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Rotation of cellulose ribbons during degradation with fungal cellulase

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Abstract

Degradation of bacterial cellulose with a commercial cellulase, Celluclast 1.5 L (Novo Nordisk), from the fungus *Trichoderma reesei*, causes a rotational movement of the cellulose microfibrils. Purified cellulases (CBH I, CBH II, and EG II) do not induce rotation of bacterial cellulose, however, ratios of CBH I and EG II do cause rotation of bacterial cellulose. Equimolar amounts of CBH I or CBH II and EG II do not result in motion during degradation. Based on these observations, we provide further evidence supporting, at least on theoretical grounds, the hypothesis that cellulose chains have intrinsic chirality. As the cellulase enzymes interact with and degrade the cellulose fibrils, the crystalline structure of the cellulose is altered, allowing the linear cellulose polymers to relax into a lower energy state, thus relieving the strain induced by crystallization of the nascent β-glucan chains during the biogenesis of the microfibril. This conversion of crystalline bacterial ribbons into more relaxed conformations produces the rotation observed during the treatment of bacterial cellulose with cellulase.

Introduction

Cellulose

Cellulose, the most abundant macromolecule, is a homopolymer of β-1, 4-linked glucose molecules. High resolution electron microscopy of negatively stained cellulose in the model system, *Acetobacter xylinum* (White & Brown, 1981), has revealed a hierarchical association of 50–80 microfibrils (3.0–3.5 nm) that associate into flat structures 40–60 nm wide known as ribbons. The ribbons are highly twisted, with one 180° turn every ~700 nm (White & Brown, 1981; Hirai *et al.*, 1998). The bacteria rotate during the synthesis of cellulose I ribbons.

Careful analysis of shadowed cellulose microfibrils synthesized by *Acetobacter xylinum* have shown them to be right-handed helices (Hirai *et al.*, 2000). Cellulose microfibrils of *Micrasterias denticulata* have also been shown to be right-handed by AFM, TM-AFM, and TEM, with 700 nm intervals between twists (Hanley *et al.*, 1997). Induced circular dichroism of methylcellulose chains and cellulose oligomers indicates a helical conformation for β-1, 4 glucans (Ritcey & Gray, 1988). Furthermore, ^{13}C NMR spectroscopy has revealed that the β-1, 4 linkages between glucose residues in cellulose derivatives adopt a 35° conformation when in free solution (Buchanan *et al.*, 1989), suggesting that the glucan chains appear to be most relaxed as a 5/4 helix.

In native crystalline cellulose, the van der Waals forces and hydrogen bonding between the glucan chains may be holding the crystal together despite conformational hindrances. Hence, the crystalline native cellulose I allomorph can be thought of as being under constant internal stress.

Use of Tinopal can cause the formation of a mini-sheet in the form of a closed tube-like structure (Cousins & Brown, 1997; Haigler & Chanzy, 1988). When the dye molecules are subsequently photoisomerized by intense UV illumination, they lose their affinity for the cellulose, causing the single glucan chain sheets to collapse into microfibrils (Cousins & Brown, 1997). During this process, the cellulose undergoes a massive torsional motion. It is believed that this motion is the result of the final crystallization of the mini-sheets

into microfibrils. The removal of the dye molecules either by washing or photoisomerization allows the crystallization of the cellulose.

Cellulase

Cellulases are responsible for the reduction of cellulose microfibrils into their constituent glucose subunits (Teeri *et al.*, 1998; Tomme *et al.*, 1995). One of the most studied cellulase systems is from the fungus *Trichoderma reesei* (Teeri *et al.*, 1998). Cellulase reactions occur as a group effort of several enzymes that work synergistically to degrade cellulose for consumption by *Trichoderma*.

The binding of cellulase to its substrate is a complex process involving the interaction of the cellulose binding domain (CBD) and the catalytic core of the enzyme with the substrate (Gilkes *et al.*, 1992; Linder *et al.*, 1996; Srisodsuk *et al.*, 1997). The first stage of action involves binding of the CBD of the cellulase protein to the surface of the cellulose. Once bound, these enzymes initiate catalytic activity. For EG, the function is to cleave the β-1, 4 glycosidic bond internally, yielding a reducing end and a non-reducing end. CBH, on the other hand, may bind anywhere along the crystalline surface but initiates its catalytic activity only at a reducing chain end (CBH I) (Divne *et al.*, 1995) or a non-reducing chain end (CBH II) (Divne *et al.*, 1994). Once catalysis is initiated, the enzymes are believed to physically move along the microfibril as they progressively clip cellobiose from the reducing or non-reducing ends. This processivity has been indirectly measured through FRAP analysis (Jervis *et al.*, 1997).

