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Short communication

Assessing cellulose microfibrillar structure changes due to cellulase action

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ABSTRACT

There is a need to understand how cellulose structural properties impact productive cellulase–cellulose interactions toward solving the mechanisms of the heterogeneous reaction. We coupled biochemical studies of cellulose hydrolysis by a purified *Trichoderma reesei* Cel7A (TrCel7A) cellobiohydrolase with atomic force microscopy (AFM) to study the impact of the cellulolytic activity on the fibrillar structure of cellulose. Bacterial cellulose (BC) fibrils were hydrolyzed by TrCel7A then immobilized by hydrophobic interactions on glass for AFM imaging. Commonly used methods to culture and isolate cellulose fibrils resulted in significant oxidation of the reducing-ends but minimal oxidation along the fibrils. We observed extensive fibrillation of BC fibrils to ~3 nm microfibrils during the course of hydrolysis by TrCel7A, leaving thinned un-fibrillated recalcitrant fibrils at >80% hydrolysis extents. Additionally, this remaining fraction appeared to be segmented along the fibril length.

1. Introduction

Cellulose is of industrial importance as a renewable resource for conversion to biofuels, biochemicals (Foust, Aden, Dutta, & Phillips, 2009; Himmel et al., 2007) and as functional components in advanced materials (Lahiji, Boluk, & McDermott, 2012; Lu & Hsieh, 2010). Due to its natural recalcitrance, one of the biggest technological challenges to realizing a cellulosic bioproducts industry lies in engineering inexpensive strategies to depolymerize cellulose.

A persistent mystery surrounding cellulase hydrolysis of cellulose is the characteristic decline in hydrolysis rates over the course of the reaction. In studies using purified *Trichoderma reesei* Cel7A (TrCel7A), a reducing-end specific cellobiohydrolase, it was shown that the hydrolysis rate decline tracks with a decline in the apparent catalytic rate constant (Jalak, Kurašhin, Teugjas, & Väljamäe, 2012; Kurasin & Valjamae, 2011). Substrate properties have been speculated to be the limiting factor (Cruys-Bagger, Elmerdahl, & Praestgaard, 2012; Kurasin & Valjamae, 2011; Zhang, Wolfgang, & Wilson, 1999), but understanding is limited. Native cellulose microfibrils are linear cellulose polymers associated via extensive hydrogen bonding and hydrophobic stacking interactions to organize into crystalline lattices that exclude water (Nishiyama, Langan, & Chanzy, 2002; Nishiyama, Sugiyama, Chanzy, & Langan, 2003). Microfibrils associate into larger fibrils (White & Brown, 1981) resulting in insoluble fibrillar structures where only celluloses on the outer surfaces are accessible to hydrolysis at the solid/aqueous interface by soluble cellulolytic enzymes. Cellulose fibrils undergo macroscopic changes in the supramolecular organization (Chanzy, Henrissat, Vuong, & Schulein, 1983; Santa-Maria & Jeoh, 2010; White & Brown, 1981) and nano-scale changes in surface properties (Wang et al., 2012) due to cellulolytic action.

Here we present details of methods to characterize and immobilize cellulose fibrils to facilitate biochemical and imaging studies of cellulose fibrillar properties. With these methods, we present results showing changes in the microstructure of cellulose fibrils due to the action of a purified TrCel7A.

2. Materials and methods

2.1. Preparation of cellulose fibrils

Pellicles from *Gluconacetobacter xylinus* (ATCC 700178) cultures (Santa-Maria & Jeoh, 2010) were rinsed, then washed in 1% sodium hydroxide (4 °C, 12 h, shaking at 60 rpm) and 0.3% sodium hypochlorite (pH 4.9 adjusted with glacial acetic acid, 2 h, 70 °C, gentle agitation) and repeated as necessary. The pellicles were rinsed until the conductivity reached 0.5–5 μS/cm and stored with 0.02% sodium azide at 4 °C.

Microfibrils (4 mL of ~0.5 mg/mL) were dispersed by ultrasonication with a 1/8 in microtip (Misonix Ultrasonicator S-4000, Qsonica, LLC, Newtown, CT) at 30% amplitude and 2× 15-s pulses (220–283 J).

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Cellulose reducing end concentrations were estimated by the bicinchoninic assay (BCA) (Doner & Irwin, 1992). Carbon, hydrogen and nitrogen (CHN) contents of lyophilized BC were analyzed using air and a barley standard (1.69% N) as calibrants (TruSpec CHN analyzer, LECO Corp., St. Joseph, MI).

2.2. Measuring oxidized groups on cellulose

The carboxyl content of cellulose was measured by conductimetry (Habibi, Chanzy, & Vignon, 2006). The conductivity of cellulose suspensions (1–3 mg/mL in 40 mL) in 0.1 mM NaCl and 2 mL of 10 mM HCl was recorded (InLab® 731 conductivity probe, Mettler Toledo, Schwerzenbach, CH), then titrated with 10 mM NaOH. The concentration of carboxyl groups is equivalent to the moles of NaOH added in the nonlinear range of the titration curve (Supplementary information 1). The carboxyl content of cellulose is reported as mmole —COOH/mole glucose measured by the Anthrone assay using glucose as the standard (Morris, 1948).

