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Extraction and characterization of native heteroxylans from delignified corn stover and aspen

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Abstract Dimethylsulfoxide-solubilized polysaccharides from delignified corn stover and aspen were characterized. The biomass was delignified by two different techniques; a standard acid chlorite and a pulp and paper QPD technique comprising chelation (Q), peroxide (P), and acid-chlorite (D). Major polysaccharides in all fractions were diversely substituted xylan. Xylan acetylation was intact after chlorite delignification and, as expected, xylan from QPD-delignified fraction was de-acetylated by the alkaline peroxide step. The study of DMSO-extractable xylans from chlorite-delignified biomass revealed major differences in native acetylation patterns between corn stover and aspen xylan. Xylan from cell walls of corn stover contains 2-*O*- and 3-*O*-mono-acetylated xylan and [MeGlcA-α-(1 → 2)][3-OAc]-xyl*p* units. In addition, aspen xylan also contains 2,3-di-*O*-acetylated xylose. 1,4-β-D-xyl*p* residues substituted with MeGlcA at *O*-2 position are absent in chlorite-delignified aspen xylan. Sugar composition in accord with NMR-spectroscopic data indicated that corn stover xylan is arabinosylated while aspen xylan is not. We have shown that corn stover xylan has similar structure with xylans from other plants of *Poales* order. No evidence was found to indicate the presence of 1,4-β-D-[MeGlcA-α-(1 → 2)][Ara-α-(1 → 3)]-xyl*p* in corn stover xylan fractions.

Keywords Biomass · Biomass delignification · Plant cell wall · Glucuronoxylan · Hardwood xylan · Corn stover xylan · Xylan acetylation · Endoxylanase · Homo- and-heteronuclear NMR-spectroscopy · MALDI-TOF MS

Abbreviations

MGX	4-*O*-Methylglucuronoxylans
GAX	Glucuronoarabinoxylans
MLG	Mixed-linkage glucan
MeGlcA	4-*O*-Methylglucuronic acid
QA	QPD-delignified DMSO-solubilized HMW fraction from aspen
QS	QPD-delignified DMSO-solubilized HMW fraction from corn stover
CA	Chlorite-delignified DMSO-solubilized HMW fraction from aspen
CS	Chlorite-delignified DMSO-solubilized HMW fraction from corn stover
MW	Molecular weight
DP	Degree of polymerization
NREL	National Renewable Energy Laboratory, Golden CO, USA

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Introduction

Biomass is a composite of cellulose (~44%), hemicelluloses (~30%) and lignin (~26%) linked together via hydrogen and covalent bonds. Separation of these components for study is difficult at best. Xylan is particularly susceptible to modification during extraction, primarily through saponification of ester-linked acetyl side chains and ferulic acid cross links to lignin during extraction with alkaline reagents. This de-esterification of acetyl residues from the xylose backbone renders the xylan insoluble in aqueous solutions, while retention of these side-chains allows the xylan to retain its native, water-soluble state. In order to study xylan with as native a conformation as possible, it must first be decoupled from lignin and then extracted from the remaining biomass. We have employed two delignification methods, one with and one without an alkaline stage in order to examine the effects of de-esterification on the extracted xylan structures. Complete removal of lignin from the biomass was not the goal of either treatment, as complete removal of the lignin results in the loss of carbohydrate during the treatment with acid chlorite (Herbst 1952).

Xylans have a linear backbone comprised of β-1,4-linked D-xyl*p* residues which, depending on the origin and extraction method, may be substituted with acetyl, arabinosyl and glucuronosyl (GlcA and MeGlcA) residues. In the lignified cell walls of dicotyledons, they are 4-*O*-methyl-glucuronoxylans (MGX); in the lignified cell walls of the *Poaceae*—glucuronoarabinoxylans (GAX) similar to those in non-lignified cell walls, but with a lower degree of substitution by glycosyl residues (Vogel 2008).

In hardwoods such as aspen, beech, and birch, the backbone 1,4-β-D-xyl*p* residues are substituted with one MeGlcA per approximately every tenth such residue. The xyl*p* residues are partially acetylated at the *O*2 and/or *O*3 positions (Timell 1967). The degree of acetylation in native aspen xylan is reported to be 0.6–0.7 (Teleman et al. 2000). The xylans found in annual plants such as maize, rice, oats, sunflower, rye, wheat and barley are more structurally diverse and complex; the xylan backbone can be heavily branched with acetyl, 4-OMe-Glc*p*A, Glc*p*A, Xyl*p*, Ara*f* and Gal*p* substituents. Although xylans and ferulic acid cross-links in the primary and secondary cell walls of *Poaceae* have been studied extensively (Harris and Hartley 1980; Chase et al. 1993; Rudall and Caddick 1994; Harris et al. 1997; Smith and Harris 1999) including corn cob xylan (Hroma'dkova' et al. 1999), maize coleoptiles (Carpita and Whittern 1986; Kim and Carpita 1992) and lignin–xylan association in maize bran (Chanliaud et al. 1995; Lapierre et al. 2001), corn stover xylan did not receive as much attention. Reports on native acetylation patterns of *Poaceae* xylans are limited.

Wood and agricultural residues, as well as dedicated energy crops are the main feedstocks for renewable fuels from biomass. Although dedicated energy crops such as short-rotation woody crops and herbaceous crops (primarily tall grasses) seem to be the largest and most promising future resource of biomass (Lin and Tanaka 2006), existing agricultural residues, such as corn stover, are an obvious source of biomass especially for the near term.

Biomass recalcitrance is a key challenge in developing cost-efficient bioethanol technology. Another challenge to biomass fermentation is the high percentage of pentoses in hemicelluloses. Understanding of hemicellulose structure and properties, as well as the effects of pretreatment processes on hemicellulose and cellulose structure is essential for the rational design of pretreatment processes to achieve higher biomass conversion efficiency.

In this paper we discuss structure of DMSO-extractable xylans obtained from two different types of raw, or unpretreated, biomass—aspen and corn stover. The biomass was defatted and delignified prior to DMSO extraction in order to facilitate removal of the hemicelluloses, as they are normally covalently attached to lignin. In addition to structural details of xylans from two dissimilar species, application of two different delignification methods provides partial but complementary information into the effect of delignification procedure on xylan structure.

Experimental

Materials

Corn stover used in this study was harvested from the Kramer Farm in Wray, Colorado, in 2003. The untreated corn stover was 1/4" chopped in a Thomas Wiley cutting mill (Thomas Scientific, Swedesboro, NJ) and then milled to pass a 20 mesh screen. The

aspen sapwood (*Populus Tremuloides*) used was harvested in 1994 and was kindly provided by Wendell Johnson of the University of Minnesota at Crookston. The wood was chipped and then milled to pass a 20 mesh screen.

Delignification procedure

Extraction of soluble, native-like hemicellulose was modified from the original procedure published by Hagglund et al. (1956).

