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Structural characterization of chemically and enzymatically derived standard oligosaccharides isolated from partially purified tamarind xyloglucan

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Abstract

Several oligosaccharide fragments, ranging from 2 to 9 contiguous residues, have been isolated from purified tamarind xyloglucan using enzymatic digestion and partial acid hydrolysis. Structures were determined using matrix assisted laser adsorption ionization-time of flight (MALDI-TOF) mass spectrometry, gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS), and Dionex high pH anion exchange–high performance liquid chromatography (HPAE–HPLC). These fragments will be used to identify reaction products from xyloglucan xylosyltransferase and glucosyltransferase enzyme assays and as possible acceptor molecules for these enzymes.

Keywords: Tamarind; Xyloglucan; Cell wall; Polysaccharides; Oligosaccharide; Xyloglucan glucosyltransferase; Xyloglucan xylosyltransferase; Acceptor molecules

1. Introduction

All higher plant cells are encased in a cell wall, which not only defines the cell's shape but also contributes to the structural integrity and morphology of the entire plant. The primary cell wall, which surrounds the cells that are expanding, has been well characterized structurally (Carpita & Gibeaut, 1993; Darvill, McNeil, Darvill, & Albersheim, 1980; McCann & Roberts, 1991). The cellulose/hemicellulose network of the primary cell wall provides structural veracity as well as physically regulating wall expansion as the cell grows (Carpita & Gibeaut, 1993). Xyloglucan is the major hemicellulose polysaccharide in the plant cell wall matrix of dicots, and its general structure consists of a β-(1,4)-glucan backbone variously substituted with xylosyl and galactosyl, residues (Fry, 1989). Occasionally, an α-L-fucosyl substitution to the C-2 of a terminal galactosyl unit forms a trisaccharide side-chain (Bauer, Talmadge, Keegstra, & Albersheim, 1973). This fucose substitution is only found in xyloglucans of the primary cell wall (Hayashi, Ogawa, & Mitsubishi, 1994) and may affect the kinetics of the binding of xyloglucan to cellulose microfibrils (Levy, William, Stuike-Prill, Meyer, & Staehelin, 1991). In addition, a proportion of the xyloglucan molecules show covalent bonding to acidic pectins (Thompson & Fry, 2000).

It is now widely accepted that such xyloglucan–cellulose interactions may be principal tension bearing factors in the cell wall. Furthermore, enzyme-catalyzed modifications of xyloglucan are now considered a key process for wall expansion during cell growth (Talbott & Ray, 1992). For plant cells to expand, cellulose microfibrils need to move apart or past each other, and this movement may create the possibility for newly synthesized xyloglucan molecules to become incorporated into the network (Fry, 1989; Vissenburg, Martinez-Vilchez, Verbelen, Miller, & Fry, 2000). The enzyme-catalyzed reactions may allow reversible cell wall loosening in elongating plant cells without compromising the localized strength of the wall matrix (Fry, 1989; Hoson, Masuda, Sone, & Misaki, 1991; Vissenberg et al., 2000). In addition, an endo-β-(1,4)-glucanase (Cel12A), isolated from *Trichoderma reesei*, has recently been shown to have a similar plant cell wall extension activity (Yuan,

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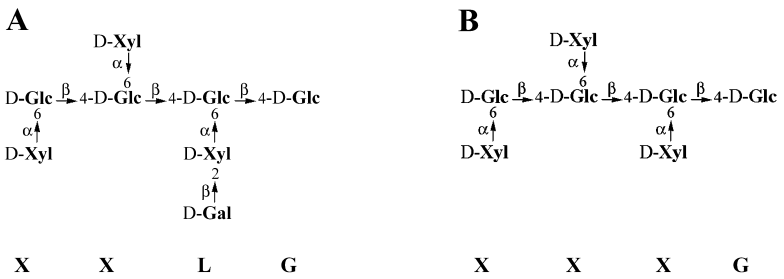

Fig. 1. A diagrammatical representation of the structural units of (A) a xyloglucan heptasaccharide (XXLG) and (B), an octasaccharide (XXXG), using the unambiguous nomenclature of Fry et al. (1993).

Wu, & Cosgrove, 2001). There are many enzymes that act upon xyloglucan within the primary cell wall, which have been identified and at least partially characterized. For example, *Endo*glucanases (EGs) (Verma, Maclachlan, Byrne, & Ewings, 1975) xyloglucan endotransglycosylases (XETs) (Fry et al., 1992; Polisensky & Braam, 1996) and exoglycosidases (Augur, Stiefel, Darvill, Albersheim, & Puigdomenech, 1995) are all up regulated during elongation of plant cells.

