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# Analysis of crystallinity changes in cellulose II polymers using carbohydrate-binding modules

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ABSTRACT

Carbohydrate-binding modules (CBMs) are a set of tools that can be used as molecular probes for studying plant cell walls and cellulose-based substrates. CBMs from enzymes of bacterial and fungal origin present a range of recognition capabilities for crystalline and amorphous cellulose. Here cellulose-directed CBMs have been used to visualize and quantify crystallinity changes in cellulose II-based polymers following NaOH treatment. Cellulose II polymers used were in the form of lyocell fibers, which are derived from eucalyptus wood pulp. The supramolecular structure, morphology, and existence of 'skin-core' model in the fiber were examined using CBM-labeling techniques. Changes in cellulose crystallinity showed maxima at 3.33 mol $dm^{-3}$ NaOH (under treatment conditions of 49 N $m^{-1}$ at 25 °C) and 4.48 mol $dm^{-3}$ NaOH (under treatment conditions of 147 N $m^{-1}$ at 40 °C); CBM methods were also suitable for quantifying changes within amorphous regions. Quantification of crystallinity changes using CBM labeling techniques was achieved in combination with image analysis, which was shown to reflect the same crystallinity changes as measured using ATR-FTIR methods. It was demonstrated that CBM-labeling techniques were able to validate the proposed 'skin-core' model of lyocell fibers, comprising a semi-permeable fiber skin and a porous core.



## 1. Introduction

Carbohydrate-binding modules (CBMs) are found in many carbohydrate-active enzymes. CBMs are non-catalytic protein modules that promote the association of the enzyme with the substrate and their main function is to increase the catalytic efficiency of the enzyme against soluble and/or insoluble substrates. In the Carbohydrate-Active enZYmes (CAZY) Database, a CBM is defined as a contiguous amino acid sequence within a carbohydrate-active enzyme with a discrete fold having carbohydrate-binding activity (Hashimoto, 2006; McCartney et al., 2006). CBMs with the capacity to bind cellulose are associated with enzymes that hydrolyze both cellulose and other cell wall polymers such as xylan, mannan, pectin, and non-cellulosic-glucans (Hogg et al., 2003; Kellett et al., 1990; McKie et al., 2001). CBMs are grouped into sequence-based families and are named after the family in which they are located (*e.g.* a family 4 CBM is designated CBM4). CBMs from families 1, 2a, 3a, 5, and 10 are classified as type A CBMs, which bind to crystalline polysaccharides, predominantly cellulose (Jamal, Nurizzo, Boraston, & Davies, 2004), as the topography of the ligand recognition site is conserved presenting a flat surface comprising predominantly aromatic residues, which interact with the multiple planar cellulose chains found in crystalline cellulose (Raghothama et al., 2000; Xu et al., 1995). Type B CBMs do not bind to the planar surface of crystalline polysaccharides but recognize isolated saccharide chains. Three well characterized examples of cellulose-binding type B CBMs are found in families 4, 17, and 28. The ligand-binding sites in these protein modules comprise extended clefts or grooves that accommodate individual glycan chains in non-crystalline regions of cellulose (Boraston, Nurizzo, et al., 2011; Jamal et al., 2004); they display similar cellulose binding properties, although competition studies indicate that these modules recognize different substructures within amorphous cellulose (Araki, Karita, Tsuchiya, Kondo, & Goto, 2010; McLean et al., 2002).

Indirect immunofluorescence labeling using recombinant CBMs is commonly used to provide a visual representation of the cellulose plant structure, particularly the variation in cell wall or other cellulose I substrates like wood tissues and Valonia cellulose recognition of both crystalline and amorphous regions (Boraston, Kwan,

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Chiu, Warren, & Kilburn, 2003; Dagel et al., 2011; Jamal et al., 2004; Kawakubo et al., 2010; Lehtio et al., 2003). The most effective binders to visualize cellulose and other plant cell walls are CBM3a and CBM28 due to their known affinities to crystalline and amorphous cellulose respectively (Jamal et al., 2004; McCartney et al., 2006). On the basis of previous research (Blake et al., 2006; McCartney et al., 2006; McCartney, Gilbert, Bolam, Boraston, & Knox, 2004), this method has been employed first time as a tool for quantitative and microscopic analysis of changes in cellulose II-based polymers on the supramolecular level, and to monitor its changes, for example, caused by NaOH treatment.