Speculating that cellulose is produced under strain, we investigated the motions of the bacterial cellulose substrate during interaction and degradation with cellulase using time-lapse microscopy. If, as this report suggests, β-1, 4 glucan polymers produced by *Acetobacter xylinum* have internal stress built into the nascent crystalline microfibrils, then these results will provide a better understanding of the dynamic interactions of cellulosic materials at both macro- and microscopic levels.

Materials and methods

Cellulose

Cellulose used in this experiment was synthesized by *Acetobacter xylinum* strain AY201 (ATCC 23769). Pellicles were removed from culture tube, washed in 0.5 M NaOH, and then frozen with liquid nitrogen and ground with a mortar and pestle. Another strain of *Acetobacter*, NQ5 (ATCC 53582), was also tested. This strain of *Acetobacter* undergoes periodic reversals of ribbon synthesis and produces a thicker bundle of cellulose ribbons than AY201 strains. 'Pretreated' cellulose was made by incubation of AY201 cellulose with 16.9 μM EG II.

Cellulase

The cellulase mixture denoted 'Complete cellulase' in this work is a dilution of the commercial product Celluclast 1.5 L^{TM} which is an extract of *Trichoderma reesei* (courtesy of Dr. Martin Schuelin, Novo Nordisk Bioindustrials, Inc., Danbury, CT; ID# 101187). A stock solution was made by diluting 1 vol Celluclast into 4 vol 50 mM acetate buffer (pH 5.0). The total enzyme concentration of this diluted Celluclast was 4.2% protein (w/v), as determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard. A 1% Celluclast solution was made by diluting 1 vol Celluclast into 19 vol 50 mM acetate buffer (pH 5.0). Purification of CBH I, CBH II, and Endoglucanase II (EG II) was achieved by a combination of column chromatography as previously reported (Amano *et al.*, 1996; Shiroishi *et al.*, 1997).

Treatment of cellulose with cellulase

One milligram of cellulose, prepared as above and suspended in pH 5.0 acetate buffer, was placed onto a microscope slide. Varying quantities and types of cellulases were added to the cellulose (Table 1), and a coverslip was placed on the slide. The reactions were performed at 23°C on the stage of a Zeiss Universal microscope and observed for 1 h.

Light microscopy

A Zeiss Universal microscope with phase contrast optics was used to image reactions. Time-lapse images of the reactions were captured with an Optronics CCD camera, and recorded onto a Panasonic optical disk recorder (ODR) at four frames per minute (15 s intervals). After capture, selected sequences from the ODR were digitized and stored on a computer using a Matrox Meteor PCI frame grabber and Image Pro Plus software. Images were saved in JPEG format, and Adobe Premiere was used to combine the JPEG

Table 1. Assay of cellulose rotation during enzyme treatment

Enzyme(s) + 1 mg AY201 Cellulose	Rotation
5 µl 1.0% Celluclast	++
5 µl 4.2% Celluclast	+
10 µl Heat denatured 4.2% Celluclast	−
5 µl 4.2% Celluclast + 10 µg Methyl Cellulose	−
1 µg CBH I	−
1 µg CBH II	−
1 µg EG II	−
EG Pre-treated Cellulose + 0.5 µg CBH I	−
EG Pre-treated Cellulose + 1 µg CBH II	−
0.5 µg CBH I + 0.5 µg CBH II	−
0.5 µg CBH II + 0.5 µg EG II	−
0.5 µg CBH I + 0.5 µg EG II	−
0.8 µg CBH I + 0.8 µg EG II	−
0.8 µg CBH I + 0.5 µg EG II	+
0.8 µg CBH I + 0.4 µg EG II	+
0.8 µg CBH I + 0.16 µg EG II	++
0.8 µg CBH I + 0.08 µg EG II	+

− No rotation; (+) slow rotation; + rotation; ++ intense rotation.

images into an AVI movie. For web presentation, the AVI was converted into Mpeg format.

Results

Rotation of cellulose ribbons was induced by the addition of the diluted Celluclast solution (1.0% and 4.2% total protein), and various ratios of purified CBH I and EG II (Table 1). Figure 1 shows the rotation of bacterial cellulose fibrils after treatment with diluted Celluclast. Images were collected at 15 s intervals using phase contrast optics. Every 10th image is shown in Figure 1. The entire set of figures spans about 20 min (the time-lapse movie of this sequence is on the Internet and can be found at: http://www.botany.utexas.edu/facstaff/facpages/mbrown/movies/movies.htm). This sequence shows a right-handed 360° rotation of a bacterial cellulose ribbon. In the field of view, some cellulose appears to dissolve and not undergo any rotation.

