin Carbon Films.** Micronets were manufactured as described (17). Thin carbon films were produced by indirect carbon evaporation methods similar to those of Whiting and Ottensmeyer (18). Micronet/thin carbon grids were prepared individually by cutting 1-mm squares from carbon-coated mica and floating the thin carbon films onto a water surface while monitoring through a dissecting microscope. Floating films were lifted from the water surface by raising a micronet-covered grid from beneath the film.

**Electron Microscopy.** Micronet/thin carbon grids with adhering cells and cellulose were incubated in separate drops of cellulase solutions within a moist chamber at room temperature. The cellulase solutions used in these experiments were as follows: (solution A) complete cellulase enzyme system, 1.0 mg/ml in 50 mM citrate buffer (pH 4.8); (solution B) purified endoglucanase IV, 0.17 mg/ml or 0.5 mg/ml in 50 mM citrate buffer (pH 4.8); and (solution C) purified cellulbiohdydrolase I (D), 0.5 mg/ml in 50 mM citrate buffer (pH 4.8).

After cellulase incubation, grids were washed with several drops of 50 mM citrate buffer and negatively stained with 1% uranyl acetate in 50 mM citrate buffer (pH 4.8). Bacintrin was used as a spreading agent in the negative stain (19). All preparations were examined with a Hitachi HU-11E electron microscope operating at 75 kV. Micrographs were taken at a magnification of ×72,000. For calibration purposes, a 52,000 line/inch grating replica and cationized ferritin molecules were used.

**RESULTS**

**Cellulose Substrate.** Knowledge of the macromolecular organization of cellulose in A. xylinum is a prerequisite for interpretation of the progressive enzymatic hydrolysis of cellulose. Negative staining of A. xylinum cellulose revealed about 50-80 3.0- to 3.5-nm microfibrils aggregated into ribbons between 40 and 60 nm wide. The ribbons were twisted with a periodicity of 1 μm (Fig. 1a). Several of these microfibrils may have coalesced into discrete bundles that subsequently aggregated into a full ribbon (Fig. 1d). The ribbon is thus composed of several levels of hydrogen-bonded structures. Glucan chains crystallize into microfibrils, intermicrofibrillar hydrogen bonding holds individual microfibrils into bundles, and these bundles are hydrogen-bonded along their surfaces to form the ribbon (20). Bundles of microfibrils occasionally separated from the ribbon and exhibited twisting, similar to the composite ribbon. The minimal width of a separated bundle was 3 nm, and the maximal width was usually a multiple of 3 nm (Fig. 1c and e).

**Initial Binding of Enzyme to Substrate.** In samples incubated for several seconds in cellulase solutions, the ribbon structure was obscured by a coating of particles, with diameters ranging from 3 to 7 nm, bound to the ribbon surface (Fig. 1f). Negative staining of the enzyme system alone revealed particles within the same diameter range (Figs. 1 and 2). Mean diameters (±SEM) of bound and unbound particles were 5.4 ± 0.2 nm and 5.4 ± 0.1 nm, respectively.

Fig. 3c illustrates the appearance of individual enzyme particles bound to the cellulose surface. Although it is difficult to assign exact three-dimensional shapes to the enzymes, it appears that these particles are approximately spherical. At the level of resolution determined by the negative stain procedures used, it has not been possible to resolve surface features of the individual particles.

Grids were also incubated in cellulases that had been exposed to protease. Normally, ribbons were completely degraded within 30 min of exposure to the complete cellulase system. However, cellulase ribbons were still present after 60 min of incubation with protease-treated cellulase. In control experiments, cellulases incubated at pH 7.5 and returned to pH 4.8 degraded ribbons within 30 min.

**Degradation by Complete Cellulase System.** Incubation of A. xylinum cellulose in solutions of T. reesei cellulases for various lengths of time resulted in the establishment of certain patterns of degradation. Ribbons that were incubated in cellulases for 5 min were coated with particles (Fig. 1g). One of the first distinct changes in ribbon structure was the spaying of ribbons into bundles of microfibrils. After 10 min of exposure to cellulases, spaying into microfibril bundles became more prominent (Fig. 1h). Bends and splits within the bundles were numerous. Some fragmentation of ribbons and their bundles was observed during these intermediate stages of degradation (Fig. 1j).

