CHAPTER SEVEN

Methods for Structural Characterization of the Products of Cellulose- and Xyloglucan-Hydrolyzing Enzymes

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Contents

Abstract

Structural characterization of oligosaccharide products generated by enzymatic hydrolysis of plant cell wall polysaccharides provides valuable

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Methods in Enzymology, Volume 510
ISSN 0076-6879, DOI: 10.1016/B978-0-12-415931-0.00007-0

information about the enzyme's activity and substrate specificity. In this chapter, we describe some of the chemical, chromatographic, and spectroscopic methods that we routinely use to isolate and characterize oligosaccharides formed by enzymatic fragmentation of cellulose and xyloglucan. These include techniques to determine glycosyl residue and glycosyl linkage compositions by gas chromatography and mass spectrometry. We also illustrate the use of electrospray ionization with multistage mass spectrometry, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and nuclear magnetic resonance spectroscopy to perform detailed structural analysis of these oligosaccharides.

1. Introduction

The polysaccharide-rich plant cell wall provides structural support to cells, tissues, and organs and is a recalcitrant barrier that wards off diverse biotic and abiotic challenges. All land plant cell walls consist of several structurally and functionally independent networks that together form recalcitrant composite structure that is nevertheless biologically responsive and pliable upon developmental and environmental cues. The networks consist of cellulose microfibrils tethered by cross-linking glycans which are embedded in a pectin and hemicellulosic matrix and may be further fortified by structural proteins and/or polyphenolic substances together with small amounts of inorganic compounds and hydrophobic molecules (Carpita and Gibeaut, 1993; O'Neill and York, 2003). Cell wall composition and architecture vary among plant species and also between tissues and developmental stages within the same plant. With the growing desire to use plant biomass as a renewable carbon-rich substrate for industrial and biomedical applications, there is an impetus for structural characterization of cell wall polysaccharides. Linkage-specific glycosyl hydrolases (GHs) have become a powerful tool for such studies. GHs are also the major players required to depolymerize and ultimately convert the plant cell wall into sugars for downstream processes. For a recent review describing the enzymes involved in deconstruction of plant cell wall polysaccharides, see Gilbert (2010).

One feature that is consistent among all land plant cell walls is the presence of cellulose, which is the most abundant component of these walls. Cellulose is composed of 1,4-linked β-D-glucosyl residues that form linear chains which are interconnected by hydrogen bonds and van der Waals forces to form rigid and insoluble cellulose microfibrils (Harris *et al.*, 2010; O'Neill and York, 2003). Effective and complete cellulose hydrolysis requires the combined action of several types of enzymes including endo-β-1,4-glucanases (EC 3.2.1.4), exo-β-1,4-glucanases or cellobiohydrolases

(E.C. 3.2.1.91), and β-glucosidases (E.C. 3.2.1.21) from a variety of GH families (Cantarel *et al.*, 2009; Gilbert, 2010; http://www.cazy.org/). Recent evidence suggests that some cellulose-degrading fungi also utilize an oxidoreductive cellulose-depolymerizing system that functions synergistically with the canonical hydrolytic cellulase system and utilizes the combined activities of cellobiose dehydrogenase (CDH, EC1.199.18) and GH61 copper-dependent oxidases (Langston *et al.*, 2011; Quinlan *et al.*, 2011).