Pre-extraction of biomass

The defatting/extraction of corn stover and aspen were carried out as follows. Approximately 300 g of 20 mesh biomass was extracted in a polypropylene thimble by Soxhlet using 80% ethanol overnight to remove lipids and other extractives. The defatted biomass was further extracted by Soxhlet with acetone and the resultant solids were air dried before delignification.

Acid chlorite delignification

Delignification by acid chlorite was carried out according to Saarnio (1954). Sodium chlorite (0.67 g $NaClO_2$/g biomass) is added to extractives-free biomass in a large zip-top plastic bag at a 10% consistency in water with mixing. After thorough wetting of the biomass, acetic acid is added at a concentration of 1 mL glacial acetic acid/g biomass. The bag containing the bleaching mixture is sealed and placed in a 60 °C water bath for 2 h with regular mixing. Chlorine dioxide gas is generated and regular venting of the vessel by opening the bag is required.

After acid chlorite delignification, the mixture is filtered by Buchner funnel and washed with deionized water until all traces of the bleaching mixture have been removed. The acid chlorite delignification step is carried out three times with washing in between each bleaching step. Water is pressed from the filter cake to an approximate 25% solids level.

QPD delignification

Delignification by hydrogen peroxide/chlorine dioxide (QPD) was carried out according to bleaching sequences developed by NREL. Chemical additions are calculated based on the percent solution in water. All reactions are carried out in heavy zip-top bags large enough to be well in excess of the amount of biomass being processed.

Chelation (Q) Stage

Metal ions that may interfere with hydrogen peroxide delignification are removed by chelation. HEDTA (*N*-(2-hydroxyethyl) ethylenediaminetriacetic acid) is added in a 1.2% solution to biomass in water at a 3% consistency for 1.5 h at 60 °C. After the chelation treatment, the pulp is washed with water as above.

Peroxide delignification (P) stage

Following chelation, the biomass is bleached with hydrogen peroxide. The sequence of addition of each chemical must be carefully followed to provide good dissolution and mixing of the chemicals prior to heating. As with the acid-chlorite step, peroxide delignification can be carried out in plastic bags or glass vessels, however the reaction container must be vented in some manner.

The water necessary for a 10% biomass consistency is weighed out, taking into account the amount of water present in the hydrogen peroxide solution and the residual water in the biomass after washing. Enough water is added to the biomass to make a 10–25% solution and the mixture is stirred in a fume hood. Dry $MgSO_4$ (0.05%) and DTPA (diethylenetriaminepentaacetic acid) (1.2%) are added to the biomass from the Q stage, as is the most of the remaining water. Some water is held back for rinsing glassware and making the NaOH solution.

Enough sodium silicate to make up a 3% solution is added and the pulp is well mixed. The silicate is approximately 14% base and 27% SiO_2. Enough NaOH to make a 3% solution is dissolved in some of the remaining water and added to the bag. The remaining water held back is used to rinse the mixing beaker with the rinsate being added to the biomass and the pulp again is mixed.

Enough H_2O_2 is added as a 30% solution to make a 5% solution. The pulp is again mixed. At this point the pulp changes from a very dark brown to a much lighter yellow color. The bag or vessel is sealed at this point.

The mixture is heated to 95 °C for 3 h with mixing every 15 min. Samples with high lignin contents will produce a vigorous exotherm at 95 °C and may result in foaming of the solution. Vessel or bag size should be chosen so that any foaming experienced will not overflow the vessel. If plastic bags are used, the bag should be vented on a regular basis to avoid rupture. Rigid vessels should also be vented periodically. The pulp is washed with water continuously at the end of 3 h until the wash water no longer gives a color with phenolphthalein indicator.

Chlorine dioxide (D) stage

The D stage is the same as above but is only performed one time.

DMSO extraction of hemicelluloses

The delignified material (either method) was washed extensively with water and air-dried prior to DMSO extraction. Dimethyl sulfoxide was added to the dried biomass at a ratio of 18 mL per g biomass. The mixture was stirred at room temperature overnight with an overhead stirrer at ~40 rpm and the solids were removed by filtration through 70 μm polypropylene cloth. The polysaccharides in the DMSO extract were precipitated by diluting the DMSO to 80% (v/v) with 100% ethanol. Concentrated HCl was added in a ratio of 0.5 mL HCl/L of final solution. The solution was mixed overnight at 4 °C with an overhead stirrer at approximately 20 rpm in a 12 L separatory funnel. After stirring, the solution was allowed to precipitate and settle overnight at 4 °C. The precipitate was collected by draining the separatory funnel and filtration through 70 μm polypropylene filter cloth at 4 °C. A gel like material was collected and washed and dried sequentially using 100% ethanol and diethyl ether.

The ethanol-precipitated DMSO-extracted material was dialyzed through 20 kDa MWCO membrane and retentates were designated as QA (QPD-delignified DMSO-soluble HMW fraction from aspen), QS (QPD-delignified DMSO-soluble HMW fraction from corn stover), CA (chlorite-delignified DMSO-soluble HMW fraction from aspen) and CS (chlorite-delignified DMSO-soluble HMW fraction from corn stover).

Glycosyl composition analysis

Methyl glycosides were first prepared from dry sample by methanolysis in 1 M HCl in methanol at 80 °C (18–22 h). The samples were then per-*O*-trimethylsilylated by treatment with Tri-Sil (*Pierce*) at 80 °C for 30 min (Merkle and Poppe 1994). Glycosyl composition analysis was performed by combined gas chromatography/mass spectrometry (GC/MS) of the per-*O*-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis.

GC/MS analysis of the TMS methyl glycosides was performed on an HP 6890 GC interfaced to a 5975b MSD, using an *AllTech* EC-1 fused silica capillary column (30 m × 0.25 mm ID). Response factors for individual sugars were calculated using peak area of the internal standard inositol in a standard sugar mixture. Amount of total carbohydrate was calculated relative to internal standard. Based on the response factors, molar percentage of each sugar was calculated relative to total carbohydrate (100%).

Analytical high pressure anion-exchange chromatography

A CarboPac PA1 Column 4 × 250 mm (*Dionex Corp, Sunnywale CA*), connected to Dionex LC System with autosampler and ED 40 electrochemical detector coupled with Peaknet integration software (*Dionex*) was used for HPAEC analyses. 10 μL of 1 mg/mL solutions of each sample in water was injected onto the column. The buffers used were: A: 100 mM sodium hydroxide, B: 1 M sodium acetate in 100 mM sodium hydroxide, and the gradient was programmed as follows: 0–5 min 0–5% linear B, 23 min 12% B, 30 min 20% B, 31–41 min 100% B, 45–55 min 100% A.

Matrix-assisted laser-desorption time-of-flight mass spectroscopy (MALDI-TOF MS)

A 2 μL of sample solutions (~10 μg/μL treated with DOWEX 50Wx8 cation exchange resin, NH_4^+ form)

were mixed with equal amount of matrix (2% α-dihydroxybenzoic acid in 50% aqueous acetonitrile). The mixture was loaded on a 100-well MS plate, allowed to crystallize and analyzed on Voyager-DE MALDI-TOF MS instrument in linear positive mode. MS instrument configurations were as follows: acquisition mass range 300–3,000 Da, accelerating voltage 25 kV, grid voltage 93%, extraction delay time 175 ns, 100 laser shots/spectrum and 300 shots were collected for each data set.

