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Selective chemical oxidation and depolymerization of (*Panicum virgatum* L.) xylan with switchgrass oligosaccharide product analysis by mass spectrometry†

Michael J. Bowman[1]*, Bruce S. Dien[1], Patricia J. O'Bryan[1], Gautam Sarath[2] and Michael A. Cotta[1]

[1]USDA, Agricultural Research Service, National Center for Agricultural Utilization Research, Bioenergy Research Unit, 1815 North University Street, Peoria, IL 61604, USA
[2]Grain, Forage, and Bioenergy Research Unit, Agricultural Research Service, U.S. Department of Agriculture, 314 Biochemistry Hall, UN-L East Campus, Lincoln, NE 68583-0737, USA

Xylan is a barrier to enzymatic hydrolysis of plant cell walls. It is well accepted that the xylan layer needs to be removed to efficiently hydrolyze cellulose; consequently, pretreatment conditions are (in part) optimized for maximal xylan depolymerization or displacement. Xylan consists of a long chain of β-1,4-linked xylose units substituted with arabinose (typically α-1,3-linked in grasses) and glucuronic acid (α-1,2-linked). Xylan has been proposed to have a structural function in plants and therefore may play a role in determining biomass reactivity to pretreatment. It has been proposed that substitutions along xylan chains are not random and, based upon studies of pericarp xylan, are organized in domains that have specific structural functions. Analysis of intact xylan is problematic because of its chain length (> degree of polymerization (d.p.) 100) and heterogeneous side groups. Traditionally, enzymatic end-point products have been characterized due to the limited products generated. Analysis of resultant arabino-xylo-oligosaccharides by mass spectrometry is complicated by the isobaric pentose sugars that primarily compose xylan. In this report, the variation in pentose ring structures was exploited for selective oxidation of the arabinofuranose primary alcohols followed by acid depolymerization to provide oligosaccharides with modified arabinose branches intact. Switchgrass samples were analyzed by hydrophilic interaction chromatography (HILIC)-liquid chromatography (LC)-mass spectrometry/mass spectrometry (MSMS) and off-line nanospray MS to demonstrate the utility of this chemistry for determination of primary hydroxyl groups on oligosaccharide structures, with potential applications for determining the sequence of arabino-xylo-oligosaccharides present in plant cell wall material. Published in 2011 by John Wiley & Sons, Ltd.

As focus on the conversion of plant biomass to liquid transportation fuels becomes more intense due to limited petroleum resources, an increased understanding of constituent cell wall polysaccharides from herbaceous energy crops will provide benefits for the efficient conversion to monosaccharide components. Typical grasses are composed of approximately: cellulose (20–30 %), xylan (20–40 %), lignin (minor), and pectin (5 %) in the primary cell wall; and cellulose (35–45 %), xylan (40–50 %), lignin (20 %), and pectin (0.1 %) in the secondary cell wall.[1] Xylan is a heterogeneous biopolymer, consisting of repeating 1,4-β-linked xylose residues with potential acetyl or arabinose substitutions at either the 2-*O* or 3-*O* positions or both. Further complexity of the chains comes from additional substitutions (hexose, hexuronic acids, and/or phenolics).[2,3] It has been proposed that substitutions along xylan chains are not random and, based upon studies of wheat,[4,5] barley,[5] and rye [6] pericarps, are organized in domains that have specific structural functions. In contrast, the view that the xylan structure occurs in repeating units due to the enzyme complexes responsible for biosynthesis has also been expressed.[2,7,8]

Xylan plays a role in determining the reactivity of switchgrass (SG) to pretreatments and final sugar yields. For example, when switchgrass is pretreated with dilute acid, the xylan component displays bimodal kinetics where the slow reacting component (20–35 %) largely determines the severity of the reaction conditions applied.[9] Determining structural features present in xylan from biofuel feedstocks will allow for targeted chemical and/or enzymatic approaches for effective saccharification. In addition to the potential benefits of xylan structural knowledge on biomass conversion, many other aspects of the grass life-cycle including biomass production yield, plant development, pathogen resistance, and forage digestibility are likely influenced by xylan structure.

The size and heterogeneity of cell wall polysaccharides make intact sequence analysis infeasible, if not impossible,

* *Correspondence to:* M. J. Bowman, USDA, Agricultural Research Service, National Center for Agricultural Utilization Research, Bioenergy Research Unit, 1815 North University Street, Peoria, IL 61604, USA.
E-mail: michael.bowman@ars.usda.gov

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with current technologies. Traditional analysis of cell wall polysaccharides involves complete depolymerization followed by compositional analysis; however, the extended pattern is lost. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been used for the detection of xylo-oligomers from various sources: corn cob,[10] wheat pericarps,[11,12] and rye pericarps;[13] however, due to the lack of standards for large and complex oligomers, identification and quantification is difficult. Additionally, the resolution of HPAEC-PAD may not be sufficient to separate isomeric components of the digest mixture, as was observed in arabino-oligosaccharides from beets.[14] New methods that would permit depolymerization to oligosaccharides without compromising branching sugars followed by analysis for the determination of oligosaccharide branching are needed to efficiently elicit xylan structures.

Mass spectrometry (MS) provides a useful tool for the analysis of carbohydrate oligosaccharides, including the ability to analyze small sample quantities and the ability to determine structural information via the fragmentation patterns of the molecules.[15] Reports are limited to studies of commercially available linear xylo-oligosaccharides[16] and arabino-xylo-oligosaccharide (AXO) purified fractions from enzymatically depolymerized (by the action of xylanase from *T. viride*) wheat bran arabinoxylanase[17,18] and, more recently switchgrass,[19] as a source of branched AXOs, with complete AXO characterization by nuclear magnetic resonance (NMR). Two reports using LC/MS analysis of enzymatically depolymerized wheat and oat spelt AXOs have demonstrated the identification of arabinose side chains by diagnostic elimination ions[20,21] using LC/matrix-assisted laser desorption/ionization (MALDI) time-of-flight/time-of flight (TOF/TOF). The characteristic elimination ions are the result of high energy collision-induced dissociation (CID) and are likely instrumentation specific.