Xyloglucans (XGs) are branched hemicellulosic polysaccharides that have a linear backbone of 1,4-linked β-D-glucosyl residues. Stretches containing two to five of these residues are consecutively substituted at O-6 with α-D-xylosyl residues. The xylosyl residues themselves may be further extended with additional sugars leading to side chain structures that can vary significantly among plant species (Hoffman *et al.*, 2005; Peña *et al.*, 2008; York *et al.*, 1990, 1996). Due to the structural complexity of XGs, a nomenclature system has been devised to describe different side chain substitutions (Fry *et al.*, 1993). Briefly, the letter G represents an unsubstituted Glc*p* residue. When the Glc*p* is substituted at O-6 with α-D-Xyl*p*, this is termed the X side chain, which may be substituted with a β-D-Gal*p* (L side chain), which itself may be substituted with an α-L-Fuc*p* (F side chain). Many endo-β-1,4-glucanases that hydrolyze cellulose are also able to cleave the XG backbone. However, several XG-specific endo-β-1,4-glucanases (XG endohydrolases, XEH, EC 3.2.1.151), which are members of the CAZy glycoside hydrolase families GH5, GH7, GH12, GH16, GH44, and GH74, have been identified (Ariza *et al.*, 2011; Cantarel *et al.*, 2009; Eklöf and Brumer, 2010; Gilbert, 2010; Gloster *et al.*, 2007; Irwin *et al.*, 2003; Pauly *et al.*, 1999). Some members of family GH74 have been shown to be oligoxyloglucan reducing end-specific cellobiohydrolases (EC 3.2.1.150) (Bauer *et al.*, 2005; Yaoi and Mitsuishi, 2002). Together these enzymes have become valuable tools for determining the fine structures of XGs and XG oligosaccharides.

Complete structural analysis of enzyme-generated oligosaccharides requires the determination of many features including monosaccharide composition and interglycosidic linkages, molecular mass, anomeric configurations, glycosidic sequence, and the presence and position of side chains. In this chapter, we describe some of the methods that are used to isolate and characterize the oligosaccharides formed by enzymatic fragmentation of cellulose and XG polysaccharides. The procedures described here can be used on a wide variety of polysaccharides from various sources. These methods are also applicable with minor modification for the characterization of oxidized cellodextrin products of GH61s and related enzymes. We describe how different techniques, including mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, are used to determine the fine structure of enzymatically generated oligosaccharides.

2. Preparation of Substrates

Microcrystalline cellulose (Avicel) and cellulose derivatives (e.g., carboxymethyl cellulose) are substrates typically used to determine cellulase activity. These and several other plant cell wall polysaccharides are available from different chemical companies. Megazyme (http://secure.megazyme.com/Homepage.aspx), in particular, offers plant cell wall polysaccharides (e.g., XGs, xylans, and mannans), as well as purified cello- and other oligosaccharides.

Methods to isolate cell walls from plant tissues and the protocols to extract and purify individual wall polysaccharides have been previously described (Hoffman *et al.*, 2005; York *et al.*, 1986).

Oligosaccharide substrates can be prepared by chemical or enzymatic hydrolysis of the respective polysaccharides and purified using methods described in Section 3. In-house purification of oligosaccharides may be time-consuming, but typically yields compounds that are more homogenous than commercially available products.

3. Purification of the Oligosaccharide Products

This review is focused on structural characterization of enzymatically produced oligosaccharides, and not enzyme assay procedures. Heterogeneity of plant cell wall components and the GHs involved in their metabolism and breakdown ultimately translate to a variety of experimental details in the assay conditions. The procedures described here are applicable to a wide variety of polysaccharides from various sources, including insoluble cellulose and soluble XG. Many of these procedures rely on the differential solubility of the substrate and oligosaccharide products.

3.1. Isolation of soluble products generated by enzymatic hydrolysis of insoluble substrates

This protocol is used for the isolation of soluble products from an insoluble substrate. For example, the isolation of low molecular weight cello-oligosaccharides from Avicel cellulose.

1. Separate the soluble products from the insoluble material by centrifugation for 15 min at $3600 \times g$. Collect the supernatant and transfer to a clean tube. To maximize the product yield, wash the solid residue with a small amount of water or buffer, centrifuge again, and pool the supernatants.

2. Inactivate the enzyme by heating the supernatant for 10 min in a boiling water bath.
3. Lyophilize the solution.

Further cleanup of the lyophilized reaction products can be largely circumvented if volatile buffers are used. We routinely use ammonium formate or ammonium acetate buffers (in the range of 10–50 m*M*) in our enzyme assays because of their volatility as well as their optimum buffering ranges (pH 4–6), which coincides with the pH optimum of many plant cell wall-degrading GHs. Alternatively, ammonium bicarbonate buffer can be used for enzymes requiring neutral to slightly alkaline pH (pH 6–9).

