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Carbohydrate Research 304 (1997) 11–20

CARBOHYDRATE RESEARCH

Structural characterization of novel L-galactose-containing oligosaccharide subunits of jojoba seed xyloglucans

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Received 5 May 1997; accepted 30 June 1997

Abstract

Jojoba seed xyloglucan was shown to be a convenient source of biologically active xyloglucan oligosaccharides that contain both L- and D-galactosyl residues [E. Zablackis et al., *Science*, 272 (1996) 1808–1810]. Oligosaccharides were isolated by liquid chromatography of the mixture of oligosaccharides generated by treating jojoba seed xyloglucan with a β-(1 → 4)-endoglucanase. The purified oligosaccharides were reduced with $NaBH_4$, converting them to oligoglycosyl alditol derivatives that were structurally characterized by a combination of mass spectrometry and 2-dimensional NMR spectroscopy. This analysis established that jojoba xyloglucan oligosaccharides contain the novel side-chain [α-L-Gal*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)-], which is structurally homologous to the fucose-containing side-chain [α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)-] found in other biologically active xyloglucan oligosaccharides. © 1997 Elsevier Science Ltd.

Keywords: Xyloglucan; L-Galactose; Oligosaccharide; Oligosaccharin; Jojoba

1. Introduction

Xyloglucan is the predominant hemicellulosic component of the primary cell walls of most higher plants [1–3]. The xyloglucan backbone is composed of (1 → 4)-linked β-D-Glc*p* residues, approximately 75% of which are substituted at O-6 with α-D-Xyl*p* residues [4,5]. In xyloglucans produced by non-solanaceous dicots, many of the α-D-Xyl*p* residues are themselves substituted at O-2 with either β-D-Gal*p* or α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*.

Xyloglucans have the ability to noncovalently cross-link cellulose microfibrils, forming a XG–cellulose network that acts as a load bearing structure within the growing cell wall [4–6]. The unbranched β-D-Glc*p* residues of xyloglucans are susceptible to endolytic cleavage by plant enzymes, and it has been

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PII S0008-6215(97)00200-0

proposed that these enzymes lead to a loosening of the cell wall that is necessary for plant cell growth [4,5,7,8]. The unbranched β-D-Glc*p* residues are also hydrolyzed *in vitro* by various microbial endoglucanases, permitting the polysaccharide to be broken down into xyloglucan oligosaccharides (XGOs) whose structures can be completely characterized [9,10].

Several XGOs have been shown to have regulatory properties, and are therefore classified as oligosaccharins [11]. One such fucose-containing XGO (the nonasaccharide XXFG, see *Nomenclature* in the Materials and methods section) has been shown [12] to inhibit, with an optimum concentration of 10^{-8}–10^{-9} M, the 2,4-dichlorophenoxyacetic acid-stimulated growth of pea-stem sections *in vitro*. The α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl*p* side-chain appears to be necessary for this inhibition, but the biochemical mechanism for this activity is not known.

The *Arabidopsis thaliana* mutant *mur1* incorporates very little fucose into the primary cell walls of its aerial tissues and thereby represents a useful system for studying the role of the fucosyl residue in the regulatory activity of XGOs [13]. An unusual XGO (the decasaccharide XLJG) was purified from *mur1* xyloglucan and structurally characterized [13]. XLJG represents a new class of XGOs that contain an α-L-Gal*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl*p* side-chain, which is structurally homologous to the α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl*p* side-chain present in wild-type plants. The presence of L-galactose in this structure may compensate for the loss of L-fucose (i.e., 6-deoxy-L-galactose) in the mutant *mur1* plant [13].

Due to the small size of the *A. thaliana* plant, it is technically challenging to isolate XGOs from *mur1* in amounts sufficient for complete structural characterization. Previous analyses [14] of jojoba (*Simmondsia chinensis*) seed xyloglucan indicated the presence of oligosaccharides that have a Gal–Gal–Xyl side-chain such as those found in *mur1* xyloglucan, but the absolute and anomeric configurations of the terminal Gal residue of this side-chain were not determined. This paper describes the preparation of milligram amounts of two oligosaccharides, XXJG and XLJG, from jojoba seeds. Chemical characterization of these oligosaccharides indicated the presence of the same unusual L-galactose-containing side-chain that was found in *mur1* XGOs. Of particular interest is the structural characterization of XXJG, an XGO having the same growth inhibitory activity in the pea-stem elongation assay as XXFG [13].

2. Materials and methods

Nomenclature.—Primary structures of xyloglucan oligosaccharides are expressed as a series of uppercase letters, each of which represent an individual β-D-Glc*p* residue in the backbone and its pendant side-chains [15]. The letter "G" represents a β-D-Glc*p* with no side-chains attached. The letters "X", "L", and "F" represent β-D-Glc*p* residues substituted at O-6 with a terminal α-D-Xyl*p* residue, the diglycosyl side-chain β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)-, and the triglycosyl side-chain α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)-, respectively. We assign the letter "J" (mnemonic Jojoba) to represent β-D-Glc*p* with the novel α-L-Gal*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)- side-chain at O-6. Glucitol residues in oligoglycosyl alditols are indicated as "Gol". The structure of a xyloglucan oligosaccharide is written by starting at the nonreducing end and listing these code letters from left to right. Thus, the oligoglycosyl alditol derivative of the novel nonasaccharide isolated from jojoba is XXJGol (Fig. 1).