NMR-spectroscopy

Samples (3–4 mg) were deuterium-exchanged by lyophilization in D_2O and dissolved in 0.2 mL D_2O. 1-D Proton and 2-D COSY, TOCSY, and HSQC spectra were acquired on a Varian Inova-500 MHz spectrometer at 28 or 70 °C using a standard Varian pulse sequence. The TOCSY mixing time was set to 80 ms. Chemical shifts were measured relative to internal acetone ($\delta_H = 2.225$ ppm, $\delta_C = 31.07$ ppm).

Endoxylanase digestion

Samples (3–5 mg each) were suspended in 50 mM ammonium acetate buffer pH 4.5 (3–5 mL), β-endoxylanase from *Aspergillus niger* with specific activity 79.3 U/mg (*Megazyme*) was added and the mixture was incubated at 40 °C. In 48 h, the enzyme was heat-inactivated (90 °C/20 min). Resulting oligosaccharides were desalted with DOWEX50 resin in NH_4^+ form to be analyzed by MALDI-TOF MS.

Deacetylation of xylan

To xylan solution (1 mg/mL) in concentrated solution of NH_4OH was added to pH ~9. The mixture was kept at 80 °C heating block. After 30 min mixture was neutralized with acetic acid and lyophilized for further analyses.

Results and discussion

Yield and glycosyl composition

Table 1 details the yield recoveries for stover and aspen after delignification by both methods. Biomass recovery after delignification by acid chlorite was relatively high for stover and aspen (88.8 vs. 81.5%, respectively), indicating a moderate amount of lignin removal. Complete or extensive lignin removal was not the goal, as this would remove additional carbohydrate and possibly change the nature of the xylan structure. Undoubtedly, some of the xylan remains bound to the remaining lignin, however we felt that minimizing potential alterations to the extracted xylan was more important than higher yields. Delignification by the QPD method resulted in much higher material removal, with biomass yields of 55.9 and 65.9% for stover and aspen respectively. This is likely due to the alkaline conditions of the QPD method extracting hemicellulose as well as a higher fraction of the lignin. Overall xylan yields were calculated based on initial

Table 2 Glycosyl composition

Glycosyl residue	QA	CA	QS	CS
Arabinose	–	–	14.3	5.8
Rhamnose	–	–	–	–
Fucose	–	–	–	–
Xylose	95.0	96.6	66.8	89.0
4-OMe-GlcA	3.8	2.3	1.3	0.6
Galacturonic acid	–	–	–	–
Mannose	–	0.4	–	–
Galactose	0.6	–	1.8	0.3
Glucose	0.7	0.6	15.8	4.2
Total	100.0	99.9	100.0	99.9
% Carbohydrate	67.91	64.86	61.44	82.74

Values are expressed as mole percent of total carbohydrate; –, None detected

Table 1 Yield of crude xylan from delignified biomass

	Delignification method	Biomass yield from bleaching (%)	Yield of xylan from bleached biomass (%)	Overall xylan yield (%)
Corn stover	Acid chlorite	88.8	7.9	39.2
Corn stover	QPD	55.9	5.0	15.9
Aspen	Acid chlorite	81.5	6.7	31.4
Aspen	QPD	65.9	7.5	28.2

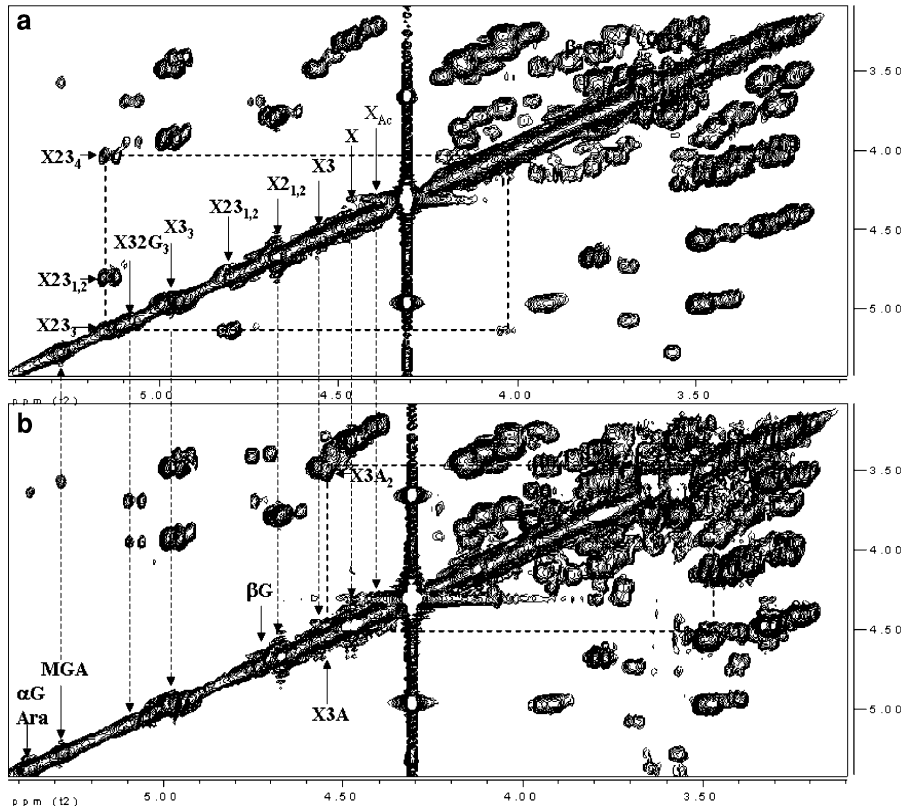

Fig. 1 COSY-spectra of CA (**a**) and CS (**b**), *X2,* 1,4-*β*-(2-OAc)-xylp; *X3*, 1,4-*β*-(3-OAc)-xylp; *X23*, 1,4-*β*-(2, 3-di-OAc)-xylp; *X32G*, 1,4-*β*-[MeGlcA-α-(1 → 2)][3-OAc]-xylp; *X2G*, 1,4-*β*-[MeGlcA-α-(1 → 2)]-xylp; *X3A* 1,4-*β*-[Ara-α-(1 → 3)]-xylp; *X* Unsubstituted 1,4-*β*-xylp; X_{Ac} Unsubstituted 1,4-*β*-xylp adjacent to acetylated xylp; *MGA,* 4-*O*-MeGlcA; *βG*, MLG. Proton numbers of a glycosyl residue are written in *subscripts*. *Cross peaks* of H1/H3, H2/H3 and H3/H4 of X23 (*rectangle* in **a**) are clearly seen in the spectrum of CA. In contrast, there are no signals of X23 in the spectrum of CS. In the COSY of CS, H1/H2 *cross peaks* of t-α-Ara*f* and X3A indicate presence of 1,4-*β*-[Ara-α-(1 → 3)]-xylp; these *peaks* are absent in aspen xylan which is not arabinosylated

