



Modifying solubility of polymeric xylan extracted from *Eucalyptus grandis* and sugarcane bagasse by suitable side chain removing enzymes

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ABSTRACT

α -L-Arabinofuranosidase (AbfB) and novel α -D-glucuronidase (Agu1B) enzymes were applied for selective hydrolysis of beechwood (*Fagus sylvatica*) xylan (Sigma–Aldrich) as well as xylans extracted from *Eucalyptus grandis* and sugarcane (*Saccharum officinarum* L.) bagasse, leading to precipitation of these carbohydrate biopolymers. Hemicellulose extraction was performed with two mild-alkali methods, Höije and Pinto. Precipitation occurred after removal of 67, 40 and 16% 4-O-methyl-D-glucuronic acid (MeGlcA) present in polymeric xylans from beechwood, *E. grandis* (Pinto) and *E. grandis* (Höije), respectively. Precipitation was maximized at Agu1B levels of 3.79–7.53 mg/g_{substrate} and hemicellulose concentrations of 4.5–5.0% (w/v). Polymeric xylan from sugarcane bagasse precipitated after removal of 48 and 22% of arabinose and MeGlcA, respectively, at optimal AbfB and Agu1B dosages of 9.0 U/g and 6.4 mg/g, respectively. Both the purity of polymeric xylans and structure thereof had a critical impact on the propensity for precipitation, and morphology of the resulting precipitate. Nano-to micro-meter precipitates were produced, with potential for carbohydrate nanotechnology applications.

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1. Introduction

Xylan represents the majority of hemicelluloses present in hardwoods (15–30% dry weight, DW), grasses and cereals (20–35% DW) and softwoods (7 and 12%, DW) (Timell, 1967). In comparison to cellulose and starch, industrial applications of isolated xylan biopolymers are limited by its high water solubility (Ebringerová, 2005). There are many potential industrial applications of such biopolymers, if produced in insoluble form (hydrogels), such as bio-films and drug delivery systems, viscosity-enhancing additives, moisture barriers in packaging films, wet-end additives in papermaking and emulsifying agents in cosmetics and food processing (Deutschmann & Dekker, 2012; Ebringerová, 2005; Höije, Grondahl, Tommeraas, & Gatenholm, 2005; Oliveira et al., 2010).

The O-acetyl-4-O-methyl glucuronoxylans present in hardwoods are substituted by 4-O-methyl glucuronic acid (MeGlcA) and acetyl groups at a ratio of 1:7:10 to xylose (Magaton,

Colodette, Pilo-Veloso, & Gomide, 2011; Spiridon & Popa, 2008; Teleman, Tenkanen, Jacobs, & Dahlman, 2002). The predominant hemicellulose in grasses is arabinoglucuronoxylan, which is substituted with arabinose side chains at a ratio of 1.3:2:10 to MeGlcA and xylose (Evtuguin, Tomas, Silva, & Neto, 2003; Izydorczyk & Dexter, 2008; Westbye, Köhnke, Glasser, & Gatenholm, 2007). The degree of polymerization, degree of substitution and type of substituents affect the water solubility and aggregation mechanisms of these xylans (Cardona, Quintero, & Paz, 2010; Fincher & Stone, 1986; Linder, Bergman, Bodin, & Gatenholm, 2003). Extracted polymers tend to precipitate when there are large, non-substituted areas on the backbone chain (Linder et al., 2003). Therefore, the viscosities and water-solubility of biopolymers can be manipulated by changing the substitution patterns on longer-chain polymers (Chimphango, van Zyl, & Görgens, 2012a; Saake, Kruse, & Puls, 2001; Westbye et al., 2007). This effect was observed with beechwood xylan at concentrations above 5% (w/v) (Ebringerová, 2005).

The water solubility and self-aggregation of biopolymers are also affected by interactions between the residual lignin present in these carbohydrates, and the MeGlcA and arabinose side groups on the backbone (Ebringerová, 2005; Westbye et al., 2007). However, such lignin interactions with the side groups may limit the accessibility of the substrate to enzymatic modifications, while also limiting possible applications. For example, the application of

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extracted xylan as a strength additive to pulp may increase the pulp Kappa number, due to high lignin content, thus increasing chemical requirements for bleaching.