Specific residues of a xyloglucan oligoglycosyl alditol are indicated by superscript lowercase that reflect the position of each residue *vis á vis* the D-glucitol moiety. The order of backbone β-D-Glc*p* residues (starting with the nonreducing terminus and progressing toward the glucitol) is thus $Glc^{c} \rightarrow Glc^{b} \rightarrow Glc^{a} \rightarrow$ Glcol. Side-chain residues are explicitly indicated by using the superscript letter of the β-D-Glc*p* residue to which the side-chain is attached (Fig. 1).

Xyloglucan oligosaccharides (XGOs).—Whole, dry jojoba seeds (15 g) were ground to a fine powder and then extracted with 500 mL of hexane with constant stirring for 12 h. The solid residue was collected by filtration through a glass microfiber filter, and traces of hexane were removed by evaporation. The resulting powder was suspended in 200 mL of distilled water, stirred for 24 h at 4 °C, and then centrifuged (18,000 × *g*, 1 h). The supernatant was then removed, and the pellet was suspended in 200 mL of 4 M KOH under constant stirring for 24 h. The resulting solution was then filtered through sintered glass, the filtrate was neutralized (pH ~ 7) with acetic acid and then centrifuged (18,000 × *g*, 30 min). The supernatant was dialyzed (6000–8000 MWCO tubing, Spectra/Por) against water (6 changes of 9 L, 4 °C) and then concentrated (~ 100 mL final volume) by rotary evaporation. Abs ethanol was added

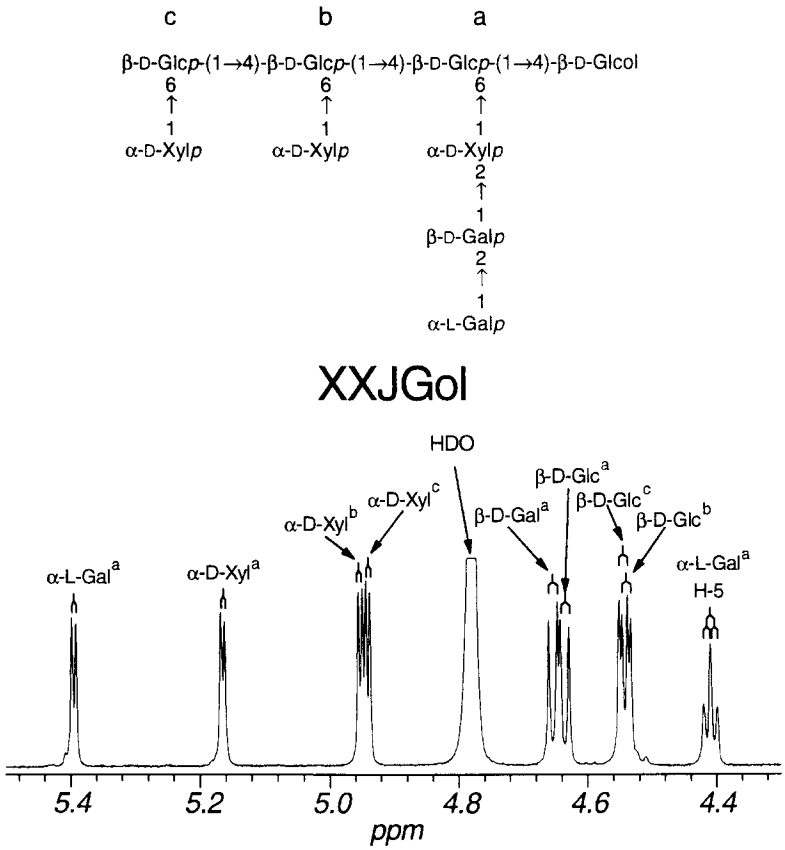

Fig. 1. Structure and partial 600-MHz ^{1}H NMR spectrum of XXJGol. All resonances in this region are assigned as anomeric (H-1) protons of the indicated residues except H-5 of the α-Gal*p* residue (δ 4.406). Lowercase letters a, b, and c indicate the positions of individual residues within the oligoglycosyl alditol.

to a final concentration of 30% (v/v) to precipitate the xyloglucan, which was collected by centrifugation (18,000 × *g*, 30 min). Residual ethanol in the pellet was evaporated under vacuum. The residue was dissolved in 10 mM NaOAc buffer and treated with endo-β-(1 → 4)-D-glucanase (EC 3.2.1.4, Megazyme, Australia, 5 U/mg of xyloglucan, 24 h, 20 °C). Anionic polysaccharides, pigments, and the endoglucanase were removed from the digested xyloglucan by passing it through a 4 × 12-cm column of Q-Sepharose fast-flow anion-exchange resin (Pharmacia) eluted with 10 mM imidazole buffer, pH 7. The eluant was concentrated (final volume 5 mL) by rotary evaporation, and desalted on a 2 × 80-cm column of Sephadex G-10 (Pharmacia).