Table 3 H1/H2 chemical shift (δ_H/δ_H ppm) assignments of differently substituted 1,4-*β-xyl* residues present in each xylan fraction (COSY and TOCSY spectra)

Fraction	X2	X3	X23	X2G	X3A	X32G
QA	–	–	–	4.64/3.49	–	–
QS	–	–	–	4.64/3.49	4.52/3.48	–
CA	4.67/4.68	4.57/3.49	4.79/4.81	–	–	4.74/3.69
CS	4.67/4.68	4.57/3.49	–	–	4.54/3.48	4.74/3.69
CS deAc	–	–	–	4.64/3.49	4.52/3.48	–
CA deAc	–	–	–	4.64/3.49	–	–

All fractions contain *β*-1,4-linked *xyl* (δ_H/δ_H 4.48/3.29 ppm). COSY of QS contains cross peaks at δ_H/δ_H 5.37/3.64 ppm indicating presence of α-amylose (*compared to NMR-spectra of standard maltooligomers*)

–, Structural unit not present

X2, 1,4-*β*-(2-OAc)-xyl*p*; *X3*, 1,4-*β*-(3-OAc)-xyl*p*; *X23*, 1,4-*β*-(2,3-diAc)-xyl*p*; *X32G*, 1,4-*β*-[MeGlcA-α-(1 → 2)][3-OAc]-xyl*p*; *X2G*, 1,4-*β*-[MeGlcA-α-(1 → 2)]-xyl*p*; *X3A*, 1,4-*β*-[Ara-α-(1 → 3)]-xyl*p*; *CS deAc*, CS deacetylated at alkaline pH; *CA deAc*, CA deacetylated at alkaline pH

xylan contents of the raw stover and aspen of 17.6 and 17.5% respectively and are similar with the exception of the stover/QPD. The high susceptibility of stover xylan and/or lignin to removal during the QPD protocol is likely due to the differences in lignin chemistry or cell wall accessibility between the two species. This is supported by similar aspen xylan yields from both delignification methods. The material generated by the acid-chlorite material was, as expected, soluble in aqueous solutions, indicating that the acetyl side groups remained intact. The QPD-extracted material was mainly insoluble, indicating that the material was deacetylated during extraction, presumably during the alkaline P step. These observations were confirmed by NMR analysis detailed below.

Glycosyl composition analyses indicated that xylan is the major component of all four fractions. Total carbohydrate content of CS is higher (82.7%) compared to others (Table 2). *Glc* content is higher in corn stover fractions due to extraction of some MLG, major hemicellulose in grass cell wall; *Ara* is absent in both aspen fractions. 4-OMe-GlcA was detected in all four fractions. With 95–96% *xyl,* aspen fractions are almost of pure xylan, stover fractions contain 66 and 89% *Xyl* with relatively higher amounts of *Glc* and *Ara* compared to aspen samples. *Ara* and *Glc* contents are higher in QPD-delignified corn stover xylan (QS) compared to chlorite-delignified fraction (CS). This is perhaps due to degradation of Ara*f* during the chlorite treatment; furanoses are more labile at acidic pH. *MeGlcA* to *Xyl* ratio is higher in aspen QPD xylan (1:25) compared to stover xylan (1:44).

Acetylation pattern in aspen and corn stover xylan

Proton spectra of chlorite delignified xylan of both species (CA and CS) showed a signal of CH_3 of acetate ester (δ_H 2.17, 2.21 ppm). COSY spectra (Fig. 1) clearly showed that after chlorite delignification, acetylation is kept intact and as expected, xylan was de-esterified by QPD-delignification.

Anomeric proton signals and H1/H2 cross peaks of 1,4-β-(2-OAc)-xyl*p* (X2), 1,4-β-(3-OAc)-xyl*p* (X3), as well as H3/H2 and H3/H4 of X3 and [MeGlcA-α-(1 → 2)][3-OAc]-xyl*p* (X32G) can be readily assigned from ^{1}H and COSY spectra of CS fraction indicating that stover xylan is monoacetylated at O2 and O3, and some *xyl* residues bear Ac at O3 and *MeGlcA* at O2 at the same time (Fig. 1; Tables 3 and 4). All these signals are present in ^{1}H and COSY spectra of CA fraction as well, though varying in intensity, indicating that aspen xylan possesses the same side chains.

The most noticeable difference in COSY spectra of CA and CS is that the cross peaks at δ_H/δ_H 5.14/4.79 and 5.14/4.05 ppm in COSY spectrum of aspen fraction are absent in corn stover spectrum (Fig. 1). This clearly points out the major difference between xylans of two species: while xylan from delignified cell walls of corn stover contains either 2*O*- and 3*O*-monoacetylated xylan and 3-OAc, 2-GlcA xylan, aspen

Table 4 Proton NMR chemical shift (δ_H ppm) assignments from ^{1}H, COSY and TOCSY spectra of CA, CS, QA and QS fractions

Glycosyl residue	1	2	3	4	5_{ax}	5_{eq}
t-α-L-Ara*f*	5.4	4.16	3.92	4.27	3.80	3.73
4-OMe-α-GlcA	5.27	3.59	3.79	3.29	4.30	
X2G	4.64	3.49	3.68	3.79	4.01	3.38
X3A	4.52	3.49	3.75	3.80	4.13	3.42
X3	4.57	3.49	4.97	3.92	3.48	4.12
X2	4.67	4.68	3.8	3.86	3.44	4.16
X23	4.79	4.81	5.14	4.03	3.53	4.2
X32G	4.74	3.69	5.07	3.95	3.48	4.15
1,4-β-xyl*p*	4.48	3.29	3.58	3.79	4.12	3.45
1,4-β-xyl*p* (adjacent to an OAc-xyl*p*)	4.42	3.22	3.54	3.76	3.38	4.05

δ_H ppm, Chemical shifts of CH_3 protons of *O*-acetyl groups at δ_H 2.16 and 2.20 ppm, CH_3 of *O*-Me at δ_H 3.47 ppm were also detected

xylan contains 2,3-diacetylated xylan in addition to monoacetylated ones. All proton chemical shift values found in ^{1}H, COSY and TOCSY-spectra (Table 4) of acetylated xylans from chlorite delignified fractions were consistent with those reported for Eucalyptus wood xylan (Evtuguin et al. 2003).

In case of chlorite-delignified samples, both fractions are water-soluble, but QPD-delignified xylans are only partially water-soluble. Acetylation is likely the factor contributing to solubility of xylan from chlorite delignified biomass.

Hemicelluloses such as xyloglucan and GAX and pectic cell-wall components often are *O*-acetylated. Examples are *O*-acetyl groups on the glucan backbone of xyloglucan or on xylan backbone of GAX and GX. The degree of *O*-acetylation of pectins varies from 0 to 90% depending on the tissue, species, and method of preparation. The role of *O*-acetyl substituents in vivo is not known, but in vitro experiments suggest that one function may be their involvement in hindering enzymatic polysaccharide breakdown. *O*-acetyl substituents also affect polysaccharide solubility and pectin's gelation properties (Pauly and Scheller 2000).