The limited MS reports may be the result of the complication that constituent monosaccharides, xylose and arabinose, are isobaric. This leads to difficulty in determination of the sequences, as glycosidic fragment ions cannot be definitively assigned as arabinose or xylose. The isobaric nature of AXOs can be overcome by the selective chemical modification of either arabinose or xylose constituents of the chain to differentiate the two pentoses. It is well accepted that the xylan chains consist of units of xylose in the pyranose form, whereas arabinose is present in the furanose form (Fig. 1(a)).[1,2] The difference in ring structure provides a target for the selective oxidation of the lone primary alcohol of the arabinofuranose branched sugars versus the secondary alcohols of the xylopyranose chain structure. There have been several reports of selective oxidation of the primary alcohols of methyl cellulose,[22] carboxymethylcellulose,[22] starch,[23,24]

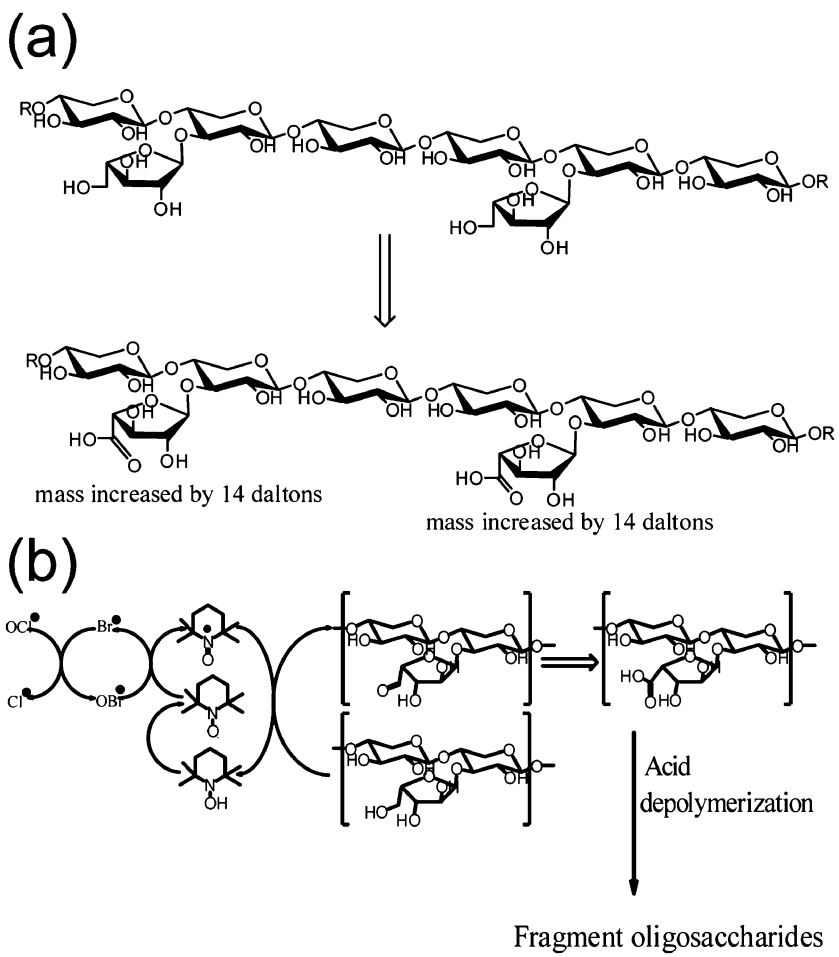

Figure 1. (a) Proposed primary alcohol to carboxylic acid structural change of arabinoxylan chains under mild oxidation conditions. (b) 4-Ac-TEMPO mechanism of oxidation showing all co-catalysts.

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pullulan,[23,24] β-cyclodextrin,[25] cellulose acetate,[23] xyloglucan,[24] inulin,[24,26] and rye-flour arabinoxylan[27] using various treatments. Oxidation is an appealing modification strategy for mass spectrometric analysis, as the conversion of the arabinofuranose primary alcohols into carboxylic acids (arabinuronic acid) will add a 14 Da mass shift to the arabinose branches, thereby allowing mass spectrometric differentiation of arabinose and xylose. The transformation of an alcohol to carboxylic acid is expected to have the benefit of increased stability to acid treatment,[27] increased signal in negative mode ionization mass spectrometry, and favorable fragmentation patterns for sequencing oligosaccharides,[28] potentially allowing for quick identification of AXO primary sequences by *de novo* sequencing without *a priori* knowledge of the products, as well as altering chromatographic properties. A technique for the modification of primary alcohol-containing polysaccharide structures by selective oxidation would serve as a complement to current enzymatic methods employed for characterization of limited AXO enzymatic endpoint products that typically rely on large-scale purification and characterization.