The salt-free, XGO-containing fractions were concentrated to 2 mL by rotary evaporation (Rotovap, Buchi), applied to a high-resolution Bio-Gel P-2 column (400 mesh), and eluted with water, as described [10]. The hexose content of the eluant fractions (1.5 mL) was determined by the anthrone assay (Fig. 2). Fractions corresponding to peaks of hexose-positive material were pooled as indicated and concentrated. The pooled fractions were further fractionated by reversed-phase HPLC on C_{18} silica gel (25.0 × 1.0 cm, Lichrosorb RP-18, E. Merck). XGOs were eluted with 15% aq MeOH (3 mL/min), detected by monitoring the refractive index (RI, Fig. 3), and analyzed by MALDI-TOF mass spectroscopy (Table 1). HPLC-purified XGOs were reduced to the corre-

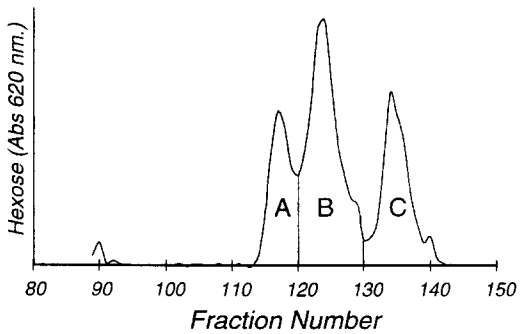

Fig. 2. Bio-Gel P-2 chromatography of oligosaccharides generated by endoglucanase treatment of jojoba seed xyloglucan. Fractions **A**, **B**, and **C** were pooled as indicated, and further separated by reversed-phase HPLC (see Fig. 3).

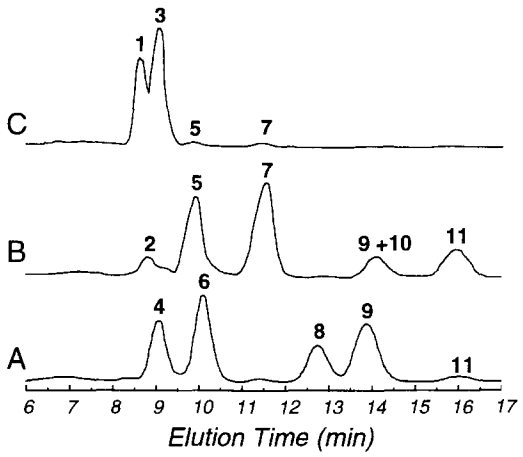

Fig. 3. Reversed-phase HPLC of Bio-Gel P-2 fractions **A**, **B**, and **C** (Fig. 2) on C_{18} silica gel eluted with 15% aqueous methanol. Individual peaks were pooled and analyzed by MALDI-TOF-MS. Each peak is labeled with a number that (referring to Table 1) indicates the structure of its most abundant component. Each oligosaccharide in the mixture gives rise to two peaks, corresponding to the two anomeric forms of its reducing Glc residue.

sponding oligoglycosyl alditols by treatment with $NaBH_4$ [16].

Partial hydrolysis of XXJG.—Peak 7 (XXJG, 0.5 mg) was dissolved in trifluoroacetic acid (1 N, 1.0 mL, 37 °C, 144 h), and the solvent was then evaporated. The resulting mixture of oligosaccharides was analyzed by MALDI-TOF-MS. These oligosaccharides were also separated by HPLC on a Phenomenex Prodigy octadecyl silica gel column (250 × 4.6 mm) eluted at 1.0 mL/min with a linear gradient from 6 to 15% aq MeOH over 60 min, detected by evaporative light scattering (SEDEX 55, Cedere, France), and then analyzed by NMR spectroscopy at 500 MHz [17].

NMR spectroscopy.—Reducing oligosaccharides and oligoglycosyl alditols (1–5 mg) were dissolved in D_2O and lyophilized to replace exchangeable protons with deuterons. NMR spectra were recorded in D_2O at 300 K (HDO line at δ 4.75 ± 0.01 relative to internal acetone at δ 2.225). 1D ^1H and ^{13}C spectra, double-quantum filtered COSY [18], TOCSY [19], and NOESY [20,21] spectra were recorded with a Bruker AMX-500 NMR spectrometer. HSQC [22] and HMBC [23] spectra were recorded with a Bruker DRX-600 NMR spectrometer using pulsed field gradients for coherence selection [24]. All 2D spectra were recorded at high resolution (< 1 Hz/pt) in the directly detected ^1H dimension, allowing chemical shifts to be measured to within less than 0.002 ppm. The mixing times for TOCSY and NOESY were 103 and 500 ms, respectively.

Absolute configuration analysis.—The absolute configurations of the monosaccharide constituents of XXJGol and of XXLG (obtained by partial hydrolysis of XXJG, see above) were determined by comparing the ^1H NMR spectra of the per-*O*-(*S*)-2-methylbutyrate derivatives to authentic standards [25]. Oligoglycosyl alditols (0.5–1.0 mg) and standard D- and L-sugars (1.0 mg) were both treated with aq CF_3COOH (2 N, 200 μL, 121 °C, 90 min), and the solvent was then evaporated. Traces of borate that

Table 1
Oligosaccharides isolated from jojoba seed xyloglucan

HPLC [a] Peak	Assigned structure [b]	Observed [c] $[M + Na]^+$	Calculated [d] $[M + Na]^+$	t_R [a] (min)
1	XXXG(α)	1084.4	1085.9	8.4
2	XXLG(α)	1246.4	1248.1	8.6
3	XXXG(β)	1085.4	1085.9	8.8
4	XLJG(α)	1572.4	1572.4	9.0
5	XXJG(α) [e]	1408.6	1410.2	9.8
6	XLJG(β)	1571.2	1572.4	10.0
7	XXJG(β)	1408.6	1410.2	11.4
8	XLFG(α)	1554.8	1556.4	12.7
9	XLFG(β)	1554.8	1556.4	13.9
10	XXFG(α)	1393.0	1394.2	14.1
11	XXFG(β)	1391.9	1394.2	16.0