An unambiguous nomenclature for xyloglucan-derived oligosaccharides (Fry et al., 1993) defines the oligosaccharide by listing the substitution pattern of glucosyl residues from the non-reducing to the reducing end of the β-(1,4)-glucan backbone. In this system G = glucose, X = xylose (assuming the presence of G), and L = galactose (assuming the presence of G and X) such that the oligosaccharides in Fig. 1 are abbreviated XXLG and XXXG. The main backbone of xyloglucan polysaccharides is made up of repeating units of XXXG heptasaccharides, presumed to be synthesized by transferase enzymes in the *trans*-Golgi cisternae and *trans*-Golgi network (McNeil, Darvill, Fry, & Albersheim, 1984; Staehelin, Giddings, Kiss, & Sack, 1990). There are two main enzyme activities involved in the synthesis of the XXXG heptsaccharide repeat unit of xyloglucan/xyloglucan glucosyltransferase (XGT) and xyloglucan xylosyltransferase (XXT) (White, Xin, & Pezeshk, 1993). XGT is thought to produce the backbone chain of xyloglucan (Campbell, Brett, & Hillman, 1988; Gordon & Maclachan, 1989), whilst XXT adds xylosyl sidechain residues to the C-6 position of the glucosyl backbone of xyloglucan (Brummell, Camirand, & Maclachlan, 1990; Hayashi & Maclachlan, 1984; Haysahi & Matsuda, 1981). Recent work by Pauly et al. (2001) has shed light onto the formation of different xyloglucans during cell elongation and when this process has abated. This work suggests that xyloglucan metabolism plays a key role in the growth and development of the primary cell wall.

In spite of this knowledge, one of the many fundamental questions in plant biology concerns the extent to which the manufacture of new cell wall components influences plant growth and development. For example, it still remains unclear whether both XGT and XXT are type-I or -II membrane proteins. It is, however, known that, under certain in vitro conditions, XGT catalyses the formation of β-(1,3)-glucan and reduces the relative abundance of β-(1,4)-glucan (White et al., 1993). In addition, a putative XXT gene family has been identified in *Arabidopsis* (Faik, Raikhel, & Keegstra, 2001). Data concerning the biochemical ramifications of the action of this enzyme on primary wall elongation will be keenly awaited.

Tamarind xyloglucan, which contains galactosyl but not fucosyl side chain residues, can be used as a model for cell wall xyloglucans due to its structural similarity (Staehelin et al., 1990). To this end, we present detailed structural analysis of an array of oligo-xyloglucan fragments obtained from purified tamarind seed xyloglucan. Such oligo-xyloglucans are needed to determine the structure of the reaction products formed during in vitro assays involving XGT and XXT. Such fragments can then be used as structural standards or as 'acceptor molecules' for further research involving the activity and action of these two important enzymes. Furthermore, we have established R_f values of most of the oligosaccharide fragments presented here using a Dionex HPAE–HPLC, which will be of great value for other researchers to identify oligosaccharide fragments using a similar system. We believe that the results presented here will, in the long run, provide additional insight into the synthesis of xyloglucan, a carbohydrate known to play an important role in the regulation of plant cell elongation.

2. Material and methods

2.1. Partially purified tamarind xyloglucan

Xyloglucan was prepared by dissolving 30 g of tamarind kernel powder (P.L. Thomas and Co., Inc., Bernardsville, NJ) in 3 l dH_2O. The solution was incubated with constant stirring for 24 h at 4 °C, centrifuged at 18,000*g* for 30 min, and the pellet was discarded. Soluble polysaccharides were precipitated overnight in 70% ethanol at 4 °C and centrifuged at 18,000*g* for 30 min. The pellet was suspended in 850 ml dH_2O, precipitated with ethanol and centrifuged a second time, and the remaining pellet was lyophilized.

2.2. Enzymatic digestion and partial acid hydrolysis (PAH)

In some experiments, the partially purified tamarind xyloglucan was digested with β-1,4-endoglucanase-II (EG-II) from *Pyrococcus furiosus* (EC 3.2.1.4; Novo Nordisk, Copenhagen) using 1 mg of this impure preparation of enzyme and 10 mg xyloglucan per ml of 50 mM glycine buffer at pH 3.0 for 1–4 h at 50 °C in a heating block. Raising the temperature to 90 °C for 5 min terminated the digestion. In addition, in order to generate the disaccharide isoprimeverose, partially purified tamarind xyloglucan was digested with Driselase (Sigma Chemical Co.) at a concentration of 1 mg impure preparation of enzyme per 10 mg xyloglucan in 1 ml 50 mM phosphate buffer at pH 7.5. The solution was incubated for 38–66 h at 40 °C in a heating block. Raising the temperature to 100 °C for 5 min terminated the digestion. Following either enzyme digestion, each sample was centrifuged at 4000*g* for 2 min and the supernatant used for further analyses. In other experiments, the isolated xyloglucan was partially hydrolyzed by incubating 10 mg of isolated xyloglucan in 0.1 M HCl for 4 h at 100 °C, followed by neutralization with 1.0 M NaOH.