EXPERIMENTAL

Reagents

Sodium chlorite, potassium hydroxide, 1-octanol, sodium hydroxide (50 % w/v), sodium acetate anhydrous, trifluoroacetic acid (TFA), acetonitrile, acetic acid, dimethyl sulfoxide (DMSO), ammonium hydroxide, xylose, arabinose, sodium cyanoborohydride, anthranilic acid, boric acid, sodium bromide (NaBr), and birch xylan were purchased from Sigma-Aldrich Co.(St. Louis, MO, USA). Sodium hypochlorite (NaOCl, 13–15 %) and acetone were purchased from Fisher Scientific (Pittsburgh, PA, USA). 4-Acetamido-2,2,6,6-tetramethyl-1-piperidinyloxy, free radical (4-Ac-TEMPO) was purchased from Alfa Aesar (Ward Hill, MA, USA). Xylooligosaccharide (XOs) mixture was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Purified xylobiose-xylohexaose standards and wheat arabinoxylan were purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland). Formic acid was purchased from Fluka Chemical (Buchs, Switzerland). Ethanol was purchased from Decon Laboratories, Inc. (King of Prussia, PA, USA).

Preparation of isolated xylan from switchgrass

Switchgrass (SG, *Panicum virgatum* L.) (50 g) at the post-flowering stage was delignified and alkaline extracted as previously described.[29] The resulting holocellulose (37.6 g, 75.2 % recovery on dry weight basis with respect to switchgrass; 429.5 mg/g glucose, 282.1 mg/g xylose, 39.5 mg/g arabinose) was saponified to isolate xylans. Holocellulose (25 g) was mixed with 250 mL 10 % potassium hydroxide (KOH) and stirred overnight at ambient temperature. The heterogeneous solution was filtered. The xylan containing filtrate was adjusted to pH 5 with glacial acetic acid. After stirring for 4 h at room temperature, the sample was centrifuged at 9000 *g* in 30 mL Corex tubes, at 20 °C for 1 h. This pellet was xylan A (8.9 % yield recovery on dry weight basis with respect to switchgrass; 104.9 mg/g glucose, 482.0 mg/g xylose, 49.1 mg/g arabinose). To the centrifuged supernatant, an additional 3 volumes of 95 % ethanol was added. The solution was chilled overnight at 4° C. The precipitate was collected by centrifugation (14 000 *g*, 20 °C, 20 min). This was xylan B (18.0 % yield recovery on dry weight basis with respect to switchgrass; 59.8 mg/g glucose, 518.4 mg/g xylose, 95.9 mg/g arabinose). Sugar profiles of holocellulose and isolated xylan A and B were determined by two-stage acid hydrolysis (NREL procedure LAP002). Monosaccharides were measured using a SpectraSYSTEM liquid chromatography system (Thermo Electron Corporation, CA, USA) equipped with an automatic sampler, column heater, isocratic pump, refractive index detector, and computer based integrator running Chromquest version 2.5 (Thermo Electron Corporation, CA, USA). Samples were injected (20 μL) onto a sugar analysis column (Aminex HPX-87 H, 300 × 7.8 mm; Bio Rad Laboratories, Inc., Hercules, CA, USA) and eluted with 5 mM sulfuric acid at 0.6 mL/min and 65 °C. Monosaccharides were identified and quantified by comparison to authentic standards.

Chemical hydrolysis of xylan for the production of xylo-oligomers

Acid depolymerization

Method 1: 100 μg of dried xylan was dissolved in 100 μL 0.1 M TFA. The vial was heated to 100 °C for 10, 20, 30, 40, or 60 min. Samples were immediately frozen, dried *in vacuo*, and resuspended in 1 mL MilliQ water for analysis by HPAEC-PAD. Method 2: 100 μg of dried xylan was dissolved in 100 μL 2 M TFA. The vial was heated to 100 °C for 60 min. Samples were immediately frozen, dried *in vacuo*, and resuspended in 1 mL MilliQ water for analysis by HPAEC-PAD.

Sodium hypochlorite oxidation

Triplicate samples (1 mg) of birch, wheat arabinoxylan, and SG xylan were treated with sodium hypochlorite (320 mM, final concentration), in the presence or absence of TEMPO (10 μg) and/or sodium bromide (1.5 mg), in 100 mM sodium hydroxide (0.5 mL final volume), for reaction times of 24, 48, 72, and 96 h at 4 °C or 25 °C with constant shaking. Samples were dried *in vacuo*, ethanol precipitated (80 % ethanol/water) twice for 14 h at −20 °C. After centrifugation, the samples were resuspended in MilliQ water at a concentration of 1 mg/mL. Aliquots (100 μg) were dried and redissolved in 1 mL MilliQ water for HPAEC-PAD analysis; additional aliquots (100 μg) were dried for acid depolymerization by method 1 or 2. Ethanol supernatants ($1/10^{th}$ of the volume) were dried and resuspended in 1 mL water for HPAEC-PAD analysis to demonstrate that the mild oxidation conditions do not release monosaccharide products.

Analysis by HPAEC-PAD

Each sample (25 μL; 2.5 μg) was analyzed by HPAEC-PAD (Dionex ACS 3000, Sunnyvale, CA, USA) utilizing a PA-100 column (Dionex) running 100 % A isocratically for 15 min followed by a gradient program to 12 % B over 20 min (A: 100 mM NaOH; B: 100 mM NaOH containing 1 M sodium

acetate) followed by 15 min of reequilibration in 100% A, based on conditions reported by Rantanen *et al.*[13] Extent of digestion was determined by comparison of xylose, arabinose, and xylooligosaccharides (xyl_2-xyl_6) released by TFA hydrolysis (at a concentration of 2 M or 0.1 M for complete hydrolysis or preferential arabinose cleavage, respectively) versus the amount of xylose, arabinose, xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose produced by acid hydrolysis of an equivalent portion of xylan.