Higher degrees of polymerization (DP) and less backbone substitution of carbohydrates are required for precipitation (Kabel, van den Borne, Vincken, Voragen, & Schols, 2007). Precipitation is usually achieved at DP values between 80 and 200 (Spiridon & Popa, 2008), although precipitation has been observed with DP as low as 10 (Chimphango, van Zyl, & Görgens, 2012b). The propensity for extracted xylan to precipitate into insoluble aggregates or hydrogels, varies with lignocellulose type, extraction methods and modifications applied (Ebringerová & Heinze, 2000; Fincher & Stone, 1986). Extraction of xylan by alkaline methods removes the acetyl groups from the *O*-acetyl-4-*O*-methyl- β -D-glucuronoxylan backbone, but preserves the MeGlcA sidegroups. For some lignocelluloses the removal of acetyl groups by alkaline extraction methods may induce precipitation (Gabriellii, Gatenholm, Glasser, Jain & Kenne, 2000; Tenkanen & Siika-aho, 2000).

Alternatively, the extraction of xylan hemicelluloses by DMSO preserves both the acetyl groups and MeGlcA, thus rendering the product highly soluble in water (Ebringerová & Heinze, 2000). The functional properties of extracted hemicelluloses for applications such as additives, coating materials, entrapment matrix, emulsifiers, dietary fibers, antioxidants and feedstock for biofuel production can thus be modified by extraction methods. For example, substituted xylan with degree of polymerization (DP) of at least 4 or more can be obtained from alkaline extraction methods. These carbohydrates can be debranched to precipitate, for application as surface modifiers for cellulosic materials (Ebringerová, 2005; Kabel et al., 2007) or to form hydrogels as entrapment matrices for slow delivery of bioactive substances (Chimphango et al., 2012a).

Precipitation of xylan hemicelluloses into hydrogels, with varying morphological properties, can be achieved by physical, chemical and enzymatic methods (Chimphango, Rose, van Zyl, & Görgens, 2012; Haimer et al., 2010; Ren & Sun, 2010; Tanodekaew, Channasanon, & Uppanan, 2006). Hydrogels were obtained by selective removal of arabinose from extracted arabinoglucuronoxylans using the α -L-arabinofuranosidase enzyme from family GH54 (AbfB), produced by expression in *Aspergillus niger* (Chimphango et al., 2012a). Similar precipitation was achieved by removal of MeGlcA from polymeric glucuronoxylans by the α -D-glucuronidase enzyme from family GH115 (Agu), expressed in *Scheffersomyces stipites* (Kolenova, Ryabova, Vrsanska, & Biely, 2010). The side-chain removal activities of AbfB and Agu enzymes are not limited to oligomeric xylans, as observed for the majority of α -D-glucuronidases and α -L-arabinofuranosidase enzymes (De Vries, Poulsen, Madrid, & Visser, 1998; Tenkanen & Siika-aho, 2000). The latter enzymes can only remove MeGlcA and arabinose sidegroups from glucuronoxylans and arabinoglucuronoxylans in the presence of xylanase to break the backbone of the carbohydrate, resulting in oligomers that remain water-soluble. However, this particular α -L-arabinofuranosidase (AbfB) removed sidegroups in the absence of xylanase, causing precipitation of various xylan substrates (Chimphango et al., 2012a). Similarly, this particular α -D-glucuronidase (Agu) removed up to 75% of the available MeGlcA of beechwood glucuronoxylan in the absence of xylanase, resulting in increased viscosity and precipitation (Ryabova, Vrsanska, Kaneko, van Zyl, & Biely, 2009). Such enzymatic precipitation is improved for extracted hemicelluloses with lower lignin content. The residual lignin may be covalently bound to hemicellulose structures, forming lignin-carbohydrate complexes (LCCs) that limit substrate accessibility to enzymes (Jeffries, 1994).