[a] Reversed-phase HPLC on C_{18} silica, Fig. 3.
[b] See *Nomenclature* in the Materials and methods section and in ref. [15].
[c] Mass-to-charge ratio (*m/z*) for quasimolecular ion observed by MALDI-TOF-MS.
[d] Calculated chemical mass of quasimolecular ion.
[e] Peak 5 contained small amounts of XXLG, $[M + Na]^+$ *m/z* = 1248.1.

had been introduced during the borohydride reduction (see above) were removed by alternately adding and evaporating 9:1 MeOH–CH_3COOH (v/v, 4 additions) and neat methanol (2 additions). (*S*)-(+)-2-Methylbutyric anhydride (100 μL, Aldrich) and pyridine (100 μL, Aldrich) were added, the reaction was incubated at 121 °C for 4 h in a sealed tube, and the solvents were then evaporated. Toluene (300 μL) was added and evaporated, and the sample was then dissolved in CH_2Cl_2 (1 mL) and extracted three times with 2 M Na_2CO_3 (2 mL). The CH_2Cl_2 solution, containing the per-*O*-(*S*)-2-methylbutyrate derivatives, was then extracted with water (2 mL) and the lower (CH_2Cl_2) phase was transferred to a clean glass tube and concentrated by evaporation. The sample was dried by adding and evaporating isopropanol (300 μL). The per-*O*-(*S*)-2-methylbutyrate derivatives were then dissolved in acetone-d_6 (Cambridge Isotope Laboratories), and their 600-MHz ^1H NMR spectra were recorded at 298 K (internal reference, acetone-d_5, δ 2.05) with a Bruker DRX-600 spectrometer.

3. Results and discussion

Isolation of xyloglucan oligosaccharides (XGOs) from jojoba seed.—XGOs were generated by endoglucanase treatment of xyloglucan extracted from defatted jojoba seed meal. Gel-permeation chromatography of the XGOs on Bio-Gel P-2 (Fig. 2) yielded three fractions, **A**, **B**, and **C**, whose most abundant components were deca-, nona- and heptasaccharides, respectively (see the next paragraph). Individual XGOs were obtained by reversed-phase HPLC of the P-2 fractions on C_{18} silica gel (Fig. 3). Each XGO gives rise to two peaks in the chromatogram because its reducing glucose residue can adopt either the α or β configuration, resulting in a pair of mutarotational isomers that are separated under the conditions used. The chromatogram can be simplified [10] by reducing the XGOs with $NaBH_4$, thereby converting them to oligoglycosyl alditol derivatives which cannot undergo mutarotation. However, the oligoglycosyl alditol derivatives of those jojoba seed XGOs that lack a fucosyl residue are difficult to separate by reversed-phase HPLC; thus fractions **A**, **B**, and **C** were subjected to reversed-phase HPLC (Fig. 3) without prior $NaBH_4$ treatment.

The primary structures of the HPLC-purified XGOs were assigned (Table 1) by analyzing their chromatographic behavior, MALDI-TOF mass spectra, and NMR spectra. Interpretation of the MALDI-TOF mass spectra is complicated by the appearance of both $[M + Na]^+$ and $[M + K]^+$ quasimolecular ions because, for example, the XXJG $[M + Na]^+$ ion has essentially the same chemical mass (*m/z* 1410) as the XXFG $[M + K]^+$ ion. However, the relatively nonpolar character of the fucosyl residue results in a significant increase in the reversed-phase HPLC retention times for XGOs containing this glycosyl constituent [10]. This makes it possible to distinguish XXJG (Peaks 5 and 7) from XXFG (Peaks 10 and 11) on the basis of their chromatographic behavior. For example, Peaks 7 and 11, corresponding to XXJG(β) and XXFG(β), respectively, were analyzed by reversed-phase HPLC under the same conditions used for their isolation (data not shown). Each of the resulting chromatograms included two dominant peaks corresponding to the α- and β-anomeric forms of the XGO, indicating that the XGO had undergone mutarotation to form the mixture subsequent to its isolation as the pure β anomer. In each case, the more thermodynamically stable β configuration was assigned to the anomer with the longer retention time, based on the relative peak areas for the two isomers. (Compare Peaks [1 and 3], [4 and 6], [5 and 7], [8 and 9], and [10 and 11], Fig. 3.)

