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Enzymatically debranched xylans in graft copolymerization.

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ABSTRACT

Wheat arabinoxylan was treated with two α -arabinofuranosidases exhibiting different mode of action to create three different polymeric substrates. These three substrate preparations were characterized by xylopyranose backbone sugars that are: (1) singly substituted by arabinose at C2 or C3, (2) doubly substituted by arabinose at C2 and C3, and (3) largely unsubstituted. All xylan preparations were grafted with glycidyl methacrylate using cerium ammonium nitrate and then evaluated in terms of graft yield and adsorption to cellulose surfaces. The highest graft yield was observed for the xylan preparation characterized by a largely unsubstituted xylopyranose backbone. Furthermore, QCM-D analyses revealed that grafted xylans exhibited a two-stage desorption pattern, which was not seen with the ungrafted xylans and was consistent with increased water sorption. Accordingly, this study demonstrates the potential of arabinofuranosidases to increase the yield and influence the viscoelastic properties of grafted xylans used as bio-based cellulose coatings.

1 INTRODUCTION

2 Hemicelluloses comprise 20–30% of the total mass of annual and perennial plants.¹ The main
3 hemicellulose fraction in hardwood trees is acetylated glucuronoxylan, which consists of a β -
4 (1→4)-linked D-xylopyranosyl (Xylp) backbone that is partially substituted at O-2 positions with
5 α -D-glucuronic acid (GlcAp) or 4-O-methyl GlcAp (MeGlcAp), and at O-2 positions and/or O-3
6 positions with acetyl substituents.^{2–4} Xylan from conifers (softwoods) and vegetative tissue of
7 monocots (e.g., cereals and grasses) similarly comprise a β -(1→4)-linked Xylp backbone with
8 varying MeGlcAp and α -L-arabinofuranosyl (Araf) contents,² whereas endosperm cell walls of
9 cereals largely comprise arabinoxylan that lack MeGlcAp substituents.⁵

10 The physicochemical characteristics and functional properties of xylans depend on their
11 molecular mass, branching and macroscopic structure. Arabinoxylans are known to exhibit high
12 intrinsic viscosity that is affected by the fraction of di-substituted over mono-substituted Xylp
13 substituents.⁶ It is also reported that arabinoxylan solubility is dependent on both molecular weight
14 and type and arrangement of xylan side groups.⁶

15 Due to the functional properties and wide availability of xylans, a broad range of applications
16 have already been considered, including use as food additives, hydrogels and aerogels for nutrient
17 and drug delivery, wet-strength additives to cellulose pulp, and films for wound dressing and
18 packaging materials.^{6–12} Barrier properties, namely oxygen, grease, and water vapor permeability,
19 along with mechanical properties are especially important to these applications.^{7,8,12–20} Selective
20 extraction of xylan-rich fractions from wood hydrolysates can yield xylan films and coatings with
21 competitive oxygen barrier properties.^{9,21} Additional performance attributes, such as water vapour
22 transmission, can also be enhanced through use of bio-based additives including lipids,¹³
23 sorbitol,^{18,22} glycerol,¹⁴ carboxymethyl cellulose and chitosan,²³ common mineral additives such

as layered silicates were also shown to significantly improve oxygen and water barrier properties of xylan films.²⁴ Alternatively, the hydrophilic nature xylans can be controlled through direct chemical modification of the xylan backbone, for example through esterification, etherification, as well as oxidative or reductive coupling, to introduce hydrophobic groups into the xylan backbone.^{7,12,25,26} Farhat et al provide a recent review of process options and modification pathways to generating water-resistant hemicellulosic materials.¹²