Identification of gene products catalyzing polysaccharide acetylation and the determination of the role of acetylation in cell wall structure and function will provide insights into the possibility of developing biomass crop varieties with significantly reduced polysaccharide acetylation and thus improving the fermentation process.

Corn stover xylan is arabinosylated as other Poaceae xylan

In accordance with the glycosyl composition data the ^{1}H spectra of CS and QS indicate presence of t-α-Ara*f* (narrow doublet at ~5.4 ppm). The peak is absent in the spectra of CA and QA (spectra not shown). In the HSQC spectrum, the proton is directly bonded to C at 108.5 ppm which is consistent with the reported values for t-α-Ara*f*. The HSQC also provided additional evidence for the presence of t-α-Ara*f* in stover xylan and the absence of it in

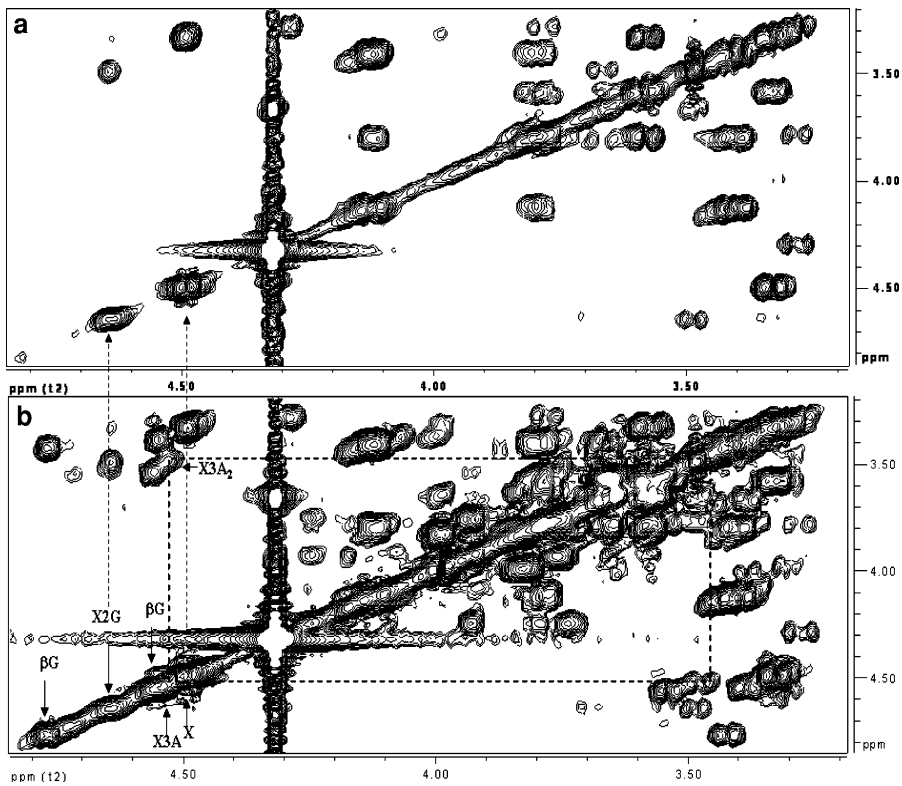

Fig. 2 COSY-spectra of QA (**a**) and QS (**b**). *Cross peak* of *X3A* unit (*rectangle* in QS, **b**) is absent in QA (**a**) indicating that aspen xylan is not arabinosylated. Spectrum of QA is much simpler compared to QS; *X2G* and 4-linked xyl*p* are major components in aspen fraction, whereas spectrum of QS is more complicated by the presence of *X3A* and MLG in addition to *X2G* and *X*. Along with GAX, MLG are a major hemicellulose in cereal and grass cell walls

aspen fractions. In contrast to the HSQC spectrum of QS, CH-5_{ax} and CH-5_{eq} (δ_C/δ_H 61.74/3.80 and 3.72 ppm) as well as CH-2 (81.6/4.16 ppm) and CH-4 (85.65/4.27 ppm) signals of t-α-Ara*f* are absent in the spectrum of QA.

The attachment t-α-Ara*f* at O3 position of xyl*p* was confirmed by the presence of chemical shifts characteristic of [Ara*f*-α-(1 → 3)]-xyl*p* (X3A) (Roubroeks et al. 2000). In COSY spectra of CS and QS, anomeric H signal at 4.52 ppm correlates with H2 at 3.45 ppm; combined results of COSY and TOCSY revealed that H3 signal is shifted downfield to 3.75 ppm compared to unsubstituted 1,4-β-xyl*p*. The same spin system is also present in TOCSY spectrum of deacetylated CS and intact QS fractions establishing that it belongs to X3A unit. Also in our (unpublished) study of GAX9—an oligomer isolated from endoxylanase digest of corn stover alkaline peroxide pretreatment liquor, it was shown by NOESY to have t-α-Ara*f* attached to the C3 of 1,4-β-xyl*p*. According to NMR-data there is no indication of [Ara*f*-α-(1 → 3)][2-*O*–Ac]-xyl*p*—a detail of GAX structure that has not been reported for *Poales* xylan.

Anomeric proton chemical shift at 5.28 ppm in ^{1}H-spectrum and a cross peak at δ_H/δ_H 5.28/3.59 ppm in COSY spectrum confirms the presence of MeGlcA in corn stover xylan. There is clear correlation between the H1 and other protons, except H5, of MeGlcA in TOCSY spectrum. Correlations between H5/H4, H5/H3 and H5/H2 (δ_H/δ_H 4.30/3.29, 4.30/3.79 and 4.30/3.59 ppm respectively) of MeGlcA enabled identification of H5 chemical shift. The HSQC spectrum of QS supports 2D homonuclear spectral data; chemical shifts of CH-1 (δ_C/δ_H 98.4/5.28 ppm), CH-4 (δ_H/δ_C 83.16/3.29 ppm), CH-5 (δ_C/δ_H 74.02/4.30 ppm) and CH_3 of OMe group (δ_C/δ_H 60.67/3.47 ppm) are in good agreement with those reported (Teleman et al. 2000; Dinand and Vignon 2001). In addition to cross peaks indicative of X32G in spectra of CS and CA, cross peak at δ_H/δ_H 4.64/3.49 ppm in the COSY spectra of deacetylated CS and CA confirms attachment of MeGlcA at O2 position of 4-β-xyl*p* (Teleman et al. 2000; Dinand and Vignon 2001; Evtuguin et al. 2003).