2.3. Structural determination of oligo-xyloglucan fragments

2.3.1. HPAE–HPLC settings

A Dionex DX300 Carbohydrate Analysis System was used with CarboPac PA-1 analytical (4×250 mm^2) and semi-preparative (9×250 mm^2) columns, using or adapting the methods of Hardy, 1989. Peaks were detected with a pulsed amperometric detector (PAD) equipped with a gold working electrode with pulse potentials (E_1–E_3) and durations (t_1–t_3) of: $E_1 = 0.05$ V ($t_1 = 360$ ms); $E_2 = +0.80$ V ($t_2 = 120$ ms); $E_3 = -0.60$ V ($t_3 = 420$ ms). Samples were eluted at 1 ml/min at 1500–1700 psi with 100 mM NaOH + 50 mM NaOAc (0–5 min) or 100 mM NaOH + 25 mM NaOAc followed by a gradient to 100 mM NaOH + 100 mM NaOAc (5–20 min gradient). After each injection, the column was re-equilibrated in 100 mM NaOH + 50 mM NaOAc or 100 mM NaOH + 25 mM NaOAc. Monosaccharides were eluted isocratically with 15 mM NaOH (Hardy, 1989).

2.3.2. Desalting of isolated oligosaccharide fragments

Oligosaccharide fragments that were isolated from HPAE–HPLC were desalted either with Dionex OnGuard-H cartridges or with a mixed bed column consisting of Bio-Rad AG 50W-X12 cation exchange resin and Bio-Rad AG 3-X4 anion exchange resin. Each Dionex OnGuard-H cartridge, which neutralizes 10 ml of 0.2 M NaOH, was flushed with two volumes of distilled water before a sample was loaded. Checking the conductivity of the elution with respect to deionized, distilled water monitored ion exchange column efficiency.

2.3.3. Glycosyl composition analysis

Ten percent of individual peaks from desalted HPAE–HPLC fractions were lyophilized and treated with 0.25 ml 2N trifluoracetic (TFA) (+0.1 mg/ml inositol for PMAA only) and incubated for 1 h at 121 °C (York, Darvill, McNeil, Stevenson, & Albersheim, 1985). TFA was evaporated with a stream of air. The sample was extracted with 1 ml of 70% ethanol and evaporated a second time with a stream of air. Samples were eluted with 15 mM NaOH on HPAE–HPLC detected with PAD for relative monosaccharide composition. Normalized percents relative to known standards from each peak were obtained.

2.3.4. Partially methylated alditol acetate (PMAA)

Eighty percent of each peak was dried and derivatized for glycosyl linkage analysis by methylation with *n*-butyllithium (Valent, Darvill, McNeil, Robertsen, & Albersheim, 1980). Reaction products dried over P_2O_5 in a vacuum oven were dissolved in 0.2 ml DMSO and 0.5 ml *n*-butyllithium was added slowly, followed by 0.5 ml of methyl iodide. Methylated samples were extracted with C_{18} solid phase filters (Alltech) on a vacuum manifold using 100% acetonitrile and absolute ethanol and the eluted samples were evaporated to dryness with a stream of air. Samples were fully hydrolyzed with 2N TFA, 121 °C, 1 h, and reduced with $NaBD_4$ (Waeghe, Darvill, McNeil, & Albersheim, 1983; York et al., 1985). Per-*O*-methylated alditols were acetylated with acetic anhydride and 1-methylimidazole as a catalyst (Blakeney, Harris, Henry, & Stone, 1983). Analysis was carried out on a Hewlett Packard gas chromatograph with a flame ionization detector. PMAAs were separated in an SP-2330 fused silica capillary column 30 m long × 0.75 mm ID × 0.20 μm film thickness (Supelco). Column oven temperature was initially held at 50 °C for 2 min, programmed to be increased 60 °C/min to 170 °C followed by a further increase of 4 °C/min to a final temperature of 250 °C and held for 2 min. Detector and injector temperatures were both 250 °C, and derivative structures were deduced as described by Carpita and Shea (1989).

2.3.5. Matrix assisted laser desorption ionization–time of flight (MALDI–TOF) mass spectrometry

Ten percent of each of the collected peaks were dried and analyzed by MALDI–TOF to determine both the hexose/-pentose ratio and the molecular weight of each oligo-xyloglucan fragment. The oligosaccharides, purified by HPAE–HPLC and lyophilized were re-suspended in 2 μl dH_2O and mixed with a matrix (2 μl) consisting of (4:1, v/v) 2,5-dihydroxybenzoic acid (DBH, 0.2 M) and α-cyanoacrylic acid (ACC, 0.06 M), both in 50% aqueous acetonitrile. The mixture (1 μl) was applied to the probe tip and the solvent immediately evaporated under vacuum (1.4×10^{-6} torr) to rapidly form homogeneous crystals. The sample was desorbed/ionized from the probe tip with a 10.58 mJ ± 1.84 μJ nitrogen laser pulse ($\lambda = 337$ nm). The

detector voltage was set at 4.75 kV and the digitizer was set to 5 mV/b.