Xylo-oligosaccharide labeling and LC/MS

Samples were derivatized with anthranilic acid using the conditions of Anumula.[30] Briefly, 200 μg of oxidized, TFA hydrolyzed xylan in 20 μL water was labeled by the addition of 200 μL methanol containing: 4 % sodium acetate trihydrate (w/v); 2 % boric acid (w/v); 3 % anthranilic acid (w/v); and 3% sodium cyanoborohydride (w/v). The reaction mixture was incubated at 80 °C for 60 min, followed by the addition of 1 mL acetonitrile. After drying, the samples were resuspended in 1 mL 80 % ethanol/water and stored at −20 °C for 10 h. Samples were centrifuged at 14 000 *g* for 10 min and the supernatant was removed. Samples were resuspened in 40 μL water to a concentration of 5 μg/μL. Acetonitrile was added to make a final concentration of 1 μg/μL prior to HILIC-LC/MS analysis. Mass spectrometry samples (10 μg injected, based on starting material) were analyzed using an LC/MS Agilent 1100 liquid chromatography system equipped with an Amide-80 (2.1 mm × 150 mm; Tosoh Biosciences, King of Prussia, PA, USA) HILIC column running a gradient elution of 20 A:80 B (buffer A: 25 mM ammonium formate (pH 3.5) in 10 % acetonitrile, buffer B: 10 % 25 mM ammonium formate (pH 3.5) in 90 % acetonitrile) to 60 A:40 B over 60 min at a flow rate of 200 μL/min with diode-array detection. Data was collected from a quadrupole orthogonal time-of-flight (QTOF) mass spectrometer (Applied Biosystems/MDS Sciex Qstar Elite) via a turbospray source using the negative mode. The instrument was tuned to a mixture of anthranilic acid labeled xylo-oligosaccharides (degree of polymerization (d.p.) 2–6) introduced by infusion to optimize the signal of xylo-oligosaccharide analytes.

Off-line nanospray of anthranilic acid labeled oligosaccharides

Oxidized SG xylan B material (500 μg) was subjected to 0.1 M TFA hydrolysis and reducing end labeling using the above conditions scaled to a 5-fold increase in starting material. An additional linear xylohexaose standard was also reducing-end labeled and purified for comparison in off-line MS analysis. After reducing-end labeling and drying *in vacuo*, the resulting tagged oligosaccharides were dissolved in 100 μL water and enriched by PepClean C18 (Pierce, Rockford, IL, USA) spin column clean-up (according to the manufacturer's protocol where formic acid was substituted for TFA, sample fractionated with water, 10 % acetonitrile/water, 50 % acetonitrile/water, and acetonitrile), the 50 % acetonitrile fraction was further enriched by size-exclusion chromatography (SEC)-HPLC using a BioSil SEC-125 column (7.5 × 600 mm; Bio-Rad, Richmond, CA, USA) using a mobile phase of 50 mM ammonium formate in water at a rate of 400 μL/min while the profile was monitored at 310 nm. Fractions showing absorbance at 310 nm were dried *in vacuo*, resuspended in MilliQ water, and dried. Resultant oligosaccharide mixtures were analyzed with a QTOF mass spectrometer (Applied Biosystems/MDS Sciex Qstar/Elite) via nanospray[31] ionization using uncoated capillaries having a 1 μm orifice (New Objective, Woburn, MA, USA). Samples were dissolved in water, then diluted in water/acetonitrile (1:1) solution to a final concentration of 5 μg/ μL, based on starting material weight. Fragmentation of the resultant tagged glycans was attained using collision energy (CAD = 5, CE = −25). Tandem mass spectra were collected isolating the 2^- charge state, as these were the most prevalent ions corresponding to singly oxidized oligosaccharide masses.

RESULTS AND DISCUSSION

Switchgrass has potential use as a bioenergy crop with numerous studies on its conversion into ethanol; however, little is known about how the xylan component affects bioconversion. Therefore, xylan was isolated, in two fractions (xylan A, xylose/arabinose ratio of 9.8:1 and more highly substituted xylan B with a 5.4:1 xylose/arabinose ratio), by traditional alkaline extraction.[29] It should be noted that the alkali-extraction method removes some of the modifications present on xylan structures, notably acetylations and ester-linked phenolics.

SG xylan B, due to its higher arabinose substitution, was used as the model for depolymerization conditions to generate oligosaccharide profiles. Dilute acid (i.e., 0.1 M TFA) preferentially removed arabinose branches versus glycosidic cleavage of 1,4-β-xylose–xylose bonds. The ratio of released arabinose to xylose and xylooligomers (up to d.p. 6), determined by HPAEC-PAD, was used to compare chemical treatments with respect to the release of branched groups versus main chain cleavage. Xylose and arabinose monosaccharide measurements were used for quantification compared to completely depolymerized sample, because the amounts of d.p. 2 to d.p. 6 released had an inverse relationship to the release of arabinose. Fifty-seven percent of total arabinose was liberated after 10 min and the subsequent rate of release was very slow (to a maximum of 73 % after 40 min), in contrast to the cleavage of xylose–xylose bonds, as measured by free xylose only. This was considerably slower and could be plotted as a linear increase from 10 min (1.1 % of total) to 40 min (12.5 % of total). In a previous report, the conversion of arabinose into arabinuronic acid led to an increase in acid stability,[27] therefore, arabinose release from 0.1 M TFA treatment can serve as an assay for oxidation of arabinose.