The structural assignment of oligosaccharides (α and β)-XXXG, (α)-XXLG, (α and β)-XLFG, and (α and β)-XXFG (i.e., Peaks 1–3 and 8–11) were confirmed by comparing their ^1H NMR spectra to those of authentic standards [10]. Peaks 4–7 represent novel XGOs that were further characterized. These XGOs were converted to their oligoglycosyl alditol derivatives by reduction with $NaBH_4$ in order to facilitate their structural characterization by NMR [10]. COSY [18] and TOCSY [19] spectra of the oligoglycosyl alditols were recorded and proton resonances were assigned to specific, isolated spin systems (i.e., individual glycosyl residues, Table 2) by tracing the scalar connectivity in these 2D spectra. This analysis confirmed the presence of the α-galactosyl residues in jojoba seed xyloglucan and resulted in a more complete assignment of the ^1H resonances of XLJGol (Peaks 4 and 6), which had been previously characterized after isolation from the *A. thaliana* mutant *mur1* [13]. The NMR spectrum (Fig. 1) of XXJGol (Peaks 5 and 7) has not previously been analyzed in detail because XXJGol is difficult to isolate from *A. thaliana*, representing less than 10% of the *mur1* xyloglucan [13]. Therefore, a thorough spectroscopic analysis of the structure of

Table 2
^{1}H assignments * for jojoba xyloglucan oligoglycosyl alditols

Residue	H-1′	H-1	H-2	H-3	H-4	H-5e	H-5a	H-6	H-6′
XXJGol									
α-L-Gal [a]	—	5.395	3.839	3.891	3.999	—	4.406	3.765	3.765
β-D-Gal [a]	—	4.655	3.771	3.891	3.917	—	3.678	3.790	3.770
α-D-Xyl [a]	—	5.165	3.677	3.778	3.643	3.712	3.577	—	—
α-D-Xyl [b]	—	4.953	3.545	3.737	3.620	3.731	3.568	—	—
α-D-Xyl [c]	—	4.941	3.542	3.731	3.610	3.713	3.548	—	—
β-D-Glc [a]	—	4.637	3.425	3.675	3.605	—	3.893	3.954	3.874
β-D-Glc [b]	—	4.541	3.405	3.670	3.730	—	3.834	4.007	3.896
β-D-Glc [c]	—	4.545	3.340	3.519	3.520	—	3.690	3.940	3.777
Glcol	3.760	3.680	3.920	3.891	3.966	—	3.966	3.749	3.860
XLJGol									
α-L-Gal [a]	—	5.399	3.840	3.892	4.000	—	4.408	3.769	3.769
β-D-Gal [a]	—	4.653	3.772	3.889	3.917	—	n.a.	n.a.	n.a.
β-D-Gal [b]	—	4.551	3.617	3.665	3.925	—	n.a	n.a	n.a.
α-D-Xyl [a]	—	5.162	3.678	3.779	3.64	3.71	3.582	—	—
α-D-Xyl [b]	—	5.171	3.669	3.922	n.a.	3.72	3.572	—	—
α-D-Xyl [c]	—	4.941	3.541	3.735	3.61	3.72	3.547	—	—
β-D-Glc [a]	—	4.646	3.432	3.695	3.611	—	3.874	n.a.	n.a.
β-D-Glc [b]	—	4.527	3.424	3.67	3.66	—	n.a.	n.a.	n.a.
β-D-Glc [c]	—	4.530	3.339	3.52	3.51	—	3.693	3.780	3.944
Glcol	n.a.	n.a.	n.a.	n.a.	n.a.	—	n.a.	3.751	3.866

* Chemical shifts in ppm, using internal acetone (δ 2.225) as a reference.
[a, b, c] See Fig. 1 and *Nomenclature* in the Materials and methods section.

XXJGol from jojoba xyloglucan was performed as described below.

The glycosyl sequence of XXJGol was determined by 2D NMR spectroscopy. The COSY and TOCSY spectra allowed most of the ^{1}H resonances (Table 2) within each glycosyl residue to be assigned by (see above). Scalar coupling constants for proton pairs within each spin system were similar to the corresponding couplings in related xyloglucan oligosaccharides [10]. ^{13}C Resonances were assigned (Table 3) by analysis of the heteronuclear single-quantum correlation (HSQC) spectrum [22], which contains a cross-peak for each directly attached carbon–proton pair. Heteronuclear multiple-bond correlation spectroscopy (HMBC, Fig. 4) [23] was used to assign long-range scalar interactions (Table 4), which allowed each glycosyl linkage in XXJGol to be characterized. For example, the HMBC spectrum includes cross-peaks (Table 4) indicating interglycosidic scalar coupling between H-1 of the α-Gal*p* residue and C-2 of the β-Gal*p* residue (Fig. 4A), and between H-2 of one of the α-Xyl*p* residues and C-1 of the β-Gal*p* residue (Fig. 4B). These scalar interactions are consistent with the side-chain structure α-Gal*p*-(1 → 2)-

Table 3
^{13}C assignments * for XXJGol

Residue	C-1	C-2	C-3	C-4	C-5	C-6
α-L-Gal [a]	99.55	69.59	70.18	69.98	71.50	62.00
β-D-Gal [a]	104.00	77.46	74.15	69.69	75.70	61.82
α-D-Xyl [a]	99.99	80.83	72.82	70.77	61.82	n.a.
α-D-Xyl [b]	99.75	72.30	73.80	70.27	62.31	n.a.
α-D-Xyl [c]	99.11	72.30	73.85	70.27	62.02	n.a.
β-D-Glc [a]	103.02	73.75	75.02	80.83	74.50	67.98
β-D-Glc [b]	103.61	73.55	74.77	80.15	74.14	66.95
β-D-Glc [c]	103.71	73.80	76.39	70.33	75.10	66.71
Glcol	63.20	73.15	70.18	80.90	72.08	62.69

* Chemical shifts in ppm, using internal acetone (δ 31.07) as a reference.
[a, b, c] See Fig. 1 and *Nomenclature* in the Materials and methods section.