2.3.6. Bio-Gel P-2 gel filtration column chromatography

The isolated oligosaccharide fragments were re-suspended in 2 ml of dH_2O and separated on a 1.5 cm × 120 cm Bio-Gel P-2 gel filtration column (Bio-Rad Laboratories, Hercules, CA, USA) using dH_2O as the elutant, with a flow rate of 1 ml per min. Eluted samples were collected in 3.45 ml fractions, with aliquots of each fraction analyzed as desired. Fractions from the void, partially-included and included volumes were pooled and lyophilized.

2.3.7. Anthrone assay

Neutral sugar content of each fraction from Bio-Rad P-2 gel filtration chromatography was determined by combining 100 μl from each fraction with 400 μl of water and 1 ml of anthrone reagent ($2\ g\ l^{-1}$ H_2SO_4). The mixture was vortexed and the absorbance read at 620 nm (Dische, 1962), using glucose as a standard.

2.4. Preparation of oligo-xyloglucan fragments

2.4.1. Preparation of oligo-xyloglucan fragments ranging from 7 to 9 residues

Following an EG-II digestion of partially-purified tamarind xyloglucan, the resultant oligosaccharides were separated on a Bio-Gel P-2 gel filtration column. Each fraction was assayed for neutral sugar content with the anthrone assay. Fractions composing the large partially-included peak were pooled and lyophilized. The resulting material was suspended in 1 ml of dH_2O and fragments were separated by HPAE–HPLC using a Dionex CarboPac PA-1 analytical column and a gradient of 100 mM NaOH/25–100 mM NaOAc. The fragments obtained were identified by comparison of relative retention times of xyloglucan products reported previously (Hardy, 1989; Hisamatsu, York, & Darvill, 1992; York, van Halbeek, Darvill, & Albersheim, 1990). Individual oligosaccharide fragments were isolated by pooling fractions from similar peaks that were separated from 12 to 15 injections of 60 μl on a Dionex HPAE–HPLC CarboPac PA-1 semipreparative column. Each peak was neutralized with 1.0 M HCl, desalted, lyophilized and stored at −20 °C until required.

2.4.2. Preparation of oligo-xyloglucan fragments ranging from 5 to 9 residues

The pooled fractions composing the large partially-included peak were subjected to a partial acid hydrolysis (PAH), neutralized and separated again on a Bio-Gel P-2 gel filtration column. Oligosaccharide fragments composed of between 5 and 9 residues were contained in the partially-included fractions, which were pooled and lyophilized. The resulting pellet was re-suspended with 1 ml of dH_2O and aliquots (10 μl) were analyzed on HPAE–HPLC as before to determine PAH efficiency. Peaks containing the individual oligosaccharide fragments ranging from 5 to 9 residues were separated as above from neighboring peaks by HPAE–HPLC using a semi-preparative column under the same conditions by collecting and pooling similar fractions from 12 to 15 injections of 60 μl. Each pooled peak was neutralized with 1.0 M HCl, desalted, lyophilized and stored at −20 °C.

2.4.3. Preparation of oligo-xyloglucan fragments containing less than 5 contiguous residues

The pooled fractions containing the oligo-xyloglucan fragments consisting of between 5 and 9 contiguous residues, resulting from EG-II digestion followed by PAH, were digested a second time with EG-II, separated on a Bio-Gel P-2 column and fractions containing the oligo-xyloglucan fragments were pooled and lyophilized. The resulting pellet was suspended with 1 ml of dH_2O and the production efficiency of oligo-xyloglucan fragments containing less than 5 residues was surveyed by injecting 10 μl aliquots onto an HPAE–HPLC analytical column, this time using a gradient of 100 mM NaOH/25–50 mM NaOAc. These peaks were isolated from each other by pooling similar fractions from 12 to 15 injections of 60 μl onto a Dionex HPAE–HPLC Semi-Prep column as before, using the same gradient as above. Each peak was neutralized with 1 M HCl, desalted, lyophilized and stored at −20 °C.

2.4.4. Isoprimeverose

Partially purified tamarind xyloglucan was digested with Driselase and separated with a Bio-Gel P-2 gel filtration column and HPAE–HPLC. Elution with 100 mM NaOH resulted in the separation of the disaccharide from monosaccharides. The disaccharide was further analysed by glycosyl composition and linkage analyses. Isoprimeverose was eluted on a 100 mM NaOH/25–100 mM NaOAc gradient for retention time comparison.