The use of TEMPO-mediated oxidation of xylan provides a method that uses inexpensive, readily available materials while generating innocuous salts as byproducts of the reaction (Fig. 1(b)). Ac-4-TEMPO had superior oxidation yields on pullulan and starch;[32] therefore, it was the radical mediator of choice for this reaction. Variations in the presence and absence of activating bromide, time, and temperature were examined as all have been reported to provide varying yields on other substrates.[22–26,32,33]

Equivalent samples of birch xylan, wheat arabinoxylan, and SG xylan were treated under oxidation conditions 1–6

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(Table 1). These three sources of xylan were selected to evaluate the variables with a diverse collection of xylan structures and degree of arabinose substitutions. When the primary alcohol of arabinofuranose was converted into an acid-stable arabinuronic acid, there was a drop in the free arabinose yield observed using 0.1 M TFA depolymerization. In SG xylan B samples the amount of free arabinose decreased, where each oxidation condition asymptotically approached different values under increasing incubation times (Table 2), indicating various degrees of arabinose oxidation. Birch samples (conditions 2–6) showed no observable change in depolymerization patterns (data not shown) due to the lack of residues containing primary alcohols in the chain structure; these results show that arabinoxylan structures are modified under the conditions used, with no effect on xylan-lacking primary alcohols. After 12 h of oxidation, the highest degree of modification occurred with the use of all three oxidation components (condition 4). The absence of the bromide catalyst (condition 3) released 50 % more arabinose as compared to condition 4 demonstrating the increased benefit of bromide catalyst to the reaction rate. When sodium hypochlorite is used exclusively (condition 2), there is no detectable difference in arabinose hydrolysis products compared to untreated xylan demonstrating the requirement of the radical mediator for oxidation.

The effect of variations in oxidation conditions 1, 2, and 4 on SG xylan B with extended reaction time (24, 48, 72, and 96 h) and temperature (4 °C and 25 °C) was examined. Oxidation of SG xylan B under conditions 5 and 6 led to a substantial reduction in the acid-catalyzed release of arabinose to 7 % of the total and 2.4 % when the oxidation occurred at room temperature (Table 2). Conditions 2 and 3 also had decreases in arabinose observed after 24 h of oxidation, with a greater conversion at room temperature. The conversion oligosaccharide products of the oxidation process are difficult to assign and quantify by HPAEC-PAD (Fig. 2) as there are no standards available and HPAEC-PAD is incompatible with on-line analysis. To confirm the complete conversion of arabinose, separate aliquots were treated with 2 M TFA, no additional arabinose was liberated, compared to 0.1 M TFA treatment, from the product of condition 5, consistent with the expected stability coming from the conversion of arabinose into arabinuronic acid.[27] In contrast, additional arabinose was liberated by 2 M TFA treated SG xylan with conditions 3 (5.2 % increase over 0.1 M TFA) and 2 (9.3 % increase over 0.1 M TFA), as seen with the incomplete 0.1 M TFA hydrolysis observed from unmodified xylan when compared to 2 M TFA (condition 1, Table 2).

Increasing the duration of the oxidation reaction (24–96 h) exhibited a slow time-dependent decrease in acid-catalyzed

Table 1. Oxidation conditions applied to xylan samples

Sample condition	Oxidation reagent	Temperature (°C)	Time (h)
Oxidation Condition 1	None	4	12
Oxidation Condition 2	NaOCl	4	12
Oxidation Condition 3	4-Ac-TEMPO, NaOCl	4	12
Oxidation Condition 4	4-Ac-TEMPO, NaOCl, NaBr	4	12
Oxidation Condition 5	4-Ac-TEMPO, NaOCl, NaBr	4	24
Oxidation Condition 6	4-Ac-TEMPO, NaOCl, NaBr	25	24

Table 2. Xylose and arabinose released from SG xylan B samples treated with varying oxidation conditions and times of 0.1 M TFA hydrolysis by HPAEC-PAD

0.1 M TFA at 100°C	20 min		30 min		40 min	
Sample condition	Percent xylose released[a]	Percent arabinose released[a]	Percent xylose released[a]	Percent arabinose released[a]	Percent xylose released[a]	Percent arabinose released[a]
Oxidation Condition 1	4.2	67.5	8.3 ± 0.5	71.4 ± 0.5	17.0	73.2
Oxidation Condition 2	6.4	64.3	14.0 ± 0.4	70 ± 2.6	22.4	70.6
Oxidation Condition 3	6.0	57.3	11.5 ± 0.1	53.3 ± 0.2	20.9	62.0
Oxidation Condition 4	6.2	37.9	10.3 ± 1.2	39 ± 1.1	12.6	43.1
Oxidation Condition 5			11.3 ± 0.4	7.0 ± 0.3		
Oxidation Condition 6			10.4 ± 0.6	2.4 ± 0.2		

[a]Compared to same amount of untreated xylan hydrolyzed with 2 M TFA for 60 min at 100°C.

release of arabinose that reached a maximum value for each condition. Conditions 5 and 6 showed the greatest effect, ultimately reaching 2 % of the total arabinose unmodified (i.e. 98 % conversion) after 96 h. Conditions 2 and 3 yielded between 14–32 % unmodified arabinose released after 96 h. However, some care must be taken as one report shows that depolymerization of cellouronic acid can occur under oxidation conditions at pH 11 with TEMPO.[34] Switchgrass xylan B showed very little degradation under the three component oxidation conditions (4-Ac-TEMPO/NaOCl/NaBr at 4 °C or room temperature after 96 h); however, conditions 2 and 3 led to a small increase of degraded material at 4 °C and the extent of degradation was nearly doubled at room temperature.