Table 1 provides data on the various enzymes added to bacterial cellulose and the effects on rotation. Two concentrations of diluted Celluclast (1% and 4.2% total protein) both induce rotation. It appears that the lower concentration of Celluclast enzyme induces a more intense rotation. Rotation of the cellulose ribbons was completely abolished after treatment with diluted Celluclast in the presence of 1% methyl cellulose.

Purified CBH I, CBH II, or EG II used alone did not induce rotation of bacterial cellulose. Even a combination of all three purified enzymes, when applied in equal microgram amounts, did not produce rotation. Only when the amount of EG II was reduced would mixtures of purified EG II and CBH I cause rotation. An upper and a lower limit of EG in the mixture was established (Table 1). Addition of CBH I to cellulose which had been pre-treated with EG II did not induce rotation (Table 1).

Discussion

Cellulose ribbons produced by *Acetobacter xylinum* in liquid culture are twisted in a right-handed manner (Hirai *et al.*, 1998). The alga *Micrasterias denticulata* also produces right-handed helical cellulose microfibrils (Hanley *et al.*, 1997). In both organisms, the spacing between twists is $\approx$ 700 nm. It is fairly obvious that the twist of the bacterial ribbon provides a plausible explanation for the rotation of the bacteria observed during time lapse videos of cellulose ribbon assembly (Colpitts, 1977; Brown, personal observations), as well as the rotation of bacterial ribbons observed during degradation with cellulase.

It is important to understand the mechanism/s leading to twisting either during biosynthesis or degradation. Two opposing views have been elaborated with respect to the twisting of cellulose during synthesis: (a) the twisting is caused somehow by the bacteria themselves during synthesis (Hirai *et al.*, 1998); or (b) the intrinsic chirality of cellulose is responsible for the twisting (Gray, 1996).

To believe that the bacteria have some torque-generating system which would be completely independent of cellulose crystallization would require a substantial quantity of supporting data. Unfortunately, this does not exist, and the studies supporting this mechanism have scant solid evidence supporting this idea. To provide support for their model, Hirai *et al.* (1998) cite evidence showing that when *Acetobacter* cells are treated with carboxymethylcellulose (CMC) during the synthesis of cellulose, they produce twisted and splayed microfibrils. They further state that this 'twisting should be produced simply by the rotation of the bacterial cell itself around its longitudinal axis'. They go on to state that by analogy, the intact