3. Results and discussion

Partially purified tamarind xyloglucan was digested in the following ways to produce an array of oligo-xyloglucan fragments: (1) Complete digestion with β-(1,4)-endoglucanase-II (EG-II), an endo-acting enzyme cleaving β-(1,4)-linkages at unsubstituted glucosyl residues in the xyloglucan backbone, produced four fragments (XXXG, XLXG, XXLG, and XLLG). (2) Partial acid hydrolysis of the oligo-xyloglucan fragments removed some sidechains to produce XGXG, XXGG, GGXG, GXXG, and XGGG (and variations containing galactose). (3) Further EG-II digestion of the acid-hydrolyzed fragments produced smaller fragments (XG, XXG, and others). (4) Complete digestion of xyloglucan with Driselase, a mixture of polysaccharide–degrading-enzymes, produced mostly monosaccharides and the disaccharide isoprimeverose; xylosyl-α-(1,6)-glucose.

All oligo-xyloglucan products were separated by gel filtration chromatography and Dionex high pH anion exchange–high performance liquid chromatography (HPAE–HPLC). Glycosyl composition of oligo-xyloglucan fragments was determined by TFA hydrolysis followed by HPAE–HPLC. Methylation analysis was used to determine glycosyl linkages present in each oligo-xyloglucan fragment, whilst matrix assisted laser desorption ionization–time of flight (MALDI–TOF) mass spectroscopy was used to determine the molecular weight and the hexose/pentose ratio (Tables 1 and 2). In these tables, we show both the standardized abbreviations for partially methylated alditol acetates as well as the "short-hand" abbreviations favored by plant scientists.

When one considers the structure of xyloglucan, it becomes clear that the presence of 6-Glc and t-Glc ought to be mutually exclusive, especially in the case of XG or XGG fragments (Table 1, peaks 2C and 2F respectively), which we show to contain both 6-Glc and t-Glc in conjunction with t-Xyl. However, our analyses consistently indicated the presence of terminally linked glucose (t-Glc) in both the XG and XGG fragments. By contrast, the GXG fragments should contain t-Glc (Table 1, peak 2F). It is probable that the t-Glc is an artifact produced during the generation of the separate fragments, and is particularly prevalent in the smaller oligo-xyloglucan fragments.

3.1. Oligo-xyloglucan fragments ranging from 7 to 9 contiguous residues

Digesting tamarind xyloglucan with EG-II produced large oligo-xyloglucan fragments. The reaction mixture was separated by Bio-Gel P-2 gel filtration chromatography and each fraction was assayed for neutral sugar content with the anthrone assay. Two large peaks were produced, the void peak contained undigested xyloglucan, while the partially included peak (Peak 1) contained xyloglucan fragments made up from less than 10 contiguous residues (Fig. 2A). The undigested xyloglucan in the void peak was not characterized, while the fractions that composed Peak 1 were pooled, lyophilized and characterized by HPAE–HPLC. Analysis of Peak 1 indicated that it contained XXXG, XLXG, XXLG, and XLLG with RF values (relative to XXXG) of 1.000, 1.160, 1.240 and 1.429 respectively (Fig. 2B). The retention times of XXXG and XLLG were reported previously (Hisamatsu et al., 1992; McDougall & Fry, 1991; York et al., 1990), as were the retention times of XLXG and XXLG (York, Harvey, Guillen, Alberhseim, & Darvill, 1993).

Pooling the appropriate fractions from a series of HPAE–HPLC separations using a semi-preparative column isolated each large oligosaccharide fragment. Neutralization, desalting and lyophilization of each peak were carried out as soon as possible to minimize oligosaccharide degradation due to the high pH of the solvents used for the HPAE–HPLC step. The purity of each peak was assessed by further analysis by analytical HPAE–HPLC (Fig. 2C).

3.2. Oligo-xyloglucan fragments ranging from 5 to 6 contiguous residues

Fractions that composed Peak 1 were pooled, lyophilized and partially hydrolyzed with 0.1 M HCl (partial acid hydrolysis; PAH) to generate smaller oligosaccharide fragments. The PAH treatment randomly removes xylosyl, galactosyl and glucosyl residues from the oligosaccharide fragments with the largest number of contiguous residues. It should be noted that this treatment would cleave all fragments which have a DP $>$ 1. In addition, PAH can also hydrolyze the glucan backbone of the oligo-xyloglucan fragments, but hydrolysis of the terminal sugar residues is thought to be kinetically favored (York, personal communication).