The present study identified the preferred dosages of the α -D-glucuronidase (Agu1B), either by itself or in combination with α -L-arabinofuranosidase AbfB, for the precipitation of water-soluble xylans through the release of MeGlcA and arabinose

side-chains. The xylan substrates from beechwood (model), *Eucalyptus grandis* and sugarcane bagasse, extracted with mild alkaline methods of Höije et al. (2005) and Pinto, Evtuguin, and Neto (2005) were included in the study (Chimphango et al., 2012b). Both extraction methods preserved the MeGlcA and arabinose side groups of polymeric xylan, thus ensuring water-solubility. The effects of substrate concentration, initial water solubility and lignin content on sidegroup removal and precipitation, were also investigated.

2. Materials and methods

2.1. Materials

Beechwood (*Fagus sylvatica*) xylan was obtained from Sigma-Aldrich. *E. grandis* chips supplied by Sappi Ngodwana pulp and paper mill (South Africa), and sugarcane bagasse (*Saccharum officinarum* L.) supplied by TSB Sugar (Mpumalanga, South Africa), were used as feedstocks for xylan extraction.

Analytical grade sugars: glucose, cellobiose, arabinose, xylose, xylobiose, rhamnose, galactose, mannose and glucuronic acid, obtained from Sigma-Aldrich, at purity levels above 99% were used as standards in sugar analysis. The enzymes used were (1) α -L-arabinofuranosidase (EC 3.2.1.55) (AbfB) with a volumetric activity of 1.1 U/ml on *p*-nitrophenyl arabinofuranoside (*p*-NPA) (Sigma) (Chimphango, Rose, et al., 2012) produced from recombinant *A. niger* and (2) crude recombinant α -D-glucuronidases (EC 3.2.1.139) (Agu1B) supernatant, endo- β -xylanase free produced from *Saccharomyces cerevisiae* (Anane, van Rensburg, & Görgens, 2013), at concentrations of 462 mg/L, respectively. The Agu1B was expressed from *S. cerevisiae* in which *Scheffersomyces stipites* CBS 6054 α -D-glucuronidase was integrated into the *S. cerevisiae* agu1B genome that showed 100% homology with the published *S. stipites* CBS 6054 gene sequence (La Grange, unpublished results).

2.2. Xylan extraction

The *E. grandis* chips were pre-milled using a Condux-Werk Wolfgang bei Hanau mill, to an average particle size of 10 mm in length. The *E. grandis* and sugarcane bagasse were milled further in a Retsch ZM 200 mill and sieved in a Vibratory Shaker Retsch AS200 to recover particle size below 425 μ m (40 mesh) but above 250 μ m (60 mesh) for chemical composition analysis. The samples that retained on 425 μ m (40 mesh) sieve were used for xylan extraction.

The xylans were extracted using two mild alkali methods, described by Höije et al. (2005) and Pinto et al. (2005), referred to as Höije and Pinto methods, respectively. The Höije method was developed for barley husks (grasses) and applied here for extraction of xylan from *E. grandis* and sugarcane bagasse, whereas the Pinto method was developed for hardwoods, and thus only applied to *E. grandis* extractions. Höije xylans were purified by dialysis (molecular weight cut off, 12–14 kDa) in water for 2 days at 20 °C. Pinto xylans were recovered by precipitation in an ethanol-methanol-water mixture at a ratio of 4:5:1. The extracts from both methods were subsequently freeze dried.

2.3. Assessing solubility of the extracted xylan

The water-solubility of extracted xylans was tested at concentrations up to 10% (w/v), by dissolving in water with agitation at 60 °C for approximately 6 h. Xylan solutions were subsequently centrifuged for 20 min at 23,600 \times g, and the phenol-sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) used to determine the xylan content in the water-soluble fraction. The soluble fraction (200 μ L) was mixed with 200 μ L of a 5% phenol solution in a small test tube with vortex. Furthermore, 800 μ L of concentrated sulfuric acid was added with vortex and the optical density

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