Accessory hemicellulases can also be used to modify xylan branching structure, and thereby alter xylan solubility, rheology, and adherence to other biopolymers. In particular, α -L-arabinofuranosidases, α -glucuronidases, acetyl xylan esterases, feruloyl esterases, as well as glucuronoyl esterases, target the branching substituents present in different xylan types.²⁷ Considering α -L-arabinofuranosidases in more detail, glycoside hydrolase (GH) family GH62 comprise enzymes that act only on mono-substituted Xylp,²⁸ and are termed AXH-m arabinoxylan arabinofuranohydrolases. Alternatively, family GH43 includes arabinofuranosidases with ability to remove Ara_f substituents from the O-3 of position of doubly substituted Xylp (i.e., AXHd3 activity).²⁹ Reducing Ara_f content in arabinoxylans was previously shown to increase the crystallinity of resulting films, which can improve water barrier properties.^{9,16,17,30,31} Moreover, by using a combination of arabinofuranosidases with distinct regioselectivity, both Ara_f to Xylp ratio as well as Ara_f position were shown to impact the water-solubility of resulting arabinoxylans and the crystallinity of corresponding films.^{16,31–33} In particular, Heikkinen et al.³³ showed that the removal of α -L-Ara_f residues (1→3)-linked to monosubstituted β -D-Xylp sugars had the greatest affect on the barrier properties of arabinoxylan based films, while Köhnke et al.³² showed that a similar treatment increased xylan adsorption to cellulose.

1 Previous studies have investigated xylan adsorption to cellulose through empirical and
2 computational analyses. For example, adsorption isotherms and QCM-D analyses were previously
3 performed to compare the association of various xylan sources (as well as other hemicelluloses)
4 to microcrystalline cellulose (Avicel), bacterial cellulose, and nano-fibrillated cellulose.^{34–36}
5 Furthermore, molecular dynamics simulations of xylan adsorption to cellulose have been
6 performed, which considered xylns from the secondary cell walls of the gymnosperm lineages
7 that are substituted with both Araf and MeGlcAp, and of xylns from Gnetophyta that contain
8 acetyl groups instead of Araf substitutions.³⁷ Both the empirical and computational analyses
9 confirmed that high molecular weight and unsubstituted xylns show highest affinity to cellulose;
10 ^{34–36} molecular dynamics simulations also predict impacts of substituent distribution on the
11 stability of cellulose associations.^{37,38}

12 In the present study, two different arabinofuranosidases were used to selectively remove distinct
13 α -L-Araf substituents from wheat arabinoxylan (WAX) prior to graft copolymerization with
14 glycidyl methacrylate (GMA). GMA was selected as the monomer for the grafting experiments as
15 it was previously shown to react well with polysaccharides, including hemicelluloses,¹⁸ and
16 because the epoxy groups introduced through GMA grafting creates sites that can be further
17 chemically modified depending on the end use.³⁹ Whereas the GH62 α -L-arabinofuranosidase
18 from *Streptomyces thermoviolaceus* (SthAbf62A) was selected to remove α -L-Araf residues
19 linked to mono-substituted β -D-Xylp sugars,⁴⁰ the GH43 α -L-arabinofuranosidase from
20 *Bifidobacterium* sp. (AXHd3) was selected to remove α -L-Araf residues (1→3)-linked to di-
21 substituted β -D-Xylp.²⁹ The effect of enzyme pretreatment on xylan co-polymer solubility, yield
22 and adsorption to cellulose was then evaluated.

1 **EXPERIMENTAL**

2 **Materials**

3 Wheat arabinoxylan (P-WAXYL; Lot 120601a and 70502a) was purchased from Megazyme
4 (Araf:Xylp ratio 38:62). The α -L-arabinofuranosidase from *Bifidobacterium* sp. (glycoside
5 hydrolase (GH) family 43; E-AFAM2; AXHd3); endo-1,4- β -D-xylanase from *Neocallimastix*
6 *patriciarum* (GH11; E-XYLNP), and β -xylosidase from *Selenomonas ruminantium* (GH43; E-
7 BXSr-3KU) were also purchased from Megazyme. The GH62 α -L-arabinofuranosidases from
8 *Streptomyces thermoviolaceus* (SthAbf62A and SthAbf62A_E213A) were provided by the
9 University of Toronto.⁴⁰ Glycidyl methacrylate (GMA) and cerium ammonium nitrate (CAN) as
10 well as 14,000 Da dialysis membrane (D9402) were purchased from Sigma-Aldrich. Ethanol was
11 procured from Altia and acetone from VWR.