We believe that the conditions used in the PAH treatment represent optimum conditions that minimize treatment time while maximizing intermediate fragment generation. Adjusting the amount of time the sample is incubated in PAH conditions can control the relative amounts of each intermediate fragment produced. The PAH fragments were neutralized and separated on Bio-Gel P-2 (Fig. 3A), resulting in a large, partially-included peak (Peak 2) representing unhydrolyzed oligo-xyloglucan fragments mixed with various xyloglucan fragments missing one or more residues. Monosaccharides removed by the PAH treatment eluted in the included volume. Fractions contained in Peak 2, which do not contain monosaccharides, were pooled and lyophilized, and the oligosaccharide components of Peak 2 were analysed by HPAE–HPLC. The data indicate that the PAH treatment substantially hydrolyzed XXXG, XLXG, XXLG and XLLG into smaller fragments and monosaccharides that eluted off the column earlier (Fig. 3B). Each oligosaccharide fragment was isolated by further HPAE–HPLC analysis. Fractions composing each peak were neutralized, desalted and checked for purity. The structure of each fragment was analyzed for glycosyl composition, glycosyl linkage, and pentose/hexose ratio (Table 1).

3.3. Oligo-xyloglucan fragments ranging from 3 to 6 residues

The acid-hydrolyzed intermediate oligo-xyloglucan fragments (Peak 2) were digested a second time with EGII to generate fragments with the fewest number of contiguous residues, which were separated on a Bio-Gel P-2 (Fig. 4A). Four individual peaks resulted from the separation of fractions from Peak 3 on a HPAE–HPLC Semi-Preparative PA-1 column (Fig. 4B). TFA hydrolysis, MALDI–TOF, and PMAA were used to elucidate the structures of each peak (Table 2). The oligosaccharide composition of Peak 3 contains the same smaller oligosaccharides that are found in

Table 1
The glycosyl composition, linkage composition, hexose/pentose ratio, proposed structure, and R_f values of the oligo-xyloglucan fragments ranging from 7 to 9 contiguous residues present in Peak 2 (Fig. 3A). The meanings of the abbreviations used for the linkage composition are as follows: t-Xyl (1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl xylose), t-Glc (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucose), 2-Xyl (1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl xylose), 4-Glc (1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucose), 6-Glc (1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucose) and 4,6-Glc (1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl glucose)

Peak number	Glycosyl composition Glc/Xyl/Gal (%)	Linkage composition	Full linkage composition	MALDI–TOF	Proposed structure	HPAE–HPLC Rf (XXXG = 1.000)
2A	50 Xyl 50 Glc	t-Xyl 6-Glc	1,5-di-*O*-acetyl-2,3,4-tri-*O*)-methyl xylose 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucose	No results	Isoprimeverose	0.208
2B	99 Glc	t-Glc 4-Glc	1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucose 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucose	No results	Cellobiose	0.24
2C	69 Glc 31 Xyl	t-Xyl t-Glc 6-Glc	1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl xylose 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucose 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucose	2 Hex:1 Pent	XG	0.393
2D	99 Glc	t-Glc 4-Glc	1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucose 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methy1 glucose	3 Hex:0 Pent	GGG	0.433
2E	No results	t-Xyl t-Glc 4-Glc 6-Glc	1,5-di-0-acetyl-2,3,4-tri-*O*-methyl xylose 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucose 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucose 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucose	3 Hex:1 Pent	GXG	0.525
2F	79 Glc 20 Xyl	t-Xyl t-Glc 4-Glc 6-Glc	1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl xylose 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucose 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucose 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucose	3 Hex:1 Pent	XGG	0.647
2G	70 Glc 29 Xyl	t-Xyl 4-Glc 6-Glc 4,6-Glc	1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl xylose 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucose 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucose 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl glucose	No results	XXG	0.695
2H	82 Glc 17 Xyl	t-Xyl t-Glc 4-Glc 4,6-Glc	1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl xylose 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucose 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucose 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl glucose	No results	GXGG or GGXG	0.724
2I	62 Glc 37 Xyl	t-Xyl t-Glc 4-Glc 6-Glc 4,6-Glc	1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl xylose 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucose 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucose 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucose 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl glucose	No results	GXXG or XGXG or XXGG	0.76
2L	No results	t-Xyl 2-Xyl t-Glc 4-Glc 6-Glc 4,6-Glc	1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl xylose 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl xylose 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucose 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucose 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucose 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl glucose	No results	GXXG or XGXG or XXGG	0.869

Table 2
The glycosyl composition, linkage composition, hexose/pentose ratio, proposed structure, and R_f values of the oligo-xyloglucan fragments ranging from 5 to 6 contiguous residues present in Peak 3 (Fig. 4A). The meanings of the abbreviations used for the linkage composition are as follows: t-Xyl (1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl xylose), t-Glc(1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucose), t-Gal (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl galactose), 2-Xyl (1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl xylose), 4-Glc (1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucose), 6-Glc (1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucose) and 4,6-Glc (1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl glucose).