3.2. Isolation of soluble products generated by enzymatic hydrolysis of soluble polymeric substrates

This protocol applies if both the substrate and the product are water soluble, but the substrate precipitates in high concentrations of ethanol. This is the case for XG and several other polysaccharides. This protocol is very simple and can often substitute for labor-intensive chromatographic techniques and is frequently used as a preliminary fractionation step even if further purification is eventually required.

1. Add ethanol to the enzymatic reaction mixture to a final concentration of 75% and incubate at 4 °C for several hours to precipitate any undigested polymeric materials. Separate the soluble products from the precipitated material by centrifugation for 15 min at $3600 \times g$. XG polysaccharides and oligosaccharides with three or more repeating structural units can be separated from smaller XG oligosaccharides with 75% ethanol. Lower final ethanol concentration yields higher-molecular weight oligosaccharides in the soluble fraction.
2. Collect the supernatant and remove the ethanol by rotary evaporation or centrifugal evaporation. Dissolve the material in water and lyophilize the solution.

Note that the temperature and time requirement as well as the final ethanol concentration for efficient precipitation vary according to the characteristics of both the substrate and the product. Empirical testing is suggested with new substances.

3.3. Purification of oligosaccharides by liquid chromatography

Liquid chromatographic techniques are used extensively to isolate and purify oligosaccharides. The choice of the chromatographic mode typically depends on the sample characteristics. We have found that

size-exclusion and reversed-phase chromatographies are versatile and are usually sufficient to obtain homogeneous oligosaccharides. Moreover, these methods employ volatile buffers and/or organic solvents that are easily removed and thus do not interfere with subsequent structural or functional analyses.

3.3.1. Size-exclusion liquid chromatography

Size-exclusion chromatography (SEC) is used to separate molecules according to their hydrodynamic size. However, if the columns are eluted with water rather than with buffer, anionic oligosaccharides typically elute in the column void volume (Peña *et al.*, 2007). The protocol below is effectively used to separate enzymatically generated oligosaccharides from undigested high molecular weight substrate.

1. Dissolve the lyophilized enzymatic digestion products in 50 m*M* ammonium formate pH 5 (~5 mg/ml).
2. Inject aliquots (200 µl) of the solution to a Supedex-75 HR10/300 column (GE Healthcare) previously equilibrated with the same buffer. These columns typically have a capacity of ~5 mg.
3. Elute the oligosaccharides with the same buffer at a flow rate of 0.5 ml/min. The eluate can be monitored with a refractive index or an evaporative light scattering (ELS) detector.
4. Collect peak fractions and combine those that contain the oligosaccharides. Lyophilize the solution.
5. Dissolve the lyophilized material in water and lyophilize again to remove the volatile ammonium salt. Repeat this step up to three times to ensure complete removal of the ammonium formate. Alternatively, the oligosaccharides can be desalted by using LC-18 cartridges (following the instructions of the manufacturer) (York *et al.*, 1996) or by using a Bio-Gel P2 or Sephadex G-10 desalting column.

Mixtures of cello- and XG oligosaccharides can be separated into individual components using a Bio-Gel P2 (400 mesh, extra fine; Bio-Rad) column (1 × 100 cm) eluted with water using gravity flow (~2 m hydrostatic head). Fractions are collected with a fraction collector and assayed colorimetrically for the presence of carbohydrate using the phenol–sulfuric acid method (Masuko *et al.*, 2005).

3.3.2. Reversed-phase high-performance liquid chromatography

Reversed-phase high-performance liquid chromatography (RP-HPLC) is used to separate individual components from oligosaccharide mixtures based on their hydrophobic characteristics. Usually the oligosaccharides have been partially purified by SEC prior to RP-HPLC.