12 **Enzymatic preparation of wheat arabinoxylan prior to grafting**

13 Wheat arabinoxylan (WAX) was separately treated with AXHd3, SthAbf62A, and AXHd3 +
14 SthAbf62A, to generate three xylan samples, namely WAX_AXHd3, WAX_SthAbf62A, and
15 WAX_AXHd3_SthAbf62A, respectively (Figure 1). WAX_AXHd3, is characterized by β -D-
16 Xylp backbone sugars that are singly substituted by α -L-Araf(1 $\rightarrow$ 3) and α -L-Araf(1 $\rightarrow$ 2) residues.
17 In WAX_SthAbf62A, β -D-Xylp backbone sugars are doubly substituted by α -L-Araf(1 $\rightarrow$ 3) and
18 α -L-Araf(1 $\rightarrow$ 2) residues. Finally, WAX_AXHd3_SthAbf62A is characterized by a largely
19 unsubstituted β -D-Xylp backbone, and was produced by first creating WAX-AXHd3 followed by
20 treatment with SthAbf62A.

Figure 1. Schematic representation of WAX prior to enzyme treatment, after treatment with AXHd3, after treatment with SthAbf62A, and after treatment with AXHd3 and SthAbf62A.

Enzymatic xylan modifications

To prepare each of the above described xylan samples, a 1 % WAX solution was suspended in 30 mL 50 mM sodium phosphate buffer (pH 6.5); to generate WAX_AXHd3, 375 μ g (25 U) of AXHd3 were added to the solution, which was then incubated in a shaking water bath (80 rpm) at 40 °C for 24 h before adding additional 150 μ g (10 U) of AXHd3 and continuing the incubation for another 24 h. WAX_SthAbf62A was prepared as described above, except that AXHd3 was replaced by 300 μ g of SthAbf62A. Finally, WAX_AXHd3_SthAbf62A was prepared by treating WAX_AXHd3 with 300 μ g of SthAbf62A. After 24 h, an additional 300 μ g of SthAbf62A was

added and the reaction was incubated for another 24 h. Control treatments used buffer alone or the SthAbf62A E213A catalytic mutant.

Following enzyme treatment, xylan preparations were boiled for 10 min to denature the applied enzymes, and precipitated using 60 % ethanol. The xylan precipitate was recovered by centrifugation for 30 min at 15,000 rpm (SS-34 rotor, Sorvall centrifuge) and then suspended in up 50 mL milli-Q water. The ethanol precipitation procedure⁴¹ was then repeated prior to freezing in liquid nitrogen and lyophilization. In cases where the ash content was >15 %, dialysis was performed using a 14,000 Da dialysis membrane against milli-Q water prior to lyophilization.

Quantification of released arabinose

The extent of Araf released to produce WAX_AXHd3, WAX_SthAbf62A, and WAX_AXHd3_SthAbf62A was quantified using the L-Arabinose/D-Galactose Assay Kit (K-ARGA; Megazyme). Araf release was further quantified by high performance liquid chromatography (HPLC) using Aminex HPX-87P (Bio-RAD) column equipped with a de-ashing Refill Cartridge (Bio-Rad) pre-column kept at 85 °C; analytes were eluted with milli-Q water at a flow rate of 0.4 mL/min. L-Arabinose was used as the standard (0.1- 0.8 g/L) and eluted with a retention time of 24.8 min.

¹H NMR analyses

WAX samples before and after enzyme treatment were analyzed by ¹H NMR to confirm selective removal of α-L-Araf (1→3) from di-substituted Xylp backbone residues and α-L-Araf (1→2) from mono-substituted Xylp backbone residues by SthAbf62A and AXHd3, respectively. To sharpen corresponding diagnostic peaks, an endo-1,4-β-D-xylanase from *N. patriciarum* (E-XYLNP; GH11, Megazyme) was used to hydrolyze each xylan sample prior to analysis.

Specifically, 0.6 mL of 1 % xylan solutions were precipitated by adding 1.3 mL of 99.5 % ethanol and centrifugation at 15,000 rpm for 10 min. The pellet was suspended in 0.6 mL of water and again precipitated with 1.2 mL of 99.5 % ethanol. The pellet was suspended in 0.6 mL of 10 mM (3-(N-morpholino) propanesulfonic acid) (MOPS) buffer (pH 7.0) and then treated with 2.5 U of E-XYLNP at 50 °C. After 24 h, an additional 2.5 U E-XYLNP was added and the reaction was incubated for another 24 h. Reaction mixtures were subsequently boiled for 15 min to inactivate the endoxylanase, then lyophilized and dissolved in 0.6 mL of D2O twice.