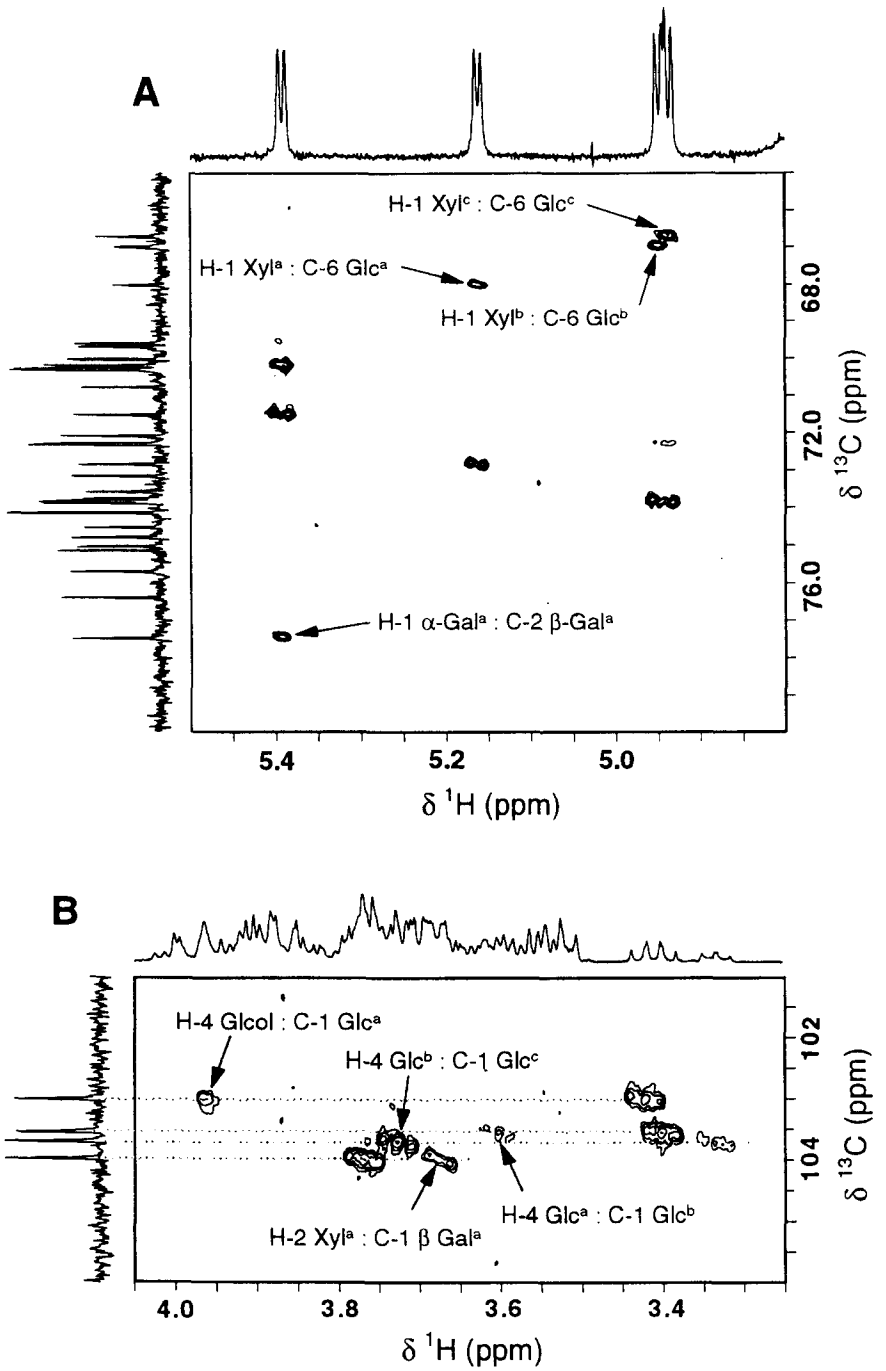

Fig. 4. Selected regions of the heteronuclear multiple-bond correlation (HMBC) spectrum of XXJGol. Cross-peaks correspond to interglycosidic scalar couplings (A) from anomeric protons of the α-linked glycosyl residues to carbons in their aglycons, and (B) from anomeric carbons of the β-linked glycosyl residues to protons in their aglycons. Additional cross-peaks (Table 4) not shown here confirm the glycosyl sequence of XXJGol.

β-Gal*p*-(1 → 2)-α-Xyl*p*. This triglycosyl side-chain is glycosidically linked to O-6 of Glc^{a} (Fig. 1), as indicated by interglycosidic scalar couplings (Table 4, Fig. 4A) between the 2-substituted α-Xyl*p* residue and Glc^{a}.