Peak number	Glycosyl composition Glc/Xyl/Gal (%)	Linkage composition	Full linkage composition	MALDI–TOF	Proposed structure	HPAE–HPLC Rf (XXXG = 1.000)
3A	99 Glc	t-Glc	1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl xylose	No results	GG	0.24
3B	28 Xyl 71 Glc	t-Xyl 6-Glc	1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl xylose 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucose	2 Hex:1 Pent	XG	0.393
3C	67 Glc 21 Xyl 10 Gal	t-Gal 2-Xyl 4-Glc 6-Glc	1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucose 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl xylose 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucose 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucose	3 Hex:1 Pent	LG	0.549
3D	69 Glc 28 Xyl 2 Gal	t-Xyl 4-Glc 6-Glc 4,6-Glc	1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl xylose 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucose 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucose 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl glucose	3 Hex:2 Pent	XXG	0.695

Fig. 2. Oligo-xyloglucan fragments ranging from 7 to 9 contiguous residues. (A) Tamarind xyloglucan digested with β-(1,4)-endoglucanase II and separated on a Bio-Gel P-2 column. Peak 1 consists of XXXG, XLXG, XXLG and XLLG. (B) Oligo-xyloglucan fragments in Peak 1 were identified by comparison of the retention times of the peaks with previously reported standards from HPAE–HPLC on a Dionex PA-1 analytical column. (C) Each oligosaccharide peak was isolated by pooling the appropriate fractions that were separated from 12 to 15 injections of 60 μl on the HPLC. The fractions were neutralized, desalted and then lyophilized. Each isolated oligosaccharide was suspended in 500 μl of water and aliquots (10 μl) were injected onto the HPLC to check for purity. An example chromatogram for the XLXG fragment is shown here.

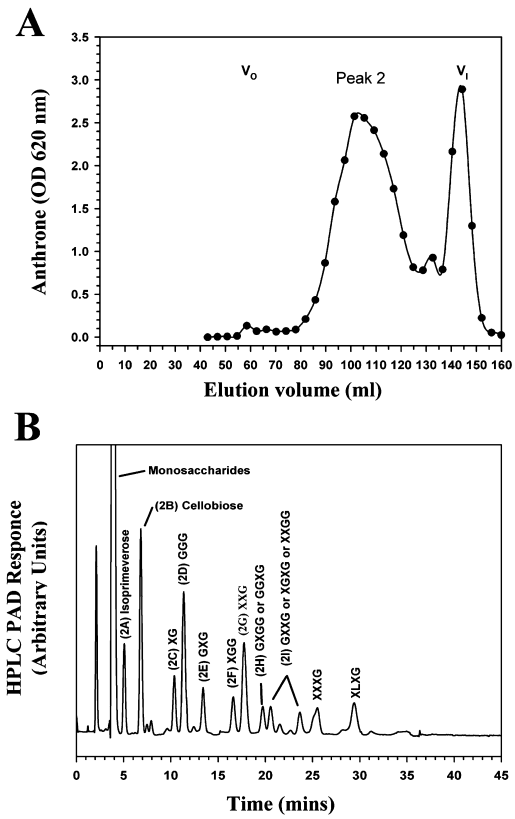

Fig. 3. Oligo-xyloglucan fragments ranging from 5 to 9 contiguous residues. (A) Bio-Gel P-2 gel filtration separation of Peak 1 following PAH. The resulting partially included Peak 2 contained XXXG, XLXG, XXLG and XLLG, in addition to many shorter oligosaccharide remnants. The peak eluted in the included volume (V_i) contained monosaccharides that were hydrolyzed from the fragments present in Peak 1. (B) The oligo-xyloglucan fragments contained within Peak 2 were pooled, lyophilized and 10 μl aliquots were separated using a Dionex HPAE–HPLC Carbo-pac PA-1 analytical column. Similar fractions representing each peak were separated from neighboring peaks by HPAE–HPLC using a semi-preparative column by collecting and pooling similar fractions from 12 to 15 injections of 60 μl. The structure of each fragment was analyzed for glycosyl composition, glycosyl linkage, and pentose/hexose ratio (Table 1).