NMR spectra were subsequently obtained in D2O at 25 °C using a Bruker Avance III 400 MHz spectrometer, with a scan number of 16, pulse angle of 30°, and relaxation delay of 12 s. The data were processed using TopSpin 3.0 software. Previously assigned peaks in ¹H NMR spectra of arabinoxylans were used for data interpretation.^{16,32,40,42–45} The methylene protons at position 2 of MOPS (2.035 ppm) were used as the internal standard. The degree of Araf substitution was determined from ¹H NMR spectra by using the ratio between the standard peak and the anomeric proton peaks at approximately (5.27-5.35 ppm) for L-Araf (1→3) in mono-substituted Xylp, (5.17-5.23) for α-L-Araf (1→2) in mono- and di-substituted Xylp, and (5.13-5.17 ppm) for α-L-Araf (1→3) in di-substituted Xylp. The ratio was then compared to an unmodified WAX sample with known arabinose content to obtain the degree of substitution (DS) of each sample.

Particle size and turbidity

The particle size distribution of each xylan preparation was analyzed by dynamic light scattering (DLS) using a Zetasizer Nano instrument (Malvern Instruments, UK). The light scattering was measured at xylan concentrations between 0.1 and 1.0 g/L to ensure sufficient scattering (> 100 kcps) while preventing multiple scattering (one photon being scattered by several particles).

Turbidity of the solutions was determined at 0.5 g xylan/L by measuring light transmittance at 700 nm wavelength, using a Helios β UV/VIS spectrometer (Unicam, UK).

Thermogravimetric analysis (TGA)

The moisture and ash content of each xylan preparation was measured by using a TGA Q500 instrument (TA Instruments, New Castle, DE). Approximately 5 mg sample was kept isothermal for 20 min first at 105 °C in a nitrogen atmosphere and then at 650 °C in an oxygen atmosphere. Dry weight and ash content were determined at the end of the isothermal periods.

Graft copolymerization

Xylans were grafted by a method described by Littunen et al.²⁶ Briefly, xylan (0.20 g) was dissolved in deionized milli-Q water (95 mL) in a round-bottom flask. Nitrogen was bubbled through the solution to remove dissolved oxygen prior to the synthesis. Dissolved CAN (0.10 g, 0.18 mmol) was injected, and the flask was heated with an oil bath to 35 °C. After 15 min, GMA (0.57 g, 4.0 mmol) was injected, and the reaction was carried out for 60 min; the subsequent separation procedure is summarized in Scheme 1. Briefly, following grafting, the reaction medium was centrifuged for 30 min at 4,000 rpm and the separated solids were washed four times with acetone, leaving the non-soluble copolymer (NS). The supernatant was precipitated with acetone using 1:4 water-acetone ratio to obtain the water-soluble copolymer (WS). The remaining acetone fraction could contain partially acetone/water-soluble soluble copolymer (AS) as well as the GMA homopolymer (HP). To separate these two products, the acetone fraction was evaporated to remove the residual water, re-dissolved in approximately 10 mL of acetone, and then centrifuged for 10 min at 4,000 rpm. The pellet comprised the copolymer that was soluble in 80 % acetone, whereas the supernatant would contain the homopolymer (HP) soluble in 100 % acetone. To quantify the

HP formed in each reaction, the corresponding fraction was precipitated with 90 % methanol and dried in vacuum at 30 °C overnight and weighed. Each experiment was repeated at least once.

Since the xylans were known to contain variable amounts of moisture and inorganic compounds, these were determined by TGA and subtracted from the total mass. The relative amount of grafted polymer (graft yield, G%) was calculated using formula 1.

$$G\% = \frac{m_{NS} + m_{WS} - m_X}{m_X} \times 100\% \quad (1)$$

where m_{NS} is the mass of the insoluble product, m_{WS} is the mass of the water-soluble product, and m_X is the corrected mass of xylan.

Since some homopolymerization was expected, the selectivity of the polymerization reaction was evaluated by calculating the copolymer fraction of all formed polymer (i.e. the graft efficiency (GE%)) using formula 2.

$$GE\% = \frac{m_{NS} + m_{WS} - m_X}{m_{NS} + m_{WS} - m_X + m_{HP}} \times 100\% \quad (2)$$

where m_{HP} is the mass of the homopolymer.