1. Dissolve the oligosaccharides or oligoglycosyl alditols (see Section 4.2) in water (~1 mg/ml) and filter the solution using a centrifugal filter device (0.2 μm).
2. Inject aliquots (200 μl) of the solution onto a Zorbax reversed-phase C-18 column (250 × 4.6 mm; Agilent Technologies Inc., Santa Clara, CA). Other manufacturers' C 18 columns are also suitable.
3. Elute the oligosaccharides at a flow rate of 1 ml/min with a linear gradient from 6% to 15% of aqueous methanol over 35 min followed by a linear gradient from 15% to 35% over 15 min. The column can be reconditioned by washing with aqueous 50% methanol for 10 min followed by washing with 6% aqueous methanol for an additional 10 min. In our laboratory, we detect the oligosaccharides using a Sedex 55 ELS detector (Sedere, Alfortville, France).
4. Fractions containing the oligosaccharides are collected and lyophilized.

4. Chemical and Structural Analysis of the Reaction Products

Several methods can be used, individually or in combination, to characterize the purified oligosaccharides. The selection of the methods depends on the scope of the experiments and the purity and previous knowledge of enzymes and substrates.

4.1. Glycosyl residue composition analysis by gas chromatography with mass spectrometric and flame ionization detection

Determination of the glycosyl residue composition of the soluble products of a reaction is usually a preliminary step in the analysis. When working with a GH of unknown specificity, composition analysis of the crude hydrolysate or purified components can provide insight into the enzyme properties, including its specificity. Neutral sugar analysis is a good starting point of analysis and is a powerful technique that gives information on both the types and amounts of monosaccharides present in the sample. The information obtained is used to guide both the analytical and purification strategies and requirements. This method to convert neutral sugars in their per-*O*-acetylated alditol (AA) derivatives is based on the protocols developed by Blakeney *et al.* (1983) and Carpita and Shea (1989). The analysis of AA was performed by gas chromatography (GC) using mass spectrometric detection (MSD) and flame ionization detection (FID). One advantage of using a MSD is that per-*O*-acetylated alditols give diagnostic mass spectra and thus can be readily distinguished from contaminating noncarbohydrate

peaks including phthalates. However, quantification using FID is preferred for determining the amounts of monosaccharides in the samples since it gives more reproducible results than MSD. Quantification by ion monitoring presents specific problems, including differences due to changes in mass tuning and ion suppression. Response factors are calculated by analysis of known standards and determination of the peak areas for each sugar derivative relative to the internal standard, myo-inositol.

1. Prepare a 20 m*M* solution of standard monosaccharides in water corresponding to the monosaccharides present in the sample (e.g., for plant cell wall materials use L-Rha, L-Fuc, L-Ara, D-Xyl, D-Man, D-Gal, D-Glc). Transfer 10–100 µl aliquots of the solution to Teflon-lined screw-cap tubes to make a series of standards with increasing concentrations of the monosaccharides and then lyophilize the samples.
2. All subsequent steps are performed in a fume hood. Transfer 200–500 µg of the oligosaccharides to Teflon-lined screw-cap tubes. Add 1 ml of 2 *M* trifluoroacetic acid (TFA) and 50 µl of 20 m*M* myo-inositol to both the samples and standards. Cap the tubes tightly and heat for 90 min at 120 °C in a temperature-controlled heating block.
3. Cool the tubes to room temperature and then evaporate the TFA at 40–45 °C using a stream of filtered air or nitrogen. Trace amounts of TFA are then removed by adding 1 ml of volatile organic solvent such as methanol or acetone and evaporated as above. The next step requires alkaline conditions, and unintentional acidification can result in incomplete reduction. Dry samples can be stored overnight if necessary.
4. Prepare a 100 mg/ml solution of sodium borohydride (or borodeuteride if isotopic labeling is required) in 1 *M* ammonium hydroxide and then dilute with 5 volumes of dimethyl sulfoxide (DMSO). Add 600 µl of the diluted solution to each sample and incubate for 90 min at 40–45 °C. Vortex every 30 min.
5. Destroy the unreacted $NaBH_4$ by adding 100 µl of glacial acetic acid and mixing thoroughly. Add 100 µl of 1-methylimidazole and 75 µl of anhydrous acetic anhydride. Mix and keep for 30 min at 40–45 °C.
6. To terminate the reaction, add 2 ml of deionized water and shake vigorously. Allow the mixture to cool to room temperature and then add 1 ml dichloromethane and shake the mixture for 1 min. Centrifuge for 5 min at 3000 × *g* and discard the upper aqueous layer.
7. Add 2 ml of deionized water to the organic layer and shake the mixture for 1 min. Centrifuge for 5 min at 3000 × *g* and again discard the aqueous layer. Repeat this step four more times to ensure complete removal of water-soluble salts and DMSO, which can interfere with the downstream analysis.
8. Concentrate the organic layer to dryness by a stream of air or nitrogen and dissolve the residue in 100–200 µl of dichloromethane or acetone.
9. The alditol acetate derivatives are analyzed using Hewlett Packard 5890A gas chromatograph coupled to a Hewlett Packard 5970 mass