The assignment of glycosidic linkages identified by HMBC spectroscopy was confirmed by 2D nuclear Overhauser spectroscopy (NOESY) [20]. Typically, the anomeric proton of a glycosidically linked sugar residue spends a significant portion of the time in close proximity (i.e., $r < 3$ Å) to the proton attached to the aglyconic carbon atom [26]. For example, the glycosidic linkage β-Gal*p*-(1 → 2)-α-Xyl*p* brings H-1 of β-Gal*p* residue very close to H-2

Table 4
HMBC cross-peaks due to long-range heteronuclear scalar interactions within XXJGol

^{13}C Resonance	Interacting ^{1}H resonances			
C-1 α-Gal [a]	H-5 αGal [a]	H-2 βGal [a,d]		
C-5 α-Gal [a]	H-1 αGal [a]	H-6 αGal [a]		
C-1 β-Gal [a]	H-2 βGal [a]	H-2 Xyl [a,d]		
C-2 β-Gal [a]	H-1 αGal [a,d]	H-1 βGal [a]		
C-3 β-Gal [a]	H-2 βGal [a]	H-4 βGal [a]		
C-5 β-Gal [a]	H-1 βGal [a]	H-6 βGal [a]	H-6′ βGal [a]	
C-1 Xyl [a]	H-6 Glc [a,d]	H-6′ Glc [a,d]	H-5e Xyl [a]	H-5a Xyl [a]
C-3 Xyl [a]	H-1 Xyl [a]	H-2 Xyl [a]	H-5e Xyl [a]	
C-4 Xyl [a]	H-3 Xyl [a]	H-5e Xyl [a]	H-5a Xyl [a]	
C-5 Xyl [a]	H-1 Xyl [a]	H-4 Xyl [a]		
C-1 Xyl [b]	H-6 Glc [b,d]	H-6′ Glc [b,d]	H-5e Xyl [b]	H-5a Xyl [b]
C-5 Xyl [b]	H-1 Xyl [b]	H-4 Xyl [b]		
C-1 Xyl [c]	H-6 Glc [c,d]	H-6′ Glc [c,d]	H-5e Xyl [c]	H-5a Xyl [c]
C-5 Xyl [c]	H-1 Xyl [c]	H-4 Xyl [c]		
C-1 Glc [a]	H-4 Glcol [d]	H-2 Glc [a]		
C-5 Glc [a]	H-4 Glc [a]	H-6 Glc [a]		
C-6 Glc [a]	H-1 Xyl [a,d]	H-4 Glc [a]		
C-1 Glc [b]	H-4 Glc [a,d]	H-2 Glc [b]		
C-5 Glc [b]	H-4 Glc [b]	H-6 Glc [b]		
C-6 Glc [b]	H-1 Xyl [b,d]	H-4 Glc [b]		
C-1 Glc [c]	H-4 Glc [b,d]	H-2 Glc [c]		
C-3 Glc [c]	H-4 Glc [c]	H-2 Glc [c]		
C-5 Glc [c]	H-4 Glc [c]	H-6 Glc [c]		
C-6 Glc [c]	H-1 Xyl [c,d]	H-4 Glc [c]		
C-1 Glcol	H-2 Glcol			
C-5 Glcol	H-4 Glcol	H-6 Glcol		

[a, b, c] See Fig. 1 and *Nomenclature* in the Materials and methods section.
[d] Cross-peak corresponds to an interglycosidic coupling.

of the α-Xyl*p* residue. The distance dependent dipolar interaction of these two protons gives rise to a negative cross-peak in the NOESY spectrum of XXJGol, confirming the presence of the β-Gal*p*-(1 → 2)-α-Xyl*p* linkage. The observation of an interglycosidic NOE does not constitute unambiguous evidence of a specific glycosidic linkage because the distances between protons on two glycosyl residues is acutely dependent on the torsional geometry of the linkage. Nevertheless, interglycosidic cross-peaks in the NOESY spectrum of XXJGol (Table 5) were consistent with the structural assignments based on the HMBC spectrum.

The absolute configurations of the monosaccharide constituents of XXJGol were determined by an empirical method based on 1D NMR spectroscopy of

Table 5
NOESY cross-peaks due to dipolar interactions within XXJGol

^{1}H Resonance	Interacting ^{1}H resonances			
H-1 α-Gal [a]	H-2 βGal [a]	H-2 αGal [a]	H-1 βGal [a,d]	
H-5 α-Gal [a]	H-4 αGal [a]	H-3 αGal [a]	H-6 αGal [a]	H-6′ αGal [a]
H-1 Xyl [a]	H-1 βGal [a,d]	H-2 Xyl [a]	H-6 Glc [a,d]	H-6′ Glc [a,d]
H-1 Xyl [b]	H-6 Glc [b,d]	H-2 Xyl [b]		
H-1 Xyl [c]	H-2 Xyl [c]	H-6 Glc [c,d]	H-6′ Glc [c,d]	
H-1 β-Gal [a]	H-1 αGal [a,d]	H-1 Xyl [a,d]	H-2 Xyl [a,d]	H-3 βGal [a]
H-1 Glc [a]	H-3 Glc [a]	H-4 Glcol [d]	H-6′ Glcol [d]	
H-1 Glc [b]	H-4 Glc [a,d]	H-3 Glc [b]		
H-1 Glc [c]	H-4 Glc [b,d]	H-3 Glc [c]	H-5 Glc [c]	

[a, b, c] See Fig. 1 and *Nomenclature* in the Materials and methods section.
[d] Cross-peak corresponds to an interglycosidic NOE.

their per-*O*-*S*-methylbutyrate (SMB) derivatives [25]. The 600-MHz ^{1}H NMR spectrum (data not shown) of the SMB derivatives of the mixture of monosaccharides generated by acid hydrolysis of XXJGol closely matched the spectrum of the SMB derivatives of a standard mixture containing L-Gal, D-Gal, D-Xyl, D-Glc, and D-Glcol in the ratio 1:1:3:3:1, indicating that XXJGol contains equimolar amounts of L-Gal and D-Gal.