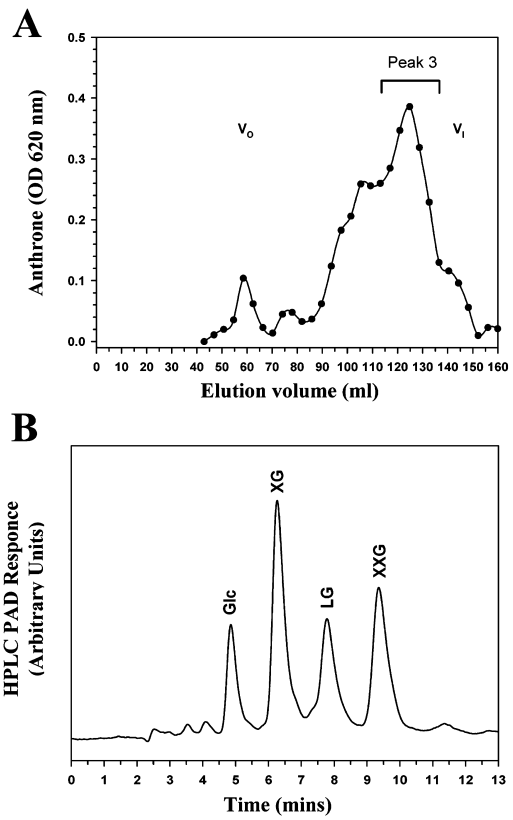

Fig. 4. Oligo-xyloglucan fragments ranging from 3 to 6 contiguous residues. (A) Partially hydrolyzed fragments from Peak 2 were subjected to further digestion by β-(1,4)-endoglucanase II and separated on a Bio-Gel P-2 gel filtration column. (B) The oligo-xyloglucan fragments contained within Peak 3 were pooled, lyophilized and 10 μl aliquots were separated using a Dionex HPAE–HPLC Carbo-pac PA-1 analytical column. This analysis revealed that Peak 3 contained four distinct oligosaccharide fragments. Similar fractions representing each of these four distinct oligosaccharide fragments were further separated by HPAE–HPLC using a semi-preparative column by collecting and pooling similar fractions from 12 to 15 injections of 60 μl. The structure of each fragment was analyzed for glycosyl composition, glycosyl linkage, and pentose/hexose ratio (Table 2).

Peak 2 with the exception of the LG fragment, which was only found in Peak 3.

3.4. Disaccharide (Isoprimeverose)

Isoprimeverose was isolated and identified by complete digestion of tamarind xyloglucan with Driselase and separation from monosaccharides on a P-2 column (Fig. 5). Analysis of Peak 4 by HPAE–HPLC confirmed a mixture of mono- and disaccharides. Glycosyl composition analysis showed the disaccharide consisted of a 1:1 ratio of glucosyl and xylosyl residues. Methylation showed t-Xyl and 6-Glc linkages only, again confirming the structure of isoprimeverose (Table 1, Peak 2A).

4. Summary

This work was successful in isolating and identifying various smaller oligosaccharide fragments from tamarind xyloglucan. Results are easily reproducible resulting in (relatively) pure oligo-saccharides for standards and substrates. Furthermore, we have established R_f values of most of the oligosaccharide fragments presented here using a Dionex HPAE–HPLC, which will be of great value for other researchers to identify oligosaccharide fragments using a similar system. Our MALDI–TOF analysis did not yield data for every peak analyzed. We believe that our desalting step may have produced some highly acidic fractions, which subsequently degraded prior to analysis. Nevertheless, these isolated oligo-xyloglucans can be used as standards to identify reaction products from xyloglucan synthase enzyme assays and to determine suitable acceptor molecules for these enzymes. In addition, the

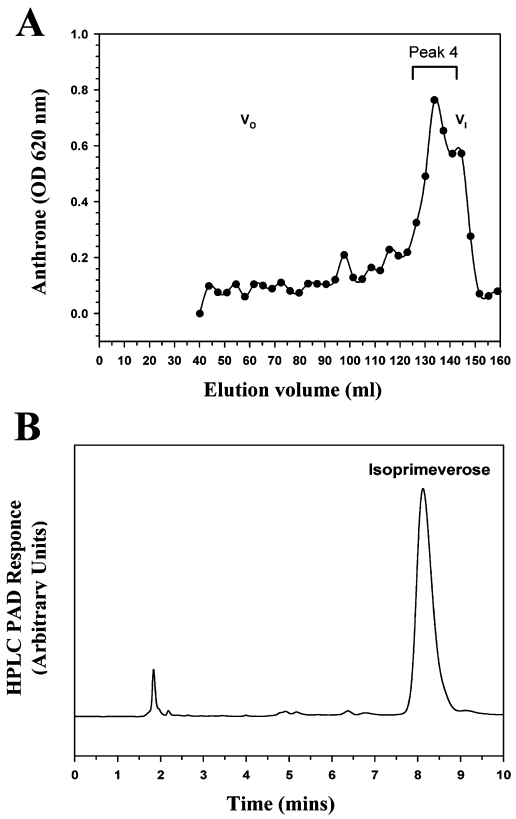

Fig. 5. (A) Tamarind xyloglucan digested with Driselase and separated by Bio-Gel P-2 gel filtration column chromatography. (B) The presence of isoprimeverose and monosaccharides are indicated in Peak 4.

oligo-xyloglucans may be derivatized with 8-aminopyrene-1,3,6-trisulfonic acid (Mort & Chen, 1996) and used in an capillary zone electrophoresis based assay as standards and substrate for xyloglucan synthase activity (Cavalier, Marry, & White, 2001).

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