selective detector (MSD) or an Agilent 7890A gas chromatograph with a flame ionization detector. In both cases, derivatized monosaccharides are separated on a SP 2330 column (30 m × 0.25 mm, 0.25 μm film thickness, Supelco) with helium as the carrier gas and the following oven temperature gradient: 80 °C held for 2 min, 80–170 °C at 30 °C/min, 170–240 °C at 4 °C/min, 240 °C held for 20 min.

4.2. Converting oligosaccharides to their corresponding oligoglycosyl alditols

For some applications, oligosaccharides are converted to their corresponding oligoglycosyl alditols prior to analysis. There are several advantages to working with alditols rather than native carbohydrates. First, unlike native reducing carbohydrates, oligoglycosyl alditols do not have reducing end α and β anomeric protons and thus have less complex NMR spectra. In some modes of chromatography, the anomeric forms can separate, and the chromatograms become unnecessarily complex. Also, oligoglycosyl alditols are less likely to undergo base-catalyzed β-elimination during per-*O*-methylation reactions (Waeghe *et al.*, 1983).

1. All steps are performed in a fume hood. Transfer 250–500 μg of the oligosaccharides to a Teflon-lined screw-cap tube. Add 250 μl of a solution of 1 *M* sodium borohydride (or borodeuteride if isotopic label is required) in 1 *M* ammonium hydroxide and keep for at least 1 h at room temperature.
2. Terminate the reaction by dropwise addition of glacial acetic acid until the release of hydrogen gas stops. Then, add 0.5 ml of 10% (v/v) acetic acid in methanol and evaporate the solvents using a stream of air or nitrogen gas to remove borate as its volatile trimethyl ester. Repeat the acetic acid/methanol treatment three times. Add 0.5 ml of anhydrous methanol and concentrate to dryness to remove traces of acetic acid; repeat three times.

4.3. Glycosyl-linkage composition analysis

A fundamental step in understanding the fine structure of an oligosaccharide is to determine how the individual sugars are linked to one another. One well-established method for this is to methylate all the free (unsubstituted) hydroxyl groups of the intact oligosaccharides prior to acid hydrolysis. The hydroxyl groups generated during the hydrolysis to monosaccharides and reduction to the alditols are then *O*-acetylated to form the partially-*O*-methylated alditol acetate (PMAA) derivatives. The fragmentation pattern of these derivatives during electron ionization (EI) MS provides information about the type of monosaccharide (i.e., hexose, pentose, deoxyhexose) as well as the position of the *O*-methyl and *O*-acetyl groups, which can then be

used to deduce the positions that the original sugars were linked (Carpita and Shea, 1989).

4.3.1. Preparation of the solid NaOH/DMSO reagent

Methylation of oligosaccharides using solid NaOH in DMSO is a faster and less hazardous approach than some other methylation procedures. This method is based on the protocol developed by Ciucanu and Kerek (1984).

1. All steps are performed in a fume hood. Combine 100 µl of aqueous 50% (w/v) NaOH and 200 µl of anhydrous methanol in a screw-cap tube and vortex to give a clear solution. Add 1 ml of anhydrous DMSO, vortex and then centrifuge for 5 min at 3600 × *g*.
2. Remove and discard the supernatant. Add 1 ml of anhydrous DMSO, vortex and centrifuge as before.
3. Repeat Step 2 five times to prepare an opalescent NaOH pellet. Suspend the pellet in 200–300 µl of anhydrous DMSO and use immediately for the subsequent methylation reaction.