To determine whether the observed acid stabilization of the arabinose residue was the result of the expected oxidation event, MS analysis of the two-stage oxidation/acid-depolymerization products was performed. SG xylan samples were reducing-end labeled with anthranilic acid as it has successfully been used for negative mode on-line mass spectral analysis.[35–37] Samples were then analyzed by HILIC-LC/MS. The resulting data (Fig. 3) show that an oxidation had occurred by the corresponding 14 Da mass shifts (labeled d.p. 12 (Ox)-d.p. 16 (Ox)) from the linear xylo-oligosaccharide

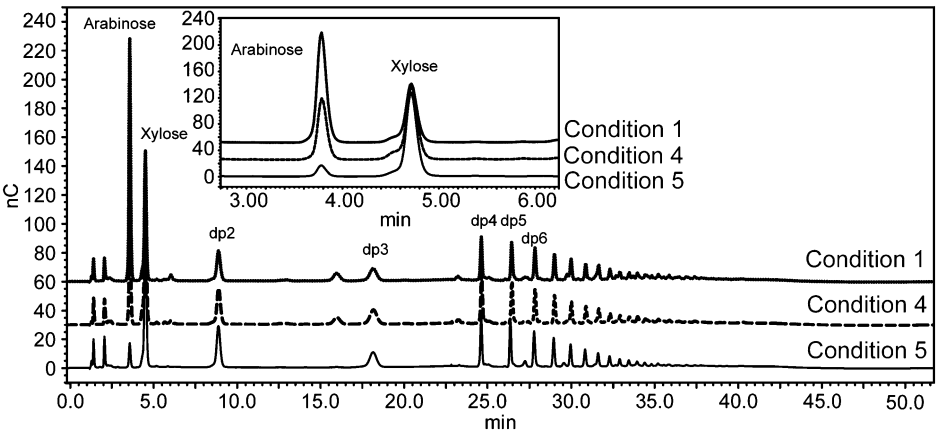

Figure 2. HPAEC-PAD chromatogram of oxidized/acid-depolymerized SG xylan B samples. Stacked chromatograms of oxidation conditions 1, 4, and 5 showing xylose release versus arabinose release after 12 h of oxidation for conditions 1 and 4 and 24 h for condition 5.

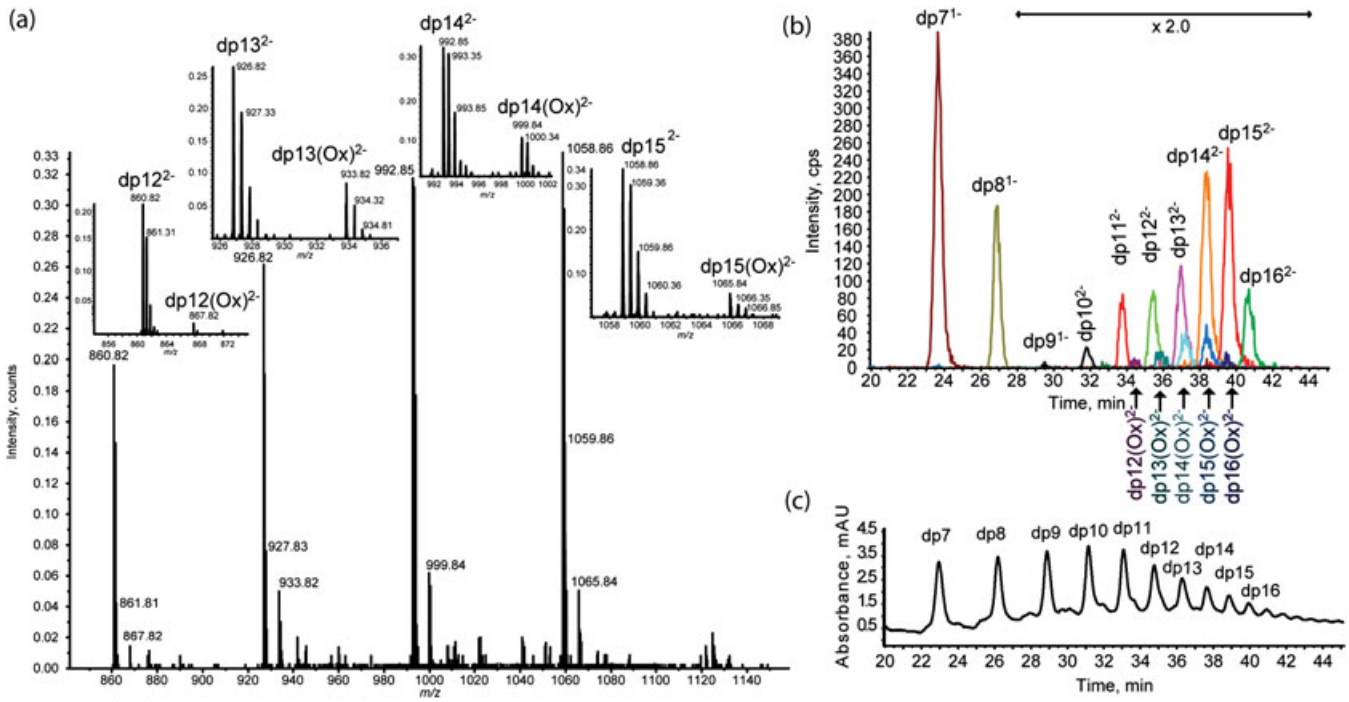

Figure 3. Negative mode HILIC-LC/MS of 0.1 M TFA treated oxidized SG xylan B (10 µg injected based on starting material) with anthranilic acid reducing-end label. (a) Mass spectrum summed from retention times 34 to 39 min. (b) Extracted ion chromatograms of d.p. 7–16 including oxidized d.p. 12(Ox)–16(Ox). The portion between 28 and 44 min is magnified two-fold for visualization of the oxidized xylooligosaccharides. (c) UV absorbance trace monitored at 310 nm.