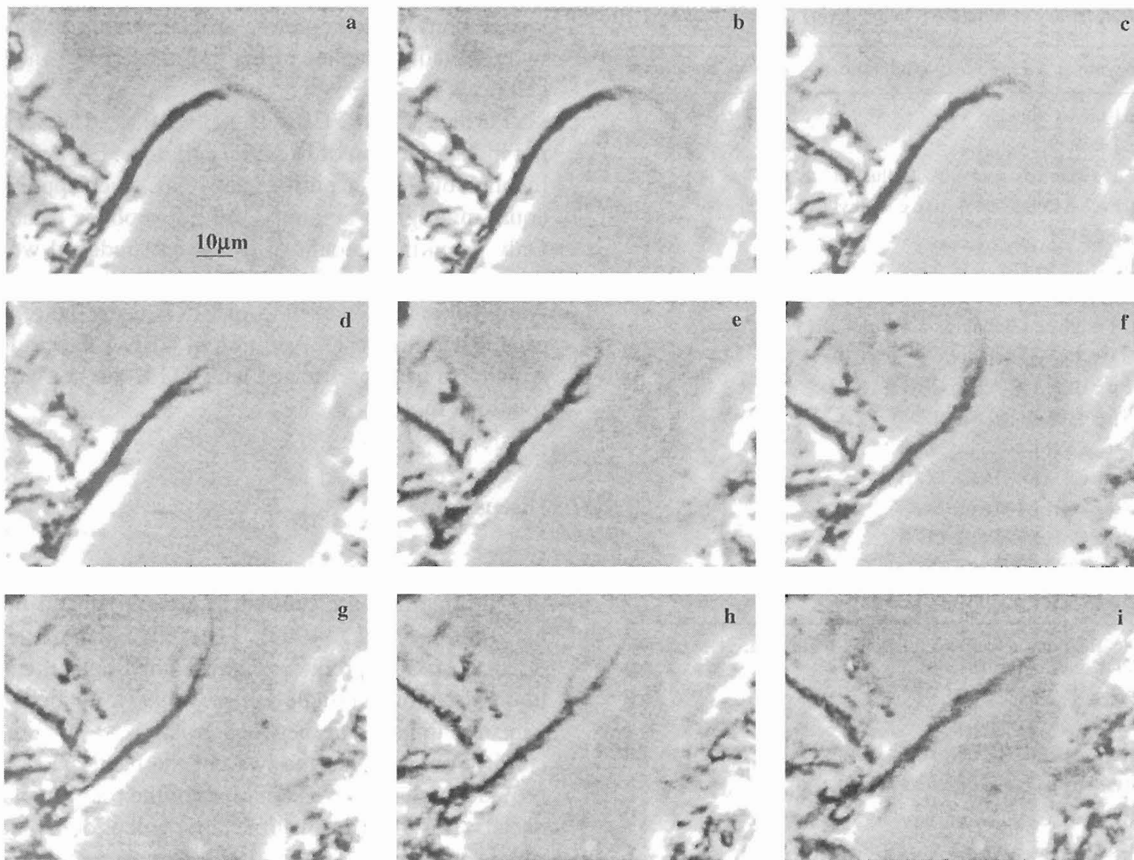

Figure 1. A time-lapse sequence showing the rotation of bacterial cellulose during degradation by complete cellulase. The central aggregation microfibril ribbons is undergoing rotational motion as the time course progresses. Approximately 150 s intervals are represented between each image.

ribbon twisting is also a result of the action of the bacterial cells themselves (Hirai *et al.*, 1998). These authors do have some interesting results and explanations regarding the effects of polymeric additives on the biosynthesis of cellulose under specified experimental conditions; however, these results cannot easily be interpreted to explain an independent motility factor controlling rotation of the bacteria, which in turn regulates the twist of microfibrils and ribbons.

On the other hand, Haigler and Chanzy (1988) make the following statement about the rotation of *Acetobacter* cells during synthesis: 'It should be emphasized that since the bacterium has no flagella or other mechanisms of locomotion, the twist of the cellulose and the rotation of the bacterial cell about its axis must be attributed to the cellulose molecules themselves or to their interaction at the cell surface'. Gray (1996) and Hanley *et al.* (1997) also support this second concept. To quote the latter, 'The chiral nature of the cellulose molecular chain is thus reflected in the physical and mechanical properties of cellulose materials (Gray, 1996), but the relationships between expressions of chirality at different morphological levels (molecule, microfibril, cell wall. etc.) remain unclear'.

Furthermore, when *Acetobacter* cells grown on agar generate cellulose, they do not move in a straight line (Brown, personal observations). Instead, they move in a spiral path and generate cellulose ribbons which are not twisted. Theoretically, this spiral ribbon could be converted into a twisted ribbon by extending the ends of the ribbon away from each other into a linear orientation. It is possible that charge interactions between the agar, the cellulose, and the bacterial cells initiate the spiral synthesis pathway, and then the chirality of the cellulose molecules forces the bacterium to curve into a spiral as it extrudes cellulose. This indicates that it is not the rotation of the bacterial cell

itself which produces twist in the cellulose ribbon, but it is the chirality of cellulose which causes bacteria to rotate.

The current study has shown that bacterial cellulose rotates as it is degraded by *Trichoderma* cellulases. This is the reverse of the process described by Cousins and Brown (1997). Rotation of cellulose can be thought of as a visual indication of the helix to crystal (photoisomerization) and crystal to helix (cellulase degradation) transitions. It should be emphasized that, in our case, the rotational motions of cellulose ribbons are associated with the degradation process of cellulose. On the other hand, Cousins and Brown (1997) demonstrated the collapse of a tube with a helical seam into a helical arrangement of splayed microfibrils. Rotation by this strain-relief model is possible only if there is internal stress present within the dye-coated cellulose tube. Furthermore, the rotation of cellulose aggregates observed during photoisomerization suggests that the force of crystallization is itself enough to straighten (at least partially) some of the bacterial cellulose.

In the biosynthesis of native cellulose I, the structure of the terminal complex facilitates the crystallization of glucan chains in a parallel fashion. It is possible that the nascent glucan chains co-crystallize before they can relax into their preferred conformations, thus locking stress within the crystal. This is supported by evidence which shows that helical cellulose mini-sheets straighten as they begin to form hydrogen bonds with each other following photoisomerization of cellulose-bound Tinopal dye molecules (Cousins and Brown, 1997).