4.3.2. Methylation of oligosaccharides

1. All steps are performed in a fume hood. Suspend 100–200 µg of the lyophilized oligosaccharide or oligoglycosyl alditol in 200–500 µl of anhydrous DMSO in a screw-cap vial. Sonicate or heat gently (60 °C) to increase solubility if necessary.
2. Add ~200 µl of freshly prepared NaOH in DMSO (from Section 4.3.1) and 10 µl of water, which is added to limit oxidative degradation (Ciucanu and Costello, 2003). Stir the mixture for 15 min at room temperature.
3. Add 300 µl of methyl iodide (caution, this reagent is toxic) and keep the mixture for 15 min at room temperature, then cool the reaction mixture in ice, add 1 ml of water and vortex. Remove excess methyl iodide by carefully bubbling air (or nitrogen) through the reaction mixture.
4. Add 1 ml of chloroform, vortex, centrifuge for 5 min at 3000 × *g*, and then transfer the lower organic layer to a clean tube.
5. Wash the combined organic phase three times with 1 ml of deionized water. Evaporate the chloroform and dissolve the residue containing the methylated materials in methanol.
6. The per-*O*-methylated glycans are converted to their PMAA derivatives and analyzed by GC-MS as described in Section 4.1, using sodium borodeuteride. The symmetry of some alditols (e.g., xylitol and galactitol) makes it impossible to unambiguously determine the location of the *O*-acetyl and *O*-methyl groups of some PMAA derivatives (e.g., 1,3,4,5-tetra-*O*-acetyl-2-*O*-methyl xylitol and 1,2,3,5-tetra-*O*-acetyl-4-*O*-methyl xylitol) unless the C1 is labeled by reduction with

deuterium. The positions of the *O*-methyl and *O*-acetyl substituents in per-*O*-methylated alditol acetates are readily determined from the primary fragment ions present in their EI mass spectra. A collection of spectra of partially methylated alditol acetate derivatives generated from diverse glycosidically linked sugars is available at http://www.ccrc.uga.edu/specdb/ms/pmaa/pframe.html or in the literature (Carpita and Shea, 1989).

4.4. Analysis of oligosaccharides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis is used to determine the mass distribution of the different species present in a mixture of oligosaccharides and is routinely used to monitor enzymatic reactions. The technique requires small amounts of sample (0.1–0.5 μg) to obtain good spectra and is rapid and relatively tolerant to the presence of low concentrations of salts and other contaminants. Another advantage of this technique is that the oligosaccharides can be analyzed without derivatization.

1. Mix 1 μl of 0.5–1 mg/ml oligosaccharide solution in water with an equal volume of matrix solution (20 mg/ml 2,5-dihydroxybenzoic acid in aqueous 50% methanol) on the MALDI target plate and then dry with a stream of warm air (hair dryer).
2. Record a positive ion MALDI-TOF mass spectrum (e.g., using a Bruker Microflex LT mass spectrometer and workstation). Spectra generated by 10–200 laser shots are typically collected and averaged.

In the MALDI-TOF mass spectra shown in Fig. 7.1A, XG isolated from cell walls prepared from Arabidopsis inflorescence stems was treated with a GH12 xyloglucan-specific endoglucanase (XEH; EC 3.2.1.151) from *Aspergillus aculeatus* (Novozymes, Bagsvaerd, Denmark) purified as described (Pauly *et al.*, 1999). The XG oligosaccharides formed were then treated (Fig. 7.1B) with an GH74 oligoxyloglucan reducing end-specific xyloglucanobiohydrolase (OREX; EC 3.2.1.150) overexpressed in *Pichia pastoris* and purified as described (Bauer *et al.*, 2005, 2006). Both reactions were performed for 16 h in 50 m*M* ammonium formate pH 5, at 25 °C.