At 20 min of cellulase incubation, only a few bundles of microfibrils or amorphous fragments were observed (Fig. 1k). The remaining strands were low in contrast, indicating that little negative stain was trapped by these remnants. Amorphous fragments, which may represent the final stages of degradation prior to complete solubilization, were frequently associated with the remaining strands (Fig. 1l). Statzyme reagent indicated that approximately 3 mg of glucose per dl was liberated after 30 min of cellulase incubation. No recognizable ribbon bundles, microfibrils, or fragments were found in incubations longer than 20 min.

**Degradation by Purified Cellulase Components.** A. xylinum cellulose incubated in purified cellulbiohdydrolase I (D) up to 30 min exhibited no change in ribbon structure, except that ribbons were coated with particles (Fig. 3a). Incubation of cellulase in purified endoglucanase IV at a protein concentration of 0.17 mg/ml produced no observable breakdown of ribbon structure after 30 min. A higher protein concentration of 0.5 mg/ml did produce some spaying of microfibril bundles during the first 30 min of incubation (Fig. 3b). Numerous recognizable ribbons were observed even after 60 min of incubation in both.
purified cellulobiohydrolase and purified endoglucanase; however, no ribbons were observed after 30-min incubations with both purified enzymes simultaneously present.

**DISCUSSION**

Cellulose is a unique substrate for an enzyme because it is a large, crystalline, insoluble macromolecule. Whereas most enzymes have soluble substrates, the cellulase enzymes must diffuse to the surface of their insoluble substrates before catalytic activity is possible. The use of thin carbon films, negative staining, and high-resolution electron microscopy have permitted the visualization of the macromolecular fine structure of cellulose, the binding of cellulases to cellulose, and changes in the fine structure of cellulose as it is degraded.

Several lines of evidence presented indicate that the particles observed on the surface of cellulose ribbons are, in fact, cellulase enzymes. (i) Release of glucose into solution during incubation of cellulose ribbons in cellulase solutions is concurrent with the disappearance of observable cellulose. (ii) Particles appear on the surface of cellulose ribbons immediately after they are introduced into cellulase solutions, and those particles remain even after washing with buffer. This indicates that the particles are tightly bound to the cellulose surface. (iii) There is an overlap in the size range of the particles observed in negatively stained cellulase solutions and the particles observed on the cellulose surface (Fig. 2). Furthermore, the mean particle sizes are almost identical. Reported molecular weights for the various cellulase enzymes are 28,000 for the β-glucosidase, 37,000–52,000 for endoglucanase, and 53,000 for cellulobiohydrolase (6, 8, 21), so some variation in observed enzyme diameter would be expected. (iv) Incubation of cellulases with nonspecific proteases results in a loss of enzyme activity. Ribbons are present even after 60-min incubations in protease-treated celluloses.

The general pattern of cellulose degradation begins with an initial splitting of the ribbon along its long axis into bundles of microfibrils, followed by a thinning of these bundles until they are dissolved (Fig. 1). The specificities of the individual cellulase enzymes are known (7, 12). Endoglucanase cleaves unbroken gluca chains, whereas cellulobiohydrolase requires a free, nonreducing gluca chain end for substantial catalytic activity. This suggests that in the initial stages of degradation, endoglucanase would be the most active enzyme because few nonreducing ends are available for cellulobiohydrolase to act upon. Bundles appear when ribbons are treated with endoglucanase only, but not when ribbons are treated with cellulobiohydrolase only (Fig. 3 a and b). Endoglucanase must provide the primary degradative action by hydrolyzing glucosidic bonds on the microfibril surfaces, thereby disrupting the organization of gluca chains required to maintain hydrogen bonding between bundles. This leads to the observed splaying. During this process, cellulobiohydrolase undoubtedly begins to cleave cellulose from newly created chain ends, eventually eroding away the original crystalline surface, completely disrupting the forces that hold the microfibril bundles into the ribbon configuration, and exposing new surfaces of gluca chains. Cellulobiohydrolase thus accelerates the breakdown of ribbons into bundles. The initial splaying of bundles in the presence of cellulase supports the morphological suggestion (Fig. 1 b–c) that ribbons are composed of discrete bundles of microfibrils, where the weakest bonds in the composite ribbon may well be the hydrogen bonds between these bundles (20). Furthermore, bundles do not seem to slip apart into individual microfibrils, perhaps because the hydrogen bonding within bundles is stronger than that between bundles. Splayed bundles are thinned down by the combined actions of endoglucanase and cellulobiohydrolase, which result in the gradual erosion of the outer surfaces of the microfibrils.