The recognition of cellulose in the context of cell walls by diverse families and types of CBM has not been systematically examined. It has been previously demonstrated that CBMs directly coupled to fluorophores or with His tag appendages can be used to investigate the localization of these proteins when bound to target polymers in the context of cell wall composites (Blake et al., 2006; McCartney et al., 2006, 2004), wood tissues (Kawakubo et al., 2010) or Valonia cellulose crystallites (Dagel et al., 2011). Herein, alkali-treated lyocell fibers, a cellulose II-based polymer, were examined. Lyocell fibers are derived from eucalyptus wood pulp and consist of a linear 1,4-β-glucan polymer where the units are able to form highly ordered structures (crystalline cellulose II), as a result of extensive interaction through intra- and intermolecular hydrogen bonding of the three hydroxyl groups in each cellulose unit as well as low ordered structures (amorphous cellulose). Lyocell has a high degree of crystallinity (up to 70–80%) which is a consequence of higher orientation during stretching and formation of fibers; lyocell fibers have the thinnest and longest crystallites, even the amorphous regions are oriented along the fiber axis (Crawshaw & Cameron, 2000). Lyocell fibers have a microfibrillar structure because a portion of the molecular chains aggregate to form microcrystals while recrystallizing along the chains, whereas the remaining chains exist in the amorphous phase as links between these two phases (Okano & Sarko, 1984). In the crystalline regions of cellulose II polymers, the layered structure is very regular, so the length of hydrogen bonds between molecules is the same (Kolpak & Blackwell, 1976; Kono & Numata, 2004; Langan, Nishiyama, & Chanzy, 1999). Current knowledge assumes the structure of cellulose II is an anti-parallel arrangement of cellulose chains with some inter-sheet hydrogen bonding, generally leading to a perfectly distributed symmetrical structure, and a C(6) conformation of $CH_2OH$ group is of *gauche-trans* conformation (gt) as well as a mixed conformation of *gauche-trans* and *trans-gauche* (tg) may be observed at significance level of better than 5% resulted from X-ray refinement (Klemm, Philipp, Heinze, Heinze, & Wagenknecht, 1998). Although, the different assessments are available like that based on molecular dynamics (MD) simulations done by Kroon-Batenburg, Bouma, and Kroon (1996). Therein, the authors propose the structure of regenerated cellulose fibers as an anti-parallel mixed conformation of gt and gt, which has slightly higher the average relative potential energy than that of a mixed gt and tg conformation. They also suggested that favorable arrangements of mercerized structure is a parallel mixed conformation of gt and gt. Alkali treatment has a substantial influence on morphological, molecular and supramolecular properties of cellulose, causing changes in crystallinity, pore structure, accessibility, stiffness, unit cell structure and orientation of fibrils in cellulosic fibers (Široký et al., 2008). Treatment with alkali can improve mechanical and chemical properties of cellulose fibers such as dimensional stability, fibrillation tendency, tensile strength, dyeability, reactivity, luster and fabric smoothness. Factors such as the concentration of NaOH, treatment temperature, applied tension, residence time, source of cellulose, physical state of cellulose (fibril, fiber, yarn or fabric), and degree of polymerization have an effect on the properties and degree of change upon treatment (Heinze & Wagenknecht, 1998; Široký, Blackburn, Bechtold, Taylor, & White, 2010).

Previous research has described how studies of crystallinity and morphology of cellulose have progressed, and how changes in cellulose II crystallinity and morphology due to sodium hydroxide treatment may be analyzed using techniques such as ATR-FTIR (Široký et al., 2010) or dye sorption (Široký, Blackburn, Bechtold, Taylor, & White, 2011). Recently within our research group a similar investigation has been achieved for cellulose I (two species of cotton) using a similar attempt to quantify obtained fluorescence micrographs of bound CBM2a, CBM3a, CBM4-1, and CBM17 (Kljun et al., 2011); it was demonstrated that the CBMs used displayed a greater ability to detect early crystallinity changes in cotton subject to treatment with 0–8 mol $dm^{-3}$ sodium hydroxide, in comparison with analysis by XRD and ATR-FTIR.

The work herein describes the use of immunoassay labeling techniques using CBMs in combination with fluorescence microscopy to monitor changes to the crystallinity of cellulose II polymers when treated with increasing concentrations of aqueous sodium hydroxide solution (0.00–7.15 mol $dm^{-3}$) under varied temperature (25 or 40 °C) and tension (49 or 147 N $m^{-1}$); crystallinity changes are quantified in combination with image analysis methods. The results of bound CBMs are compared with crystallinity changes monitored using Attenuated Total Reflectance Fourier-Transform Infrared (ATR-FTIR) spectroscopy.

## 2. Experimental

### 2.1. Materials

Plain-woven (1/1 weave), desized, scoured lyocell fabrics (Tencel, 140 g $m^{-2}$, comprised of 50/1 Nm yarn) used for this observation were kindly supplied by Lenzing AG, Austria. Technical grade NaOH (*ca.* 50%, w/w) was used in formulating the alkali solutions used in treatments, with *Lyogen MC* (Clariant, Basel, Switzerland) added as wetting agent. Analytical grade acetic acid was used in formulating the neutralization liquor.

CBMs types used in this research are shown in Table 1; all CBMs were of the fold 1 (β-sandwich) family (Hashimoto, 2006; Jamal et al., 2004; McCartney et al., 2004). All CBMs were kindly supplied by Prof. Harry Gilbert, Department of Biological and Nutritional Sciences, Newcastle University; 3D structures of the CBMs are shown in Blake et al. (2006). All other chemicals were supplied by Sigma–Aldrich.

**Table 1**
Cellulose-directed CBMs used in this study.

| CBM probe | Fold family | Fold | Type | Epitope | Reference |
|---|---|---|---|---|---|
| CBM2a | 1 | β-Sandwich | A | Crystalline cellulose | Bolam et al. (1998) |
| CBM3a | 1 | β-Sandwich | A | Crystalline cellulose | Tormo et al. (1996) |
| CBM10 | 5 | OB fold | A | Crystalline cellulose | Jamal et al. (2004), Hashimoto (2006) |
| CBM4-1 | 1 | β-Sandwich | B | Amorphous cellulose | Tomme, Creagh, Kilburn, and Haynes (1996) |
| CBM17 | 1 | β-Sandwich | B | Amorphous cellulose | Boraston, Chiu, Warren, and Kilburn (2000) |
| CBM28 | 1 | β-Sandwich | B | Amorphous cellulose | Jamal et al. (2004), Hashimoto (2006) |

OB = oligonucleotide/oligosaccharide bindings.