4.5. Analysis of the reaction products by NMR spectroscopy

NMR spectroscopy is a powerful tool for studying the enzymatic hydrolysis of polysaccharides and oligosaccharides as it provides information on the catalytic mechanism and substrate specificity (Rudsander *et al.*, 2008). NMR analysis is especially useful when the enzyme activity is unknown

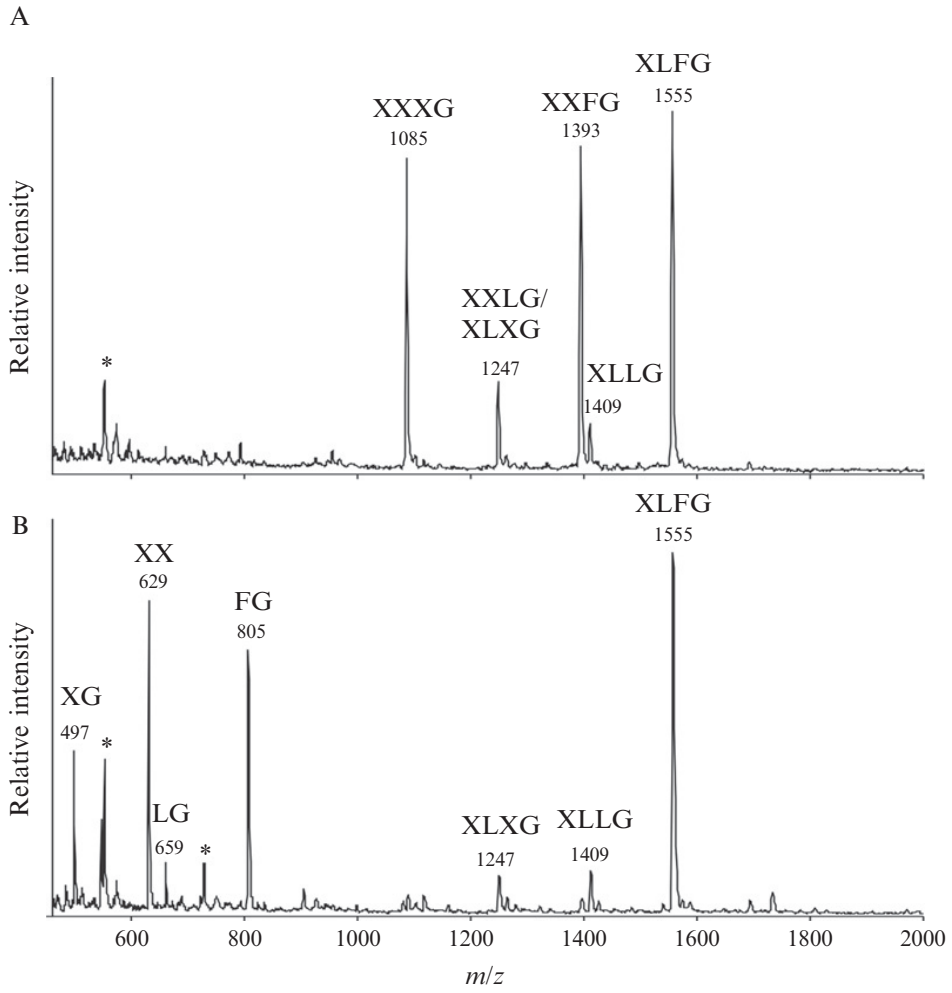

Figure 7.1 MALDI-TOF mass spectra of oligosaccharides generated from Arabidopsis xyloglucan by (A) xyloglucan-specific endoglucanase (XEH) and (B) both XEH and oligoxyloglucan reducing end-specific cellobiohydrolase (OREX). XEH hydrolyzes the glycosidic bond at the reducing end side of an unbranched glucosyl residue generating subunits with an XXXG-type branching pattern, namely XXXG, XXFG, XLFG, and the isobaric pair XXLG/XLXG. OREX further degrades xyloglucan oligosaccharides by hydrolyzing the glycosidic bond between the second and third glucosyl units from the reducing end, if the xylose at the third glucose from the reducing end is not further substituted. Thus, XXXG, XXLG, and XXFG are degraded to the common fragment XX, as well as XG, LG, and FG, respectively. XLXG and XLFG are not substrates for OREX. For detailed nomenclature of the xyloglucan side chains, see Section 1. The peaks marked with an asterisk are matrix signals.