After alkaline treatment of CS, NMR-spectroscopic profile of deacetylated fraction (Fig. 3b)

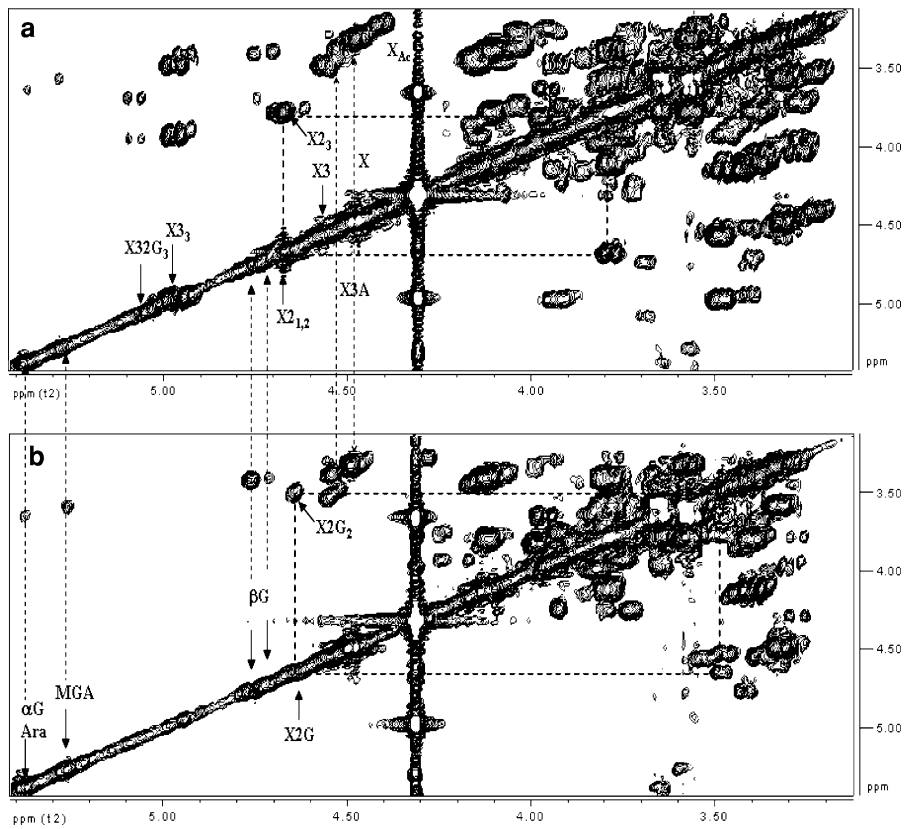

Fig. 3 COSY-spectra of CS (**a**) and its deacetylated product (**b**): cross peaks in anomeric region. **a** COSY spectrum of CS—DMSO-solubilized xylan from chlorite delignified corn stover. **b** COSY spectrum of CS after de-acetylation. *Abbreviations:* see Fig 1 legend. After de-acetylation cross-peaks belonging to acetylated xylan (*X2*, *X3*, *X23*, *X32G* and *XAc*) (**a**) have disappeared from the spectrum (**b**) and *cross-peak* characteristic of *X2G* H1/H2 (δ_H/δ_H 4.64/3.50 ppm) appeared in the spectrum of de-acetylated product

turned out to be similar to that of QPD-xylan or QS (Fig. 2b). The COSY-spectrum of deacetylated CS (Fig. 3b) became much simpler: cross-peaks indicative of 1,4-β-(2-MeGlcA)-xyl*p* (X2G) have appeared in the spectrum along with mixed-linkage glucan peaks. Comparison of NMR-spectra of intact and deacetylated CS fraction (Fig. 3a, b) indicates that [MeGlcA-(1 → 2)][Ara-α-(1 → 3)]-xyl*p* units are absent in the CS.

Analyses of endoxylanase digestion products

HPAEC analyses indicated that from factions CA and CS endoxylanase produced series of oligomers and only a few oligomers were generated from QA and QS (Fig. 4). Control HPAEC analysis of intact samples run with the same gradient shows no oligosaccharide peaks (chromatograms not shown). Based on NMR-spectroscopic data of polymeric fractions, it can be predicted that the enzyme digestion products of CA will be 2,3-di-,2- or 3-monoacetylated and 3-Ac, 2-GlcA-substituted xylan oligomers. Products of CS will be 2- or 3-monoacetylated; 3-Ac, 2-GlcA-substituted; and 3-*Ara*-substituted xylo-oligomers. Also GAX oligomers should be produced from QS, and GX oligomers should be generated from QA by endoxylanase action. Subsequent MALDI-TOF MS analyses revealed the MW of the above oligomers in the digestion mixtures (Fig. 5a–d; Table 5).

HPAEC analyses (Fig. 4) have shown that series of oligomers produced by endoxylanase action from aspen chlorite-delignified xylan (CA) are of higher DP compared to CS indicating that aspen xylan is more resistant to enzyme digestion compared to stover xylan. The higher degree of acetylation of aspen xylan likely makes it more resistant to enzyme

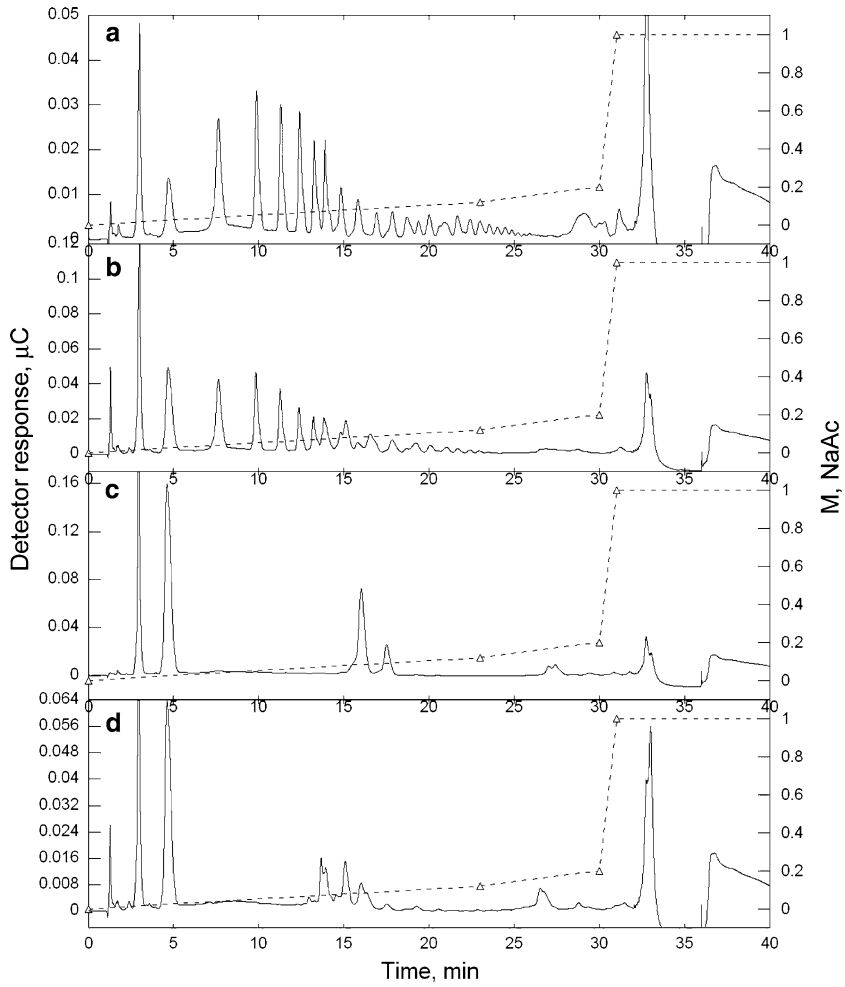

Fig. 4 HPAEC of endoxylanase digests of CA, CS, QA, QS. *Dotted lines* indicate ammonium acetate gradient concentration (*M*). **a** CA; **b** CS; **c** QA; **d** QS, **e** standard xylose (XYL1), xylobiose (XYL2) and xylotriose (XYL3). HPAEC indicates endoxylanase-produced series of oligomers from CA and CS. Note that oligomers in CA are of higher DP compared to CS indicating that compared to stover xylan, aspen xylan is more resistant to enzyme digestion due to its higher acetylation degree. Xylan from QPD delignified aspen produces fewer oligomers with DP ~9–11, QPD-stover xylan oligomers are of DP ~6–9 and 10

