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Nanocrystals of cellulose allomorphs have different adsorption of cellulase and subsequent degradation

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ABSTRACT

Suspensions of cellulose nanocrystals (CNCs) having different allomorphs were prepared from pulp and microcrystalline cellulose. Comparison found varying particle features. *In situ* observation of cellulose enzymes interacting with CNC I, II and III by atomic force microscopy (AFM) and transmission electron microscopy (TEM) found diverse adsorption of the enzymes on the CNC. Greater degradation to glucose was revealed for short rod-like CNC II and thin needle-like CNC III than CNC I, which were mainly due to their greater d-spacings for the (110) (CNC II) and (1–10) (CNC III) planes compared to the (200) spacing for CNC I. According to the model crystal structures, the surface areas for those planes where the enzymes would adsorb were also lager for CNC II and III. However, CNC III had significantly enhanced adsorption capability compared to the low affinity of CNC II. Enzymes tended to gather near hydrophobic surfaces that correspond to planes with small crystallite sizes on CNC III, and the enzymes caused breaks of nanocrystals during the degradation. Understanding the interactions between CNC allomorphs and cellulase would provide deep insights into biodegradation of crystalline cellulose as well as extensive application of CNCs.

1. Introduction

Cellulose, the main component of biomass, is the most abundant source for sustainable and renewable energy and materials (Chundawat et al., 2011a; Moon et al., 2011). Cellulose is composed of unbranched molecules of β-1, 4-glucosy repeating units tightly aggregated into microfibrils (3–5 nm in diameter) via strong intermolecular hydrogen bonds and van der Waals forces (Klemm et al., 2005; French et al., 2014). The regular and ordered microfibril aggregation constitutes the highly crystalline parts of cellulose, while the other regions with disordered arrangement of cellulose chains are often called amorphous. Several allomorphs of crystalline cellulose (I, II, III and IV) have been determined by earlier studies (French, 2014; Zugenmaier, 2001). There are two forms of native cellulose I, two-chain monoclinic cellulose Iβ (higher plants) and triclinic Iα (algae). Both can be converted to other allomorphs via thermochemical treatments, namely, cellulose II by NaOH swelling, or dissolution and regeneration (Langan et al., 2001; Zhang et al., 2005), cellulose III by amines or ammonia (Wada et al., 2004) and cellulose IV by high temperature treatment in glycerol (Gardiner and Sarko, 1985).

Tightly arranged and strongly connected crystalline regions of cellulose have stimulated great interest due to their unique physical and chemical properties. For instance, the efficiency of enzyme binding on the crystalline portion of cellulose has long been discussed (Chundawat et al., 2011b; Ciolacu et al., 2014; Teeri, 1997). Cellobiohydrolases, an exoglucanase, was reported to have greater activity on crystalline cellulose than endo-acting enzymes that are more active on amorphous phases (Lynd et al., 2002). Higher crystallinity of cellulose substrates resulted in lower digestibility by cellulase, mainly because of a well-arranged hydrogen bonding network (Chundawat et al., 2011b). In terms of diverse cellulose allomorphs, swelling of microfibrils and weaker interactions between the aforementioned hydrophobic planes caused dramatically higher enzymatic digestibility of mercerized cellulose II allomorph than original cellulose Iβ (Ling et al., 2017; Wada et al., 2010). More susceptibility of cellulose III to cellulase was also found to be due to the disordered cellulose molecular arrangements (Horikawa et al., 2013).

To more directly observe cellulose-binding modules (CBMs) of cellulase on crystalline cellulose molecules, extraction of cellulose crystalline (CNCs) from purified cellulose source is beneficial (Hu et al., 2016; Nakamura et al., 2014). The process is fulfilled via removal of amorphous parts by either acid or enzymatic hydrolysis (Klemm et al.,

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2011; Sacui et al., 2014). CNCs, also known as whiskers, are rod-like crystals with high mechanical strength and surface area, as well as low density (Brinkmann et al., 2016). Common methods for CNC preparation often lead to negative surface charges on particles that are then able to form a stable colloidal suspension (Frka-Petesic et al., 2015). Highly crystallized CNCs have stronger bindings with CBM than microcrystalline cellulose and amorphous cellulose (Guo and Catchmark, 2013). A real-time atomic force microscopy (AFM) was used to visualize the crystalline cellulose degradation by individual cellulase enzyme *Trichoderma reesei* cellobiohydrolase I (*Tr*Cel7A) (Igarashi et al., 2011). The cellulase molecules were believed to slide unidirectionally along the crystalline cellulose surface and then halted collectively. Clear visualization of enzyme adsorption and hydrolysis of CNC in suspensions was also achieved by transmission electron microscopy (TEM) combining the negative staining and metal shadowing technique (Horikawa et al., 2017). CNCs derived from *Valonia* and algae were biodegraded by cellulase enzymes in their work. Disintegration was observed to initiate from the middle part, which also showed some splitting and formation of larger bundles.

The interacting behavior between cellulase and CNC allomorphs can also be interpreted by converting samples into CNCs, though relative studies are limited. Jin et al. proposed that CNC-II has lower affinity to enzymes than CNC-I, but the softer layer conformation of the cellulose and the more reversible adsorption on CNC III may facilitate its hydrolytic activity (Jin et al., 2017). Changing the crystalline allomorph to cellulose III may increase the apparent number of accessible lanes on the crystalline surface and thus increasing the number of moving cellulase molecules (Igarashi et al., 2011). Cellulase adsorption and hydrolysis of CNCs can be enhanced by synergism with other enzymes, such as the polysaccharide monoxygenase enzyme AA9 (Hu et al., 2014; Hu et al., 2016). But the enhanced cellulose deconstruction was not helpful for cellulose II and cellulose III allomorphs that already have higher cellulase accessibility than cellulose I (Cui et al., 2014).