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ions (labeled d.p. 7–16). Interestingly, smaller quantities of oxidation products were observed in the lower d.p. numbers (2–5 and 7–8), whereas more intense single oxidation events were observed for ions corresponding to d.p. 9–16. Generation of oligomers of the highest d.p. amenable for MS/MS analysis is a long-range goal, as this will allow for the determination of the spacing between arabinose substitutions and whether they are fixed or variable. Due to the nature of endo-xylanase activity, the dominant product of end-point digestion is xylobiose, where xylose repeats are effectively converted into xylose, xylobiose, and/or xylotriose depending on the specificity of the enzyme used. Chemical depolymerization or partial enzymatic digestion (e.g. 50 %) could provide appropriate samples; however, in either case, the resultant oligomers will contain a distribution of isomers. The analysis of the distribution of isomers will allow for increased understanding of the extended structure of the xylan chains. In previous reports, HILIC has been shown to have multiple peaks for extracted ions,[38–42] with specific examples for arabino-xylo-oligomers[20,21] that are presumably isomers; however, the conditions used in this study to survey the oligomer products were not optimized for separation of each isomeric oligomer. It is further noted that with the high degree of oligosaccharide modification from this chemistry, that any AXO isomers would exist as the lower abundance oxidized ions. Conditions can be modified for isomeric separation or alternate chromatographies may be employed (e.g. graphitized carbon[42]). Additionally, a d.p. 6 corresponding to a double oxidation was also observed, demonstrating the ability to determine the number of arabinose side branches on AXOs (Fig. 4). These LC/MS data also show ions corresponding to compositions containing either oxidized xylotetraose with an oxidized arabinose and a hexuronic acid (Fig. 4, *m/z* 988.34); however, it is undetermined whether this existed as a hexuronic acid before oxidation treatment or is the result of oxidation of a hexose residue. It is also possible that this ion corresponds to a methylated hexuronic acid containing xylopentaose; therefore, care must be taken in assignment of oligosaccharides potentially containing both hexose and oxidized arabinose residues. In either case neither hexose nor hexuronic acid

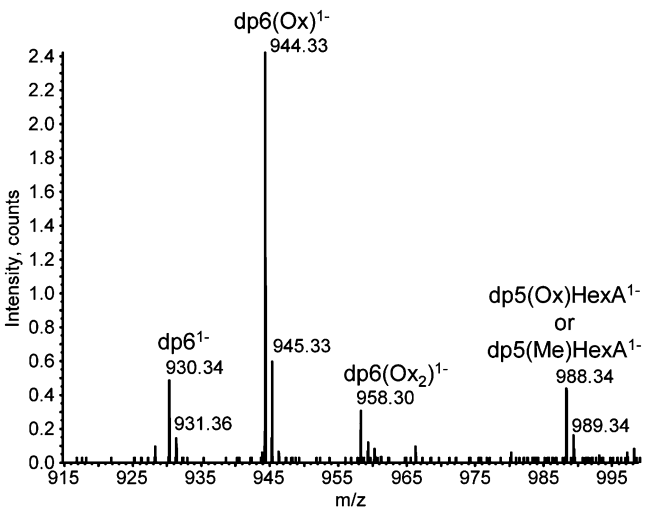

Figure 4. Negative mode MS spectrum of SG xylan B from HILIC-LC/MS summed from 20 to 21 min.

containing oligosaccharides were previously reported from switchgrass arabino-xylo-oligosaccharides from enzymatic digestion.[19] The ability to modify arabinoxylan to increase acid stability permits the use of alternate depolymerization techniques to enzymatic digestion, whereas more information may be discerned from resultant oligosaccharides due to reduced bias by enzyme substrate recognition limitations.

Although the ultimate goal of the use of this method will be to ascertain the sequences of longer oligosaccharides, a smaller oligosaccharide was selected for MS/MS analysis to demonstrate the potential utility of the 14 Da mass shift in primary alcohol-containing oligosaccharides. To obtain this confirmatory MS/MS data, a second set of samples was depolymerized and reducing-end labeled. Following a two-step enrichment protocol using C18 spin columns and a SEC step, samples were amenable to nanospray analysis. An ion corresponding to the smaller d.p. 6(Ox) oligosaccharide mass in the 2^- charge state was isolated and fragmented (Fig. 5(a)); while this ion is of low intensity, it provides a simplified fragmentation pattern to identify modified fragment ions. Nomenclature is based on Domon and Costello.[43] For emphasis, oxidized fragment ion labels include the designation (Ox) to denote those that contain a 14 Da mass shift. The prevalence of a B_1 ion corresponding to an oxidized pentose residue (*m/z* 145.05^{1-}) is the highest intensity fragment ion in the spectrum (Fig. 5(a)). The oxidized SG-derived AXO has singly charged modified C-type ions (*m/z* 559.30^{1-}, 691.35^{1-}) in addition to modified (*m/z* 273.66^{2-}/548.31^{1-}, 339.68^{2-}, 405.71^{2-}) and unmodified Y-type (*m/z* 270.18^{1-}, 402.24^{1-}, 266.68^{2-}/534.33^{1-}, 332.70^{2-}, 798.45^{1-}) ions, whereas a standard linear anthranilic acid labeled xylohexaose (Fig. 5(b)) has predominantly Y-type ions (*m/z* 266.66^{2-}/534.32^{1-}, 332.69^{2-}/666.37^{1-}, 398.73^{2-}). The presence of sequential oxidized and unmodified Y-type ions and oxidized C-type ions leads to the assignment of three modified isomers present of this composition (Fig. 5(a)). The absence of ions corresponding to structures where an arabinuronic acid is present on either the reducing end or non-reducing end of the oligosaccharide leads us to believe that only these three structures are present; however, the presence of ions B_4 (*m/z* 527.4^{1-}) and Y_4 (*m/z* 332.70^{2-}) cannot completely exclude this possibility. These ions may be due to double fragmentation of involving loss of an arabinuronic acid B-type ion (*m/z* 145.05^{1-}) and a backbone fragmentation.