The grafting was verified by Fourier transform infrared (FTIR) spectroscopy. Infrared spectra of the starting materials and all products were recorded with a Nicolet 750 Magna device as an attenuated total reflection (ATR) measurement to confirm the presence of new functional groups after modification.

Scheme 1. Product separation and purification diagram.

TLC analyses to quantify free sugars in grafted xylans.

The Araf to Xylp ratio of grafted and ungrafted xylans was determined to predict the grafted position of the GMA copolymer. Approximately 1 mg of each xylan sample in 120 μ L of 5 mM MES (2-(N-morpholino) ethanesulfonic acid sodium) buffer (pH 6.0) was incubated with 2.3 μ g of SthAbf62A, 1.35 μ g AXHd3, 0.5 μ g E-XYLNP and 0.075 μ g E-BXSR-3KU at 40° C for 24 h. BSA was also added to each reaction mixture to a final concentration of 0.17 mg/mL.

Following enzymatic digestion, 1-12 μ L of each reaction (depending on product intensity), were dried onto a silica plate for thin layer chromatography (TLC) analysis (Silica Gel 60 aluminum sheet; Merck; VWR); 1 μ L of 1 % cellobiose solution was added to each aliquot to serve as an internal reference. Reaction products were separated on the TLC plate using 1-butanol : ethanol : water (3:2:2) for 5 h, stained using the orcinol staining solution. The components of staining solution in final 500 ml were as follows: 380 ml of 99,5% ethanol, 50 ml of 95-97% H₂SO₄, 1 g orcinol (3,5-dihydroxytoluol or 5-methyl resorcinol), and brought to 500 ml using milli-Q water.

TLC spots were integrated and analyzed with Image Lab 5.1 software. Samples of arabinose (0.2%), xylose (0.2%) and cellobiose (0.1 %) were also run in parallel to track product migration and intensity. Each fraction of the grafted xylan (i.e., water-soluble, water insoluble; acetone/water-soluble soluble) were analyzed separately. The resulting Araf:Xylp ratios were then

normalized to the percent of the corresponding fraction in the total grafted xylan. The quantity of Araf and Xylp released from both grafted and untreated WAX was also verified by HPLC, as described above

Adsorption of xylans to cellulose

The adsorption of enzyme treated WAX on regenerated cellulose was studied before and after graft copolymerization. The model surfaces were prepared by spin-coating SiO₂ sensors (Biolin Scientific, Sweden) with trimethylsilyl cellulose (TMSC, DS 2.3⁴⁶) dissolved in toluene (10 g L⁻¹), and the TMSC surfaces were then regenerated using 10 % HCl vapor. Measurements by quartz crystal microbalance with dissipation (QCM-D) were performed with a Q-Sense (Västra Frölunda, Sweden) E4 instrument with controlled flow. The water-soluble copolymer fractions were used to represent the grafted xylans. The samples were dissolved in deionized water at a concentration of 0.5 g L⁻¹, passed through 0.45 µm syringe filters, and pumped at constant rate of 0.1 mL/min through the measurement chambers. The polysaccharide solutions were added after first acquiring a stable baseline with ultrapure (milli-Q) water. The instrument recorded changes in both resonance frequency and dissipation of the sensor. The fundamental resonance frequency was 5 MHz, and the overtones at 15, 25, 35, 45, 55, and 65 MHz were recorded. Adsorbed masses were calculated from the 7th overtones with the Sauerbrey equation (3). Dissipation (D) is defined by equation (4) and is proportional to the ratio between dissipated and stored oscillation energy.⁴⁷

$$\Delta m = \frac{C\Delta f}{n}, \quad (3)$$

where Δm is the adsorbed mass, C is the device sensitivity constant (0.177 mg s m⁻², reported by manufacturer), Δf is the change in oscillation frequency, and n is the overtone number.

$$D = \frac{E_{diss}}{2\pi E_{stored}}, \quad (4)$$

where E_{diss} is the energy dissipated during one oscillation E_{stored} is the energy stored.