digestion. QPD-delignified xylan, however, produces few oligomers with DP ~9–11 for aspen, DP ~6–9 for stover xylan.

There are no monoacetylated xylan oligomers in MALDI-TOF MS of CA endoxylanase digestion products (Fig. 5b), while in CS there are monoacetylated

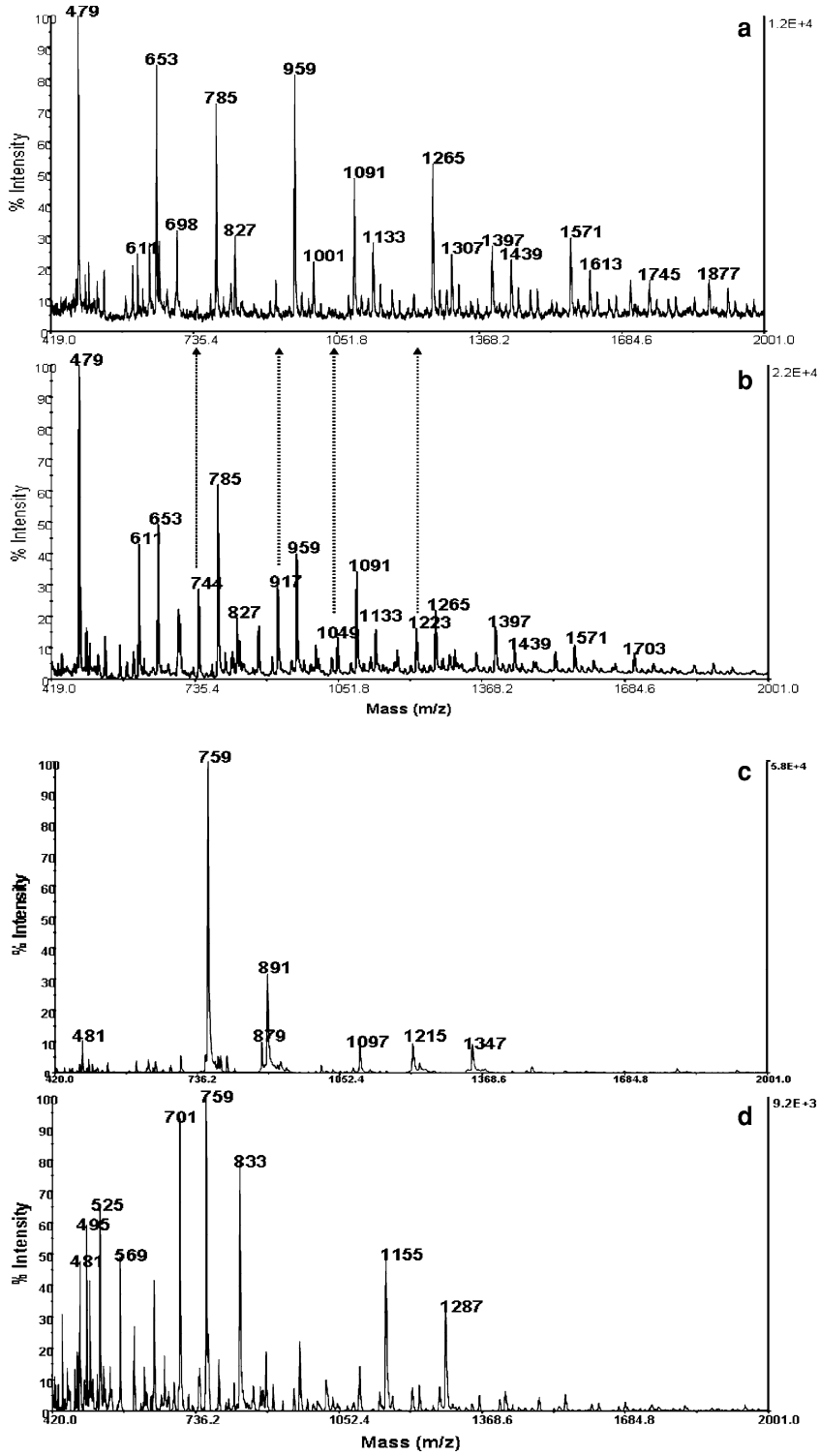

Fig. 5 MALDI-TOF MS of endoxylanase digests. **a** CA; **b** CS; **c** QA; **d** QS. All peaks are $[M+Na]^+$. *Arrows* point to the *peaks* absent in the MS of CA compared to CS; since aspen xylan contains diacetylated xyl*p* residues, there are no monoacetylated oligomer peaks for lower oligomers and six no diacetylated peaks of higher oligomers in contrast to stover xylan. See Tables 5 and 6 for the oligomer masses

Table 5 Oligosaccharides identified in endoxylanase digests of xylan from chlorite-delignified biomass by MALDI-TOF MS

Oligomers	Number of OAc groups, n/[M + Na]$^+$					
	1	2	3	4	5	6
Pen_3Ac_n	479					
Pen_4Ac_n	611	653				
Pen_5Ac_n	*744*	785	827			
Pen_6Ac_n		*917*	959	1,001		
Pen_7Ac_n		*1,050*	1,091	1,133		
Pen_8Ac_n			*1,223*	1,265	1,307	
Pen_9Ac_n				1,397	1,439	
$Pen_{10}Ac_n$					1,571	1,613
$Pen_{11}Ac_n$						1,745
$Pen_{12}Ac_n$						1,877
Pen_4Ac_nMGA	*801*					
Pen_5Ac_nMGA		975				
Pen_6Ac_nMGA		1,107	1,149	1,191		
Pen_7Ac_nMGA			*1,281*	1,323		
Pen_8Ac_nMGA	*1,329*		*1,414*	1,456	1,498	
Pen_9Ac_nMGA					1,630	
$Pen_{10}Ac_nMGA$			*1,678*			1,803
$Pen_{11}Ac_nMGA$						1,935

Italic [M + Na]$^+$ values are found only in enzymatic digest of stover fraction CS

Pen, Xyl in CA and QA fractions, Xyl or Ara in CS and QS fractions

MGA, 4-OMeGlc*p*A

lower MW xylan oligomers (up to Pen_4- and Pen_5-based) (Fig. 5a). The data is consistent with our findings by NMR-spectroscopy that in addition to monoacetylation, there are 2,3-*O*-diacetylated xyl*p* units in aspen xylan while stover xylan is only monoacetylated at either C-2 or C-3.