Aldehyde groups on cellulose were selectively oxidized to carboxylic acids by reacting 150 mg of cellulose in 50 mL of sodium acetate buffer (pH 4) and 300 mg of sodium chlorite ($NaClO_2$) for 40 h at room temperature (RT). The ketone groups on ~0.1 mg/mL of $NaClO_2$ treated cellulose was reacted with 0.01 mg/mL Alexa Fluor 488-hydrazine (AF488-Hyd) (Life Technologies, Grand Island, NY) in 200 mM sodium acetate buffer pH 5.0 and shaken at 200 rpm overnight at RT. AF488-cellulose was filtered (0.2 μm, 25 mm dia, Isopore™, MilliPore), washed, then resuspended in sodium acetate buffer. Fluorescence intensities at E_x/E_m 488/520 nm were converted to concentrations using an AF488-Hyd standard curve. The ketone content of cellulose is reported as μmole ketone/mole glucose.

2.3. Hydrolysis of BC by TrCel7A

TrCel7A was purified from Celluclast (Novozymes, Inc.) and labeled with Alexa Fluor 594 (Life Technologies, Grand Island, NY) (AF594TrCel7A) (Santa-Maria & Jeoh, 2010).

Dispersed BC was combined with 10 μmole AF594TrCel7A/g cellulose in 0.5 mL total volume, 50 mM sodium acetate pH 5.0 and rotated end-over-end at RT in the dark. Substrate-only and enzyme-only controls were prepared in parallel. All reaction components were equilibrated to RT for >30 min before the reactions were

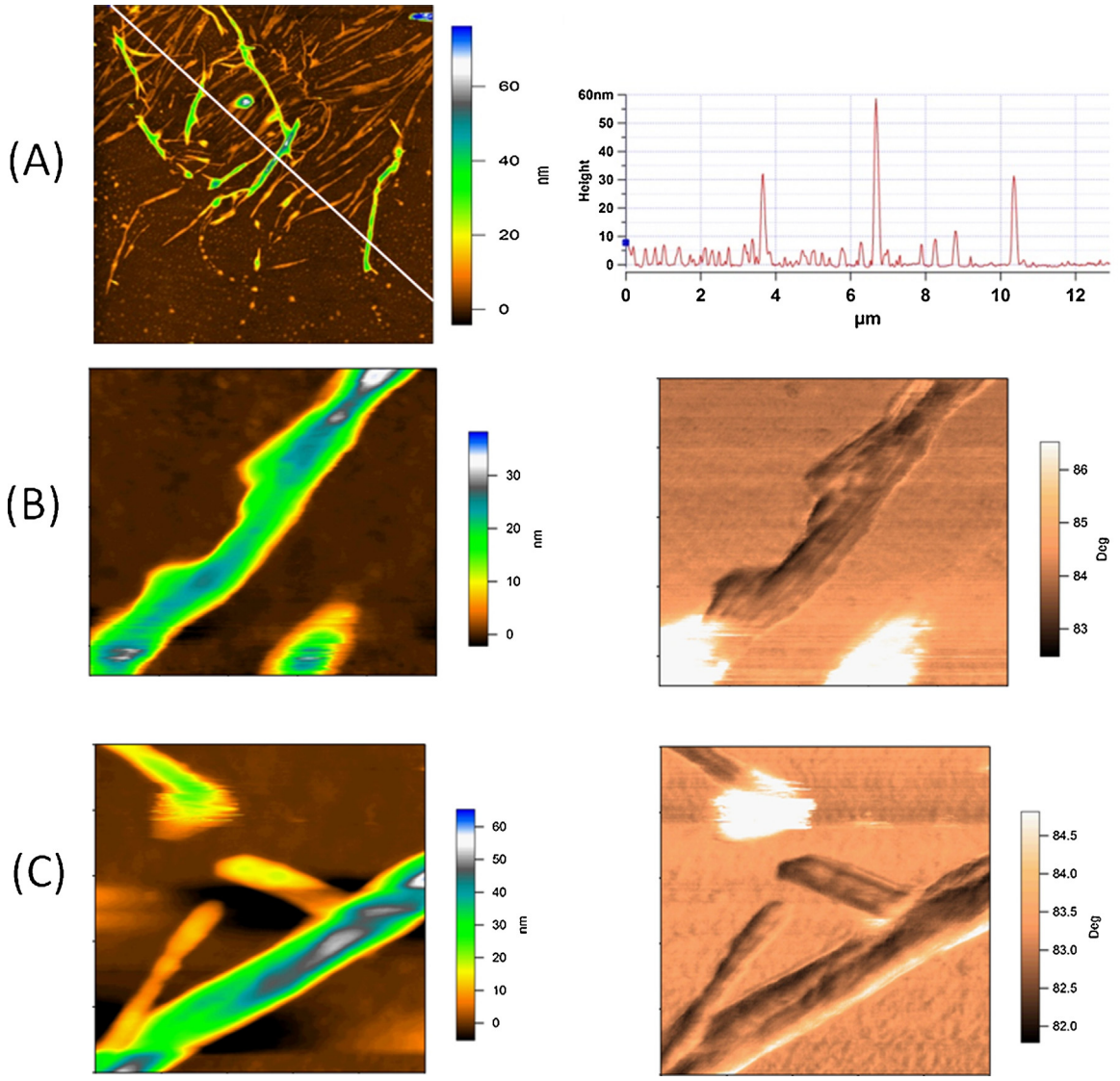

Fig. 1. ABA washed BC fibrils immobilized on glass. These images correspond to the substrate-only control and zero time point in the hydrolysis experiments. (A) 10 μm × 10 μm field of view (FOV) (dimensions of the image shown). Left: AFM height image, right: cross-section height profile corresponding to the white line in the height image; (B and C) 1 μm × 1 μm FOV. Left: height data, right: phase data.