How are stress/strain relationships involved with the rotation of bacterial cellulose by cellulases? We hypothesize that internal stress is being released during the degradation of crystalline cellulose by cellulase. This might occur if CBH I molecules are interdigitating into the cellulose and releasing strain to cause the rotation. It has been shown that treatment of cotton fibers with chemically inactivated CBH I leaves holes or depressions following removal of the cellulase molecules (Lee *et al.*, 2000) This suggests that the CBD and some of the linker could be interdigitating into the cellulose; however, the structure of CBH I is highly suggestive of a surface mode of action for these enzymes (Divne *et al.*, 1998). In addition, biochemical studies (Valjamai *et al.*, 1998) and computer simulations (Sild, 1999) suggest that CBH I and CBH II act predominantly on the surface of cellulose. We suggest as one possible explanation for surface versus penetration site of action is that the CBD and the linker region may interdigitate between the microfibrils, but leave the catalytic core on the surface to carry out the degradation of glucan-chain ends. In order for interdigitation to occur, hydrogen bonds and/or van der Waals interactions between glucan chains would have to be broken. While this model suggests that CBH I itself theoretically should induce rotation, we find that EG II is still required to allow CBH I access to the core of a microfibril.

There are several reasons why a combination of EG II and CBH I (not CBH II) could induce rotation. Differences between CBH I and CBH II which may be responsible for rotation include the different degrees of processivity of the two exo-enzymes, and their opposite affinities for the two ends of a polysaccharide chain. Although it is difficult to see how the preference of the cellobiohydrolases for opposite ends of cellulose might be responsible for CBH II's inability to induce rotation of cellulose, it is plausible to consider the differences in their processivity. CBH I has a much longer tunnel (50 Å) than CBH II (20 Å) (Henriksson *et al.*, 1995) which may translate directly into the increased processivity of CBH I. In addition, because CBH II also possesses some endo-activity (Ståhlburg, 1993), it is possible that CBH II makes too many nicks in the cellulose, thus eliminating the integrity of the backbone of the cellulose which is necessary for rotation to occur.

While both CBH I and CBH II hydrolyze cellulose from the chain ends, only CBH I appears to effectively degrade the crystalline core of a microfibril (which would be the region where the most energy for rotation is stored). On the other hand, CBH II appears to prefer amorphous regions (Chanzy *et al.*, 1983; Irwin *et al.*, 1993; Divne *et al.*, 1998). This model accounts for the difference between CBH I and CBH II; however, it does not explain the requirement of EG II for rotation. In our opinion, EG II is required to be present along with CBH I to allow access to the crystalline core of the ribbon. If the cellulose is pre-treated with EG II followed by treatment with CBH I, rotation does not occur (Table 1), suggesting that the synergy between both enzymes is required for the dynamic release of strain leading to rotation.

There are several lines of evidence to indicate that cellulose molecules are indeed right-handed helices. Individual glucan chains recently have been imaged in a novel form, called nematic-ordered cellulose (Kondo *et al.*, 2000), in which single polymer chains have been observed to be twisted. Because this cellulose

does not crystallize into ordered allomorphs, these glucan chains are in their most native and relaxed form. Also, induced circular dichroism of methylcellulose and cellulose oligomers by Congo Red suggests a helical conformation for these polymers (Ritcey & Gray, 1988). Furthermore, ^{13}C NMR relaxation studies and two-dimensional nuclear Overhauser exchange spectroscopy (NOESY) have shown that the lowest energy state of a substituted glucan chain is a 5/4 helix. This shows that the cellulose chain undergoes a 360° rotation for every five glucose residues, or approximately 38 turns per micrometer (Buchanan *et al.*, 1989). Since it is known that this is not the conformation of glucan chains within native crystalline cellulose I, either the helical nature of the specimen in the Buchanan study was due solely to the substituted nature of the glucan chain, or some forces are causing the glucan chains in native crystalline cellulose to deviate from their preferred helical conformations.

In conclusion, the results of the current study provide further evidence supporting, at least on theoretical grounds, that cellulose chains may have intrinsic chirality. Alteration in this chirality could occur when glucan chains associate during biosynthesis. The number of chains, their position at biosynthesis, and factors controlling crystallization, all might contribute to the twisting of the glucan chain aggregate, microfibril, or ribbon.

In the coming future, the dynamic properties of cellulose observed during both synthesis and degradation may lead to the development of a very inexpensive nano-motor, which could be activated by the crystallization/decrystallization cycle. Given that cellulose has piezoelectric properties, it is not unreasonable to consider the development of such a device. A future study might be aimed at determining the torque force generated during the degradation of cellulose.

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