RESULTS and DISCUSSION

Characterization of ungrafted xylan preparations

We investigated the potential to control the water solubility, graft yield, and adsorption properties of xylan-GMA copolymers through pre-enzyme treatments that selectively removed α -L-Araf branching substituents prior to the graft polymerization. Specifically, AXHd3 was used to remove α -L-Araf (1 $\rightarrow$ 3) from di-substituted Xylp backbone residues, thereby generating WAX_AXHd3 (**Figure 1**). Alternatively, SthAbf62A targeted α -L-Araf (1 $\rightarrow$ 3) in mono-substituted Xylp, generating WAX_SthAbf62A. Finally, AXHd3 and SthAbf62A were used together to generate WAX_AXHd3_SthAbf62A.

The extent and position of Araf removal was confirmed by measuring both the released Araf (**Figure 2**) and analyzing the residual xylan by ^1H NMR (**Figures 3, S1**). The analysis of released Araf confirmed that approximately 20 % of total Araf subunits were released by AXHd3, 30 % of total Araf were released by SthAbf62A, and 70 % of total Araf were removed when combining AXHd3 and SthAbf62A activities. Consistent with previous studies,⁴⁸ ^1H NMR analyses of residual xylans indicated that approximately 30 % of α -L-Araf (1 $\rightarrow$ 3) on di-substituted Xylp remained in WAX_AXHd3, whereas approximately 20 % of α -L-Araf (1 $\rightarrow$ 3) from mono-substituted Xylp remained in WAX_SthAbf62A. Similarly, WAX_AXHd3_SthAbf62A retained approximately 40 % of di-substituted Xylp and 30 % mono-substituted Xylp.

Figure 2. Ara β remaining in enzyme treated and control wheat arabinoxylan samples determined by ^1H NMR (NMR) (left x-axis; shown as bars) and Ara β released as measured by the L-Arabinose Assay Kit (right x-axis; shown as square points connected by a solid line). m(1→3) refers to L-Ara β (1→3) in mono-substituted Xylp; d(1→3) refers to α -L-Ara β (1→3) in di-substituted Xylp; m/d(1→2) refers to α -L-Ara β (1→2) in di-substituted Xylp.

Figure 3. Anomeric signals in the ^1H NMR spectra of each arabinoxylan preparations in D_2O . $m(1\rightarrow3)$ refers to $\text{L-Araf}(1\rightarrow3)$ in mono-substituted Xylp (5.27-5.35 ppm); $m/d(1\rightarrow2)$ refers to $\alpha\text{-L-Araf}(1\rightarrow2)$ in mono- and di-substituted Xylp (5.17-5.23); $d(1\rightarrow3)$ refers to $\alpha\text{-L-Araf}(1\rightarrow3)$ in di-substituted Xylp (5.13-5.17 ppm).

Selective removal of Araf substituents was expected to promote the aggregation of resulting xylan preparations, impacting their water solubility and chemical reactivity. Therefore, DLS was used to characterize the water solubility and particle size distribution of each xylan sample. Since decreased solubility would likely increase sample turbidity, visible light transmittance was also determined. Whereas the particle size of WAX and WAX_AXHd3 were similar, the particle size of WAX_SthAbf62A and $\text{WAX_AXHd3_SthAbf62A}$ were increased by 1.5 and 4 times, respectively; the double enzyme treatment also dramatically increased sample turbidity (**Figure 4**). Since particle sizes were beyond the reliable range of DLS measurements, absolute particles sizes could not be determined; however, the results were consistent with turbidity measurements and confirmed increasing aggregation with increasing, unsubstituted Xylp .

Figure 4. Average particle size (bars) and light transmittance at 700 nm (line) of ungrafted xylans.

Graft copolymerization

Enzymatically treated WAX samples were graft copolymerized with GMA in aqueous solution using identical conditions. Three product fractions were then separated based on their solubility, and the overall graft yield and efficiency were determined gravimetrically.