Since stover xylan is arabinosylated, endoxylanase digestion of stover xylan (QS) results in longer pentosyl oligomers carrying single MeGlcA compared to the shorter ones generated from aspen xylan (QA) (Table 6; Fig. 5c, d): instead of Pen_7MGA and Pen_8MGA in endoxylanase digestion products of stover xylan, Pen_6MGA_2 and Pen_7MGA_2 are present in aspen xylan digestion products. The structure of $Xyl_7AraMGA$ (GAX9) isolated from enzyme digestion products of corn stover alkaline peroxide pretreatment liquor *(our unpublished results)* indicated that *Ara* and *MeGlcA* substituents on adjacent *Xyl* residues may have inhibited the enzyme action.

Table 6 Oligosaccharides identified in MALDI-TOF MS spectra of endoxylanase digests of xylan from QPD-delignified biomass

Fraction	Oligomer	[M + Na]$^+$
QA	Pen_4MGA	759
	Pen_5MGA	891
	Pen_6MGA_2	1,215
	Pen_7MGA_2	1,347
	Pen_8	1,097
QS	Pen_4	569
	Pen_4MGA	759
	Pen_5	701
	Pen_6	833
	Pen_7MGA	1,155
	Pen_8MGA	1,287

QA, xylan from QPD-delignified aspen; *QS*, xylan from QPD-delignified stover

Table 7 Relative amount (% total *xyl*) of xylan structural units in each fraction

Xyl*p* residue	CA	CS	QA	QS
X	49.6	64.5	91.3	86.3
X2	16.0	10.4	–	–
X3	17.5	17.0	–	–
X23	11.7	–	–	–
X3G2	5.2	3.1	–	–
X2G	–	–	8.7	4.3
X3A	–	5.0	–	9.4

Percentage amount calculated based on proton NMR-spectra integration

–, None detected

No evidence was obtained to indicate presence of 1,4-β-D-(2-MeGlcA,3-Ara)-xyl*p* units in QPD-delignified corn stover.

Based on the evidences obtained, structural units and their relative amount in DMSO-soluble heteroxylans of two species delignified by two distinct methods are presented in Table 7.

Conclusion

There is limited or no report on corn stover xylan structure compared to those of other grasses and cereals such as wheat, barley, rye bran and rice straw

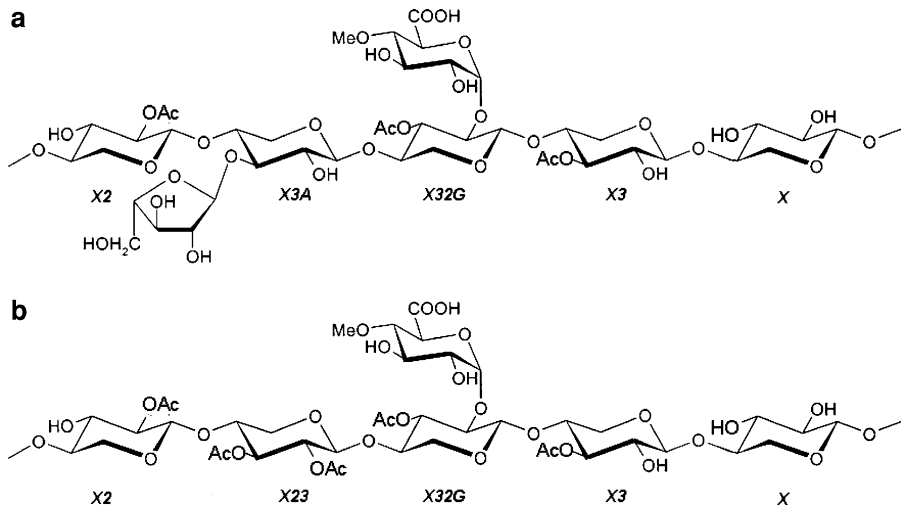

Fig. 6 Xylan structural units* in corn stover xylan (**a**) and aspen xylan (**b**). * The figure does not reflect the actual sequence the structural units are linked together in the polymer. As noted above isolated unsubstituted xyl residues may be separated by one or two substituted residues and this pattern is interrupted with longer unsubstituted sequences. *Abbreviations*: see Fig. 1 legend. Fine details of corn stover native-like xylan can be elucidated by studying individual GAX oligomer structures purified from enzyme digests or partial hydrolyzates of CS fraction

(Sun et al. 1996; Roubroeks et al. 2000; Kabel et al. 2002).

There are a few studies of corn cob xylan (Hroma'dkova' et al. 1999; Huisman et al. 2000; Lapierre et al. 2001; Zhu et al. 2006); however *O*-acetyl substituents were removed in most cases by dilute alkali treatments used. Corn cob xylan structure is generally consistent with the GAX structure reported for *Poaceae* cell walls such as bamboo (Ishii and Hiroi 1990), wheat straw (Sun et al. 1996), sorghum (Huisman et al. 2000): backbone 4-β-xyl residues are substituted with t-α-Ara*f* at *O*-3 and with MeGlcA at *O*-2 positions in addition to 2-*O*- and 3-*O*-monoacetylated *Xyl* residues. In corn stover xylan, we found no evidence of 2,3-di-*O*-acetylation or evidence that a single *Xyl* is substituted with *Ara* and OAc group at the same time.

MeGlcA side chains in stover xylan are scarcer ($\sim$1:44) compared to that of dicot or hardwood species ($\sim$1:26).

The distribution pattern of side chains in heteroxylans is suggested to be non-random and may reflect, together with the variety of primary structural features, the functional diversity of xylan in plants. Xylans from hardwood species show a broad range of MeGlcA/Xyl ratios of 1:4–16 (Ebringerová et al. 2005). Wheat arabinoxylans, also those of barley, malt and sorghum show a non-random distribution pattern of side chains. Isolated unsubstituted xylose residues are separated by one or two substituted residues and this pattern is interrupted with longer unsubstituted sequences (Gruppen et al. 1993; Jacobs et al. 2001). The data presented here for corn stover xylan structure is in good agreement with these models; CS fraction from corn stover is 2- and 3-*O*-monoacetylated glucuronoarabinoxylan which is typical of the lignified secondary cell walls of monocots.

Structural details of aspen xylan fractions obtained after chlorite delignification process are similar to those obtained by microwave treatments (Teleman et al. 2000). Compared to microwave treatment reported chlorite-delignification yields native acetylated glucuronoxylan of much higher MW.

Based on the findings presented structural units of aspen and stover xylan can be depicted as in the Fig. 6.

Fine details of corn stover native-like xylan can be elucidated by studying an individual GAX oligomer structures purified from enzyme digests or partial hydrolyzates of CS fraction.

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