Crystalline parts of cellulose that act as main cause of biomass recalcitrance to enzymes are still under controversial discussion (Chundawat et al., 2011b; Gupta et al., 2016). In this work, by using an atomic force microscope (AFM) as well as transmission electron microscope (TEM), we visualized *in situ* the adsorption and degradation by cellulase of CNCs, rather than the formerly preferred thchnique that used CNC films (Ganner et al., 2015; Strasser et al., 2016; Wang et al., 2012). Supramolecular structure and surface characterization of CNCs with varying allomorphs were also revealed.Therefore, the detailed comprehension was gained on the interaction of CNC allomorphs with cellulase.

2. Materials and methods

2.1. Materials

Two cellulose sources were chosen as raw materials to prepare CNCs. Soft wood pulp was obtained from Donghua Pulp Factory and microcrystalline cellulose (MCC), derived from cotton fiber, was purchased from Sinopharm Chemical Reagent Co. Cellulose II was obtained by soaking pulp and MCC respectively in 20 wt% NaOH at 45 °C for 1 h. Cellulose III was prepared by ethylenediamine (EDA, ≥ 99.5%) treatment at room temperature with continuous stirring for 1 day followed by methanol washing to remove remaining EDA.

2.2. CNC preparation

Three allomorphs of cellulose from both pulp and MCC were used to prepare CNC suspensions by sulfuric acid hydrolysis (Yang et al., 2013). About 2 g of cellulose were added to 100 mL of 55 wt% sulfuric acid at 60 °C for 1.5 h with mechanical stirring (400 rpm). The dispersion was diluted four-fold in water followed by rinsing with three repeated centrifuge cycles. Afterwards, samples were dialyzed against deionized water for several days, until the pH reached ~ 6. To disperse cellulose particles, the suspension was ultra-sonicated for 30 min. The achieved colloidal suspensions with nanosized cellulose I (CNC I), II (CNC II) and II I (CNC III) from pulp and MCC were adjusted to the concentration of 0.08 wt% and separately denoted as PNC I, PNC II, PNC III and MNC I, MNC II, MNC III.

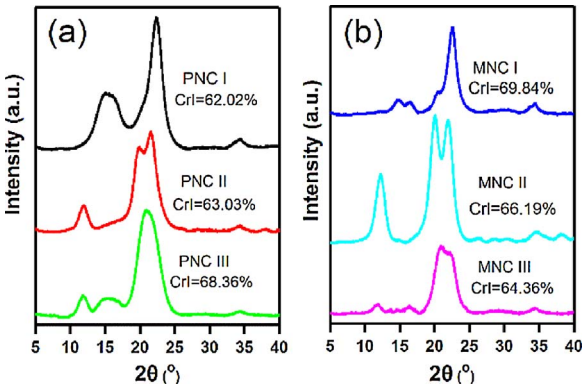

Fig 1. XRD patterns and crystallinity index of cellulose nanocrystals. (a) PNC I, PNC II and PNC III made from pulp and (b) MNC I, MNC II and MNC III made from microcrystalline cellulose.

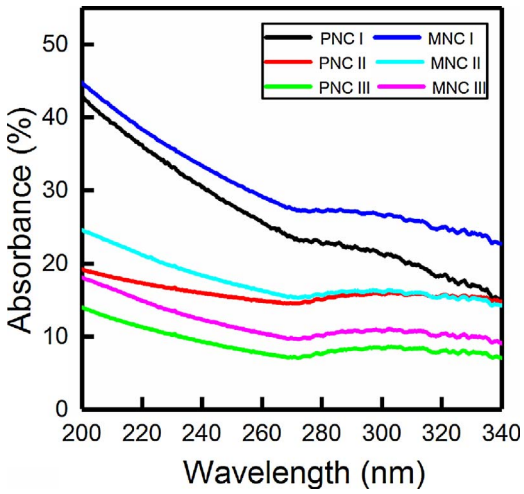

Fig. 2. UV–vis absorbance spectra of pulp and MCC derived CNC dispersions with different allomorphs.

2.3. Enzyme incubation

Commercial cellulase from *Trichoderma reesei* was purchased from Novozymes (China). The multienzyme is mainly composed of the exocellulase cellobiohydrolase I (*Tr*Cel7A) and has a cellulase activity of 160 FPU/g. It also contains β-glucosidases with the capability to cleave the cellobiose into its constituent glucose units. The 20 mL conical flasks containing 10 mL CNC suspensions with 50 FPU/g cellulose and 0.05 M sodium acetate buffer were incubated at 48 °C with air-shaking. Six replications for each CNC sample were used to test the released glucose after specific time intervals. The released glucose was analyzed by a high performance liquid chromatograph (HPLC) (Agilent 1200 series, Agilent Technologies, USA). All assays were performed in triplicate.

2.4. Characterization

The optical transmittance of each suspension was measured at wavelengths from 200 to 340 nm using a UV–vis spectrophotometer (UV2300PC, Shanghai Spectrum Instrument Ltd. China). The data were