Partial removal of Araf increased the graft yield (**Figure 5**), producing more copolymer with all enzyme-treated xylans. The most significant increase was observed for WAX_SthAbf62A, which was also characterized by comparatively high particle size. As previously observed, the lower water solubility of WAX_SthAbf62A and WAX_AXHd3_SthAbf62A improved the product yield by facilitating copolymer separation.²⁶ However, the lower water solubility of these xylan preparations also impacted their dispersion in the reaction mixture, leading to higher variance in product yields. Predictably, the water solubility of each WAX substrate was also reflected by the solubility of corresponding graft copolymers. Whereas WAX and WAX_AXHd3 mainly produced

a water soluble copolymer, the fraction of water insoluble products increased up to 14 times for WAX_SthAbf62A, and up to 26 times for WAX_AXHd3_SthAbf62A.

Figure 5. Yield by product fraction relative to the mass of xylan.

FTIR analysis of water soluble and water insoluble fractions showed characteristic absorption peaks for both xylan and poly(glycidyl methacrylate) (PGMA); the carbonyl signal of PGMA (1725 cm^{-1}) was also present in the acetone/water-soluble soluble and homopolymer fractions (**Figure 6**). The characteristic carbohydrate hydroxyl group peak at 1025 cm^{-1} was weak in the acetone/water-soluble soluble fraction, which was also characterized by a strong absorption band near 1660 cm^{-1} corresponding to carbon-carbon double bonds. This observation suggests that a substantial part of the acetone/water-soluble soluble copolymer was formed via an acid-catalyzed ring-opening reaction of free GMA with heavily grafted or short-chain xylan copolymers, rather than radical polymerization.

Figure 6. FTIR spectra of different product fractions. Abbreviations: WAX, ungrafted xylan; NS, non-soluble copolymer; WS, water-soluble copolymer; AS, acetone/water-soluble soluble copolymer; HP, homopolymer (PGMA).

TLC analysis of free sugars in grafted xylan samples

To determine whether the cerium oxidation and subsequent grafting preferentially targeted particular Araf and Xylp positions, AXHd3 and SthAbf62A along with endo-1,4- β -D-xylanase (E-XYLNP) and β -D-xylosidase (E-BXSR-3KU) were used to treat and then analyze the ungrafted and grafted WAX samples. More specifically, each of the four xylan-GMA copolymers generated in this study were simultaneously treated with AXHd3, SthAbf62A, β -D-xylanase and β -xylosidase. Ungrafted xylan was used as reference. It was hypothesized that if preferential grafting of GMA to either Araf or Xylp had occurred, then the ratio of Araf:Xylp would differ for a given xylan sample before and after the grafting reaction, assuming that enzyme activity is hindered by the graft co-polymer.

TLC analyses of enzymatically released Araf and Xylp indicated that GMA was grafted equally well to Xylp and Araf residues in each xylan sample, also confirming that cerium ion oxidation did not discriminate between main chain and branching sugars (Figure 7). Notably, sugars were also released from the acetone/water-soluble fraction, supporting the assignment of this fraction as a copolymer rather than homopolymer.

Figure 7. Araf:Xylp ratio calculated from TLC analyses. Ungrafted wheat arabinoxylan (WAX) served as reference and comprised only one fraction (black square symbols), whereas the grafted xylan (WAXg) contained three differently soluble fractions, namely insoluble, water-soluble, and acetone/water-soluble (values represented as stacked bars).

Adsorption of xylans to cellulose

Effects of substitution pattern and polymer grafting to the adsorption behavior of WAX to regenerated cellulose were studied by QCM-D. Hemicellulose adsorption to cellulose fibers can improve their tensile strength, and offers a pathway to their surface-selective functionalization by

using derivatized xylan.^{30,32,49} However, the stability of xylan adsorption to cellulose is dependent on xylan molecular weight and substitution pattern,^{32,34} xylan solubility in water, and the colloidal stability of the xylan molecules.⁴⁰ Modification of the Araf substitution patterns of WAX by using appropriate enzymes has also been reported to increase its binding capacity to cellulose and enable adjustment of the viscoelastic properties of the adsorbed layer.³²

The slope of change in dissipation (ΔD) versus change in frequency (Δf) reflects the viscoelastic properties of the adsorbed layer, where a higher slope indicates a more viscous surface layer. Accordingly, the $\Delta D/\Delta f$ data plotted in **Figure 8** distinguished untreated WAX and WAX_AXHd3, from WAX_SthAbf62A and WAX_AXHd3_SthAbf62A, where the latter xylan preparations formed more densely packed and less hydrated adsorption layers (**Figure 8**).