Our results visually confirm the widely noted synergistic action of endoglucanase and cellulobiohydrolase (10, 11, 22). Cellulose is completely dissolved in less than 30 min when the complete cellulase enzyme system or reconstituted mixtures of purified endoglucanase and cellulobiohydrolase are present in the

**Fig. 3.** (a) Cellulose ribbon incubated for 30 min in purified cellulobiohydrolase I (D). Note that particles are bound to the ribbon surface, but general ribbon structure has not changed. (×200,000; bar = 100 nm.) (b) Ribbon incubated for 30 min in purified endoglucanase IV. This ribbon exhibits some bundle splaying; however, whole ribbons are still present. (×200,000; bar = 100 nm.) (c) High-magnification view of a microfibril bundle. Circles represent possible cellulase enzyme molecules bound to the cellulose substrate. (×510,000; bar = 50 nm.)
incubation solution. However, when grids are incubated with only endoglucanase or celllobiohydrolase, ribbons are still observed even after 60 min of exposure to enzymes. Celllobiohydrolase had no noticeable effect on ribbon structure during these long incubations. This is not surprising because there should be relatively few nonreducing ends exposed on the surface of normal A. xylinum ribbons. Endoglucanase produces some splaying of ribbons into microfibril bundles (Fig. 3b), but whole ribbons are still present after 60 min of incubation. Over the relatively long period of 30–60 min, endoglucanase may break enough glucosidic bonds to weaken the microfibril surfaces that are holding the bundles into the ribbon configuration, and splaying occurs. This observation is consistent with the findings of Wood and McCrae (10) that endoglucanase increases the capacity for uptake of alkali and reduces the tensile strength of cellulose. However, although endoglucanase produces visible changes in ribbon structure, the degradative action of this enzyme when used alone is slow compared to the synergistic degradation that occurs when endoglucanase and celllobiohydrolase are simultaneously present.

Occasionally, discrete fragments and breaks along whole ribbons have been observed (Fig. 1j), suggesting that in some cases splaying may not be inherent to ribbon degradation. Fragmentation could result either from locally elevated concentrations of endoglucanase or weak areas in the ribbon itself which are degraded faster than adjacent areas (23, 24). Fragmentation, however, seems to be an exception to the generally observed pattern of degradation.

The model cellulose from A. xylinum has allowed the visualization of cellulases bound to the substrate and has provided some unique observations of the progressive events of cellulase enzymatic activity. Our observations are consistent with the mechanisms of cellulase action proposed on the basis of extensive biochemical data. Although our techniques may have approached the limits of resolution attainable by negative staining, an informative picture of the more general mode of cellulase action is beginning to appear. With our techniques, we have examined bundles of cellulose microfibrils in wall fragments of growing corn roots and have found progressive degradation similar to the splaying of bundles in A. xylinum (25).

In conclusion, it now seems that the application of low-dose darkfield electron microscopy to cellulases and their association with cellulose is feasible. By use of these methods, resolution better than 0.5 nm has been obtained, and the surface regions of enzymes have been analyzed (26). Successful observations of cellulase action in this resolution range would undoubtedly help to clarify some of the long-standing problems of the mechanism of cellulase action not obtainable with conventional electron microscopic or biochemical techniques.

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