The nonreducing terminal α-Gal*p* residue of XXJGol was initially assigned the L configuration and the 2-linked β-Gal*p* residue was assigned the D configuration based on the structural homology of XXJGol with the well-characterized nonasaccharide XXFGol [10]. All of the β-linked Gal*p* residues in previously described xyloglucans have the D configuration, and all of the terminal α-linked Fuc*p* (i.e., 6-deoxy-α-galactopyranosyl) residues in these xyloglucans have the L configuration. The α-L-Fuc*p* residues of xyloglucans are all found in side-chains with the structure α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)-, which is homologous to the structure, α-L-Gal*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)-, proposed for the side-chain in XXJGol. The only difference between these two structures is the presence or absence of a single oxygen atom in the terminal glycosyl residue. The absolute configurations of the glycosyl residues in this side-chain are thus consistent with the observed conservation of the structural features of xyloglucans. The conserved features are likely to be responsible for the biological activity of both XXFGol and XXJGol [13].

The precise locations of the D-Gal*p* and L-Gal*p* residues of XXJG were confirmed by analyzing the products obtained by partial hydrolysis of the oligosaccharide. Fragment oligosaccharides were generated by incubation of XXJG in 1 M trifluoroacetic acid for various lengths of time (up to 144 h). MALDI-TOF-MS analysis of these fragments suggested that the terminal Gal*p* residue of XXJG is acid labile because the first product formed has the same molecular mass as XXLG. Further hydrolysis (e.g., for 144 h) resulted in the appearance of small amounts of an oligosaccharide with the same molecular weight as XXXG. Therefore, oligosaccharides obtained after 144 h of hydrolysis were separated by HPLC and characterized by NMR spectroscopy. Although a gradient was used in this case to elute the HPLC column (see Materials and methods section), the oligosaccharides were eluted in the same order as depicted in Fig. 3, where isocratic elution was employed. Peaks 2 (α-XXLG), 5 (α-XXJG and β-XXLG), and 7 (β-XXJG), designated using the numbering scheme of Fig. 3, dominated the chromatogram of the partial hydrolysate. Peaks 2 and 5 had the same retention times as the α and β forms of standard XXLG from tamarind seed xyloglucan [17]. MALDI-TOF-MS of the HPLC fractions of the partial hydrolysate indicated that Peak 5 contained both XXLG ($[M + Na]^+$, *m/z* 1248) and unhydrolyzed XXJG ($[M + Na]^+$, *m/z* 1248), and that Peak 2 contained XXLG ($[M + Na]^+$, *m/z* 1248) and small amounts of XXXG ($[M + Na]^+$, *m/z* 1086). The 500-MHz ^{1}H NMR spectrum Peak 2 was essentially identical to that of authentic XXLG [17], except for the presence of low-intensity signals due to 10–15% contamination with XXXG, as predicted by MALDI-TOF-MS analysis (see above). These results clearly demonstrate that XXLG is the first product formed upon partial acid hydrolysis of XXJG. Because XXLG contains only D-Gal*p* residues in its disaccharide side-chain β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)-, the L-Gal*p* residue of XXJG must be at the terminus of a trisaccharide side-chain with the structure α-L-Gal*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*-(1 → 6)-.

The ^{1}H NMR spectra of XXFGol and XXJGol reflect the structural similarity of these oligosaccharides. For example, H-5 resonances of the terminal α-L-Fuc*p* of XXFGol (δ 4.519) and the terminal α-L-Gal*p* residue of XXJGol (δ 4.406, Fig. 1) are both shifted to significantly lower field than the H-5 resonances of terminal α-L-Fuc*p* or α-L-Gal*p* in most other oligosaccharides. This shift is probably due to conformationally induced deshielding of these protons, suggesting that the triglycosyl side-chains of XXFGol and XXJGol adopt similar molecular geometries, as expected if they have nearly identical primary structures. Thus, assignment of the L configuration to the terminal α-Gal*p* residue and the D configuration to the 2-substituted β-Gal*p* residue in XXJGol is consistent with all of the available biochemical and spectroscopic data.

4. Conclusions

Oligosaccharide subunits of jojoba seed xyloglucan were generated by endoglucanase treatment of the polysaccharide and purified. The complete primary structure of the oligoglycosyl alditol XXJGol was determined by a combination of mass spectrometry and NMR spectroscopy. The jojoba xyloglucan oligosaccharides XXJG and XLJG contain the side-chain [α-L-Gal*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-

Xyl *p*-(1 → 6)-], which is unusual because it contains both L-Gal and D-Gal. This side-chain is structurally homologous to the fucose-containing side-chain [α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl *p*-(1 → 6)-] found in biologically active xyloglucan oligosaccharides, such as XXFG, produced by diverse plant species. Jojoba seed thus represents a convenient source of biologically active, L-galactose-containing xyloglucan oligosaccharides.

Acknowledgements

This research is supported by United States Department of Energy (DOE) grant DE-FG02-96ER-20220, and by the DOE-funded (DE-FG05-93ER20097) Center for Plant and Microbial Complex Carbohydrates. The authors thank Dr. John Glushka for helpful discussions, and Dennis Warrenfeltz for technical support and maintenance of the NMR spectrometers.

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