Figure 8. Change in dissipation versus frequency during adsorption of ungrafted xylans. The black diagonal shows the border between elastic and viscoelastic domains.

Roughly halfway to maximum adsorption, the curves of all xylan types exhibited an inflection point, indicating slightly more elastic character of the outer adsorbed layer. Several earlier studies have noted similar phenomenon and some have attributed it to the formation of a secondary adlayer

1 based on xylan-xylan attraction, after saturation of the cellulose surface.^{32,35,50} In all cases,
2 approximately 70% of the xylan remained adsorbed to the cellulose surface following extensive
3 washing with water (**Table S1**). Moreover, desorption curves overlapped with corresponding
4 adsorption curves, indicating that the viscoelastic properties of the adsorbed layers were not
5 changed during rinsing.

6 Following grafting of WAX, in our study, between 30-50% of the xylan samples, remained
7 adsorbed to the cellulose surface, regardless of the enzyme treatment (**Figure 9A, Table S1**). The
8 $\Delta D/\Delta f$ data plotted in **Figure 9** also revealed an apparent two-stage adsorption/desorption
9 phenomenon (**Figure 9B**). More specifically, initial adsorption proceeded quickly producing a
10 rather rigid (elastic) layer. This was followed by the formation of a more swollen top layer, as
11 further adsorption abated. The resulting outermost layer shed rapidly during rinsing; however, the
12 underlying xylan coating was more stable and appeared to require water adsorption and/or
13 rearrangements leading to increased dissipation prior to detachment from the cellulose surface.

14 The difference in $\Delta D/\Delta f$ plots generated using ungrafted versus grafted xylans can be explained
15 by the relative hydrophobicity of the graft copolymer, which also increased xylan aggregation
16 (Figure 4). With less bound water, corresponding aggregates would form a comparatively elastic
17 layer on the underlying cellulose surface. After saturating the cellulose surface, subsequent
18 stacking of the xylan aggregates would likely lead to water entrapment, significantly increasing
19 the viscosity of the add-layer.

Figure 9. Change in dissipation versus frequency during adsorption of all grafted xylans (A), and different stages of adsorption/desorption illustrated on a single curve (B). The black diagonal shows the border between elastic and viscoelastic domains.

1 Adsorption studies with hydrophobized hemicellulose are not very common. In one other case,
2 however, fatty acids and short polydimethylsiloxanes were added to the reducing end of
3 galactoglucomannan using reductive amination, which was found to increase its adsorption to
4 cellulose by lowering water solubility while retaining affinity to the cellulose surface.⁵¹ Distinct
5 from the adlayers formed by the xylan copolymers generated herein, specific modification of the
6 reducing end of galactoglucomannan promoted bilayer formation.⁵¹ These differences highlight
7 options to regulate both the chemical functionality and organization of hemicellulose coatings.

9 CONCLUSIONS

10 In the current study, we investigated the potential to regulate the properties of arabinoxylan-
11 based copolymers through enzymatic removal of specific Araf substituents. Whereas removal of
12 α -L-Araf (1 $\rightarrow$ 3) from di-substituted Xylp did not detectably impact xylan solubility, removal α -
13 L-Araf (1 $\rightarrow$ 3) from mono-substituted Xylp subunits led to product precipitation. Moreover,
14 subsequent graft copolymerization with GMA showed that by decreasing the water solubility of
15 the xylan, higher graft yields could be achieved. TLC analysis of enzymatically depolymerized
16 reaction products also confirmed that redox initiation was equally effective with Araf and Xylp
17 substituents.

18 QCM-D analyses of xylan adsorption to cellulose showed that arabinofuranosidase treatment of
19 arabinoxylans can control the viscoelastic character of the coated layer; however, subsequent xylan
20 grafting with GMA outweighed this impact of enzyme pretreatment. Whereas the gains in xylan
21 adsorption to cellulose achieved through enzyme pretreatment could be retained when substituting
22 GMA for other functional monomers, enzyme pretreatment is expected to consistently benefit the
23 recovery of resulting xylan copolymers.

ASSOCIATED CONTENT

Supporting Information.

Full ^1H NMR spectra of all arabinoxylan preparations, and adsorbed masses of xylans measured by QCM-D.

This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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