Future work will focus on chromatographic method development for separation of isomers of various d.p. values, as well as optimizing depolymerization techniques to obtain the maximal information regarding sequences present. The ability to analyze acid depolymerized oxidized xylan from switchgrass samples by MSMS demonstrates the potential for fast characterization for isomeric AXOs with varying substitution patterns for application to herbaceous plant materials.

CONCLUSIONS

Native switchgrass xylan is difficult to chemically depolymerize while maintaining branching due to the labile nature of arabinose side groups. Therefore, a method to selectively oxidize primary alcohols of SG arabinoxylan to carboxylic acids has been developed. The oxidation conditions are mild,

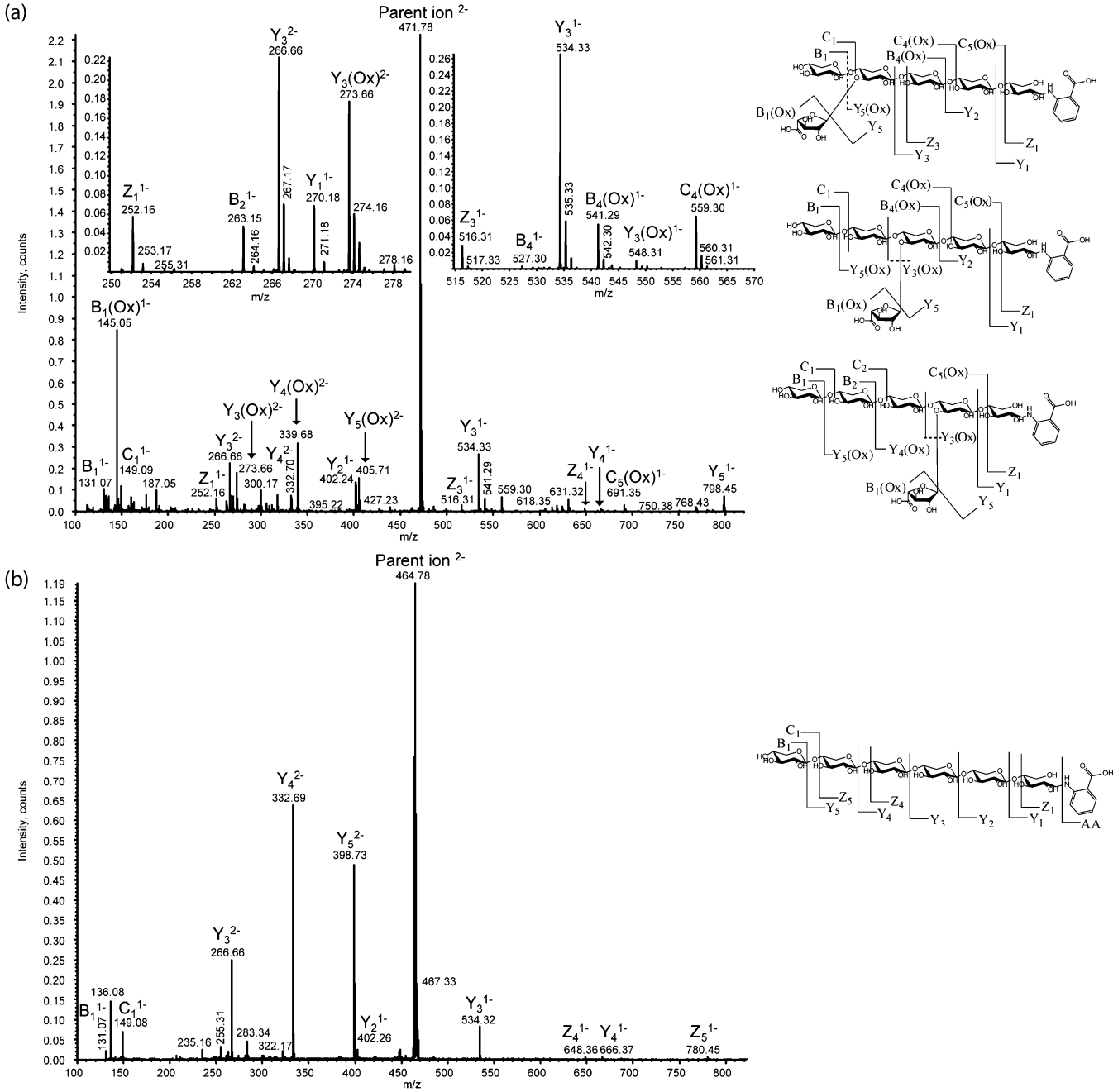

Figure 5. (a) Negative mode nanospray tandem mass spectrum of oxidized d.p. 6 (d.p. 6(Ox)) labeled with anthranilic acid from SG xylan B. (b) Negative mode nanospray tandem mass spectrum of xylohexaose standard labeled with anthranilic acid. Ions are labeled according to the nomenclature of Domon and Costello.[43]

generating no measurable carbohydrate degradation products. The method has additional characteristics that are beneficial, including: providing a mass shift that differentiates xylopyranose and arabinofuranose constituents that can be used for sequencing of oligosaccharides; increased stability of arabinuronic acids allow for alternative depolymerization techniques; providing structural variation allowing alternate chromatography techniques; and samples can be oxidized in parallel, permitting medium-throughput analysis. The strategy of mild, selective oxidation of arabinofuranose branches provides a method with applications for the determination of the extended structures of structural xylan from herbaceous energy crops by mass spectrometry.

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