or when the substrate to be analyzed is complex (e.g., plant cell wall). The enzyme-generated oligosaccharides, purified as described in Section 3, can be structurally characterized by one- and two-dimensional (1D and 2D, respectively) NMR. Analysis of the 2D spectra can be used to identify isolated spin systems and to obtain chemical shifts and coupling constants

for each type of residue in the oligosaccharide. These data are then used to determine the identity, the ring form, and the anomeric configuration of each residue. Experiments including $^{1}H—^{13}C$ HMBC provide data to deduce glycosidic linkages between the residues in the oligosaccharide.

In the case of water-soluble polysaccharides (e.g., XG) or short oligosaccharides (e.g., cello-oligosaccharides), their enzymatic hydrolysis can be monitored by NMR. One advantage of this approach is that the composition and structure of both substrates and products can be determined. Most of these substrates and products have been characterized previously, and the chemical shifts and coupling constants diagnostic for their most common structural features are available in the literature (Harjunpää *et al.*, 1996; Hoffman *et al.*, 2005; York *et al.*, 1990). In particular, a database consisting of a searchable table of ^{1}H NMR chemical shift of XG oligoglycosyl alditols is available at http://ccrc.uga.edu/db/nmr. Structural features of the residues in the oligoglycosyl alditols can be used to retrieve their chemical shifts from the database. Also, chemical shift data can be used as a query to obtain information about the residue identity and chemical environment.

4.5.1. Determination of the hydrolysis stereochemistry by real-time ^{1}H NMR

Real-time ^{1}H NMR can be used to determine the anomeric stereochemistry of enzymatic hydrolysis products, and thus distinguish between the retaining and inverting mechanisms of a GH (Rudsander *et al.*, 2008). In this technique, an enzyme is added to a solution of the oligosaccharide in an NMR tube, which is then immediately placed in the instrument. 1D ^{1}H-NMR spectra are acquired at fixed time points over the course of the enzyme reaction. The cleavage of the glycosidic bond results in the formation of a new oligosaccharide with α or β reducing glycose depending on the mechanism that the enzyme uses to hydrolyze the substrate as well as the original anomericity. If the rate of enzymatic hydrolysis is faster than the rate of anomeric interconversion, the α or β anomeric proton signal that appears in the 1D spectra will indicate the cleavage mechanism (see Fig. 7.2). The anomeric conversion of glucose, for example, at or near neutral pH in D_2O occurs at time constant of approximately 1 h (Harjunpää *et al.*, 1996). Thus, the amounts of substrate and enzyme, as well as temperature and pH, have to be suitable in order to get a workable ratio of the rates of anomeric conversion and product (new anomeric signal) generation. The presence of α and β anomeric signals from the reducing end of the initial substrate itself may complicate interpretation of such results. Thus, oligosaccharide substrates are typically converted to their corresponding oligoglycosyl alditols prior to analysis (Section 4.2) so that their NMR spectra contain no reducing end anomeric resonances.

1. Polysaccharides, oligosaccharides, or oligoglycosyl alditols (1–2 mg) are dissolved in a suitable buffer for NMR analysis at a concentration and pH/pD appropriate for the enzymatic reaction. Nonvolatile inorganic buffers such as sodium phosphate, that lack nonexchangeable protons, are preferred, so the buffering capacity is retained during lyophilization, and the NMR spectrum is not "contaminated" with additional signals that can overlap with the signals from the sample.
2. Lyophilize the solution and dissolve the dry material in 1 ml of deuterium oxide (99.9%; Cambridge Isotope Laboratories). Repeat this step to remove all exchangeable protons from the carbohydrate and buffer molecules.
3. Dissolve the dry material in 200 μl of deuterium oxide and transfer the solution to a 3 mm NMR tube. Place the tube with the oligosaccharide in the instrument (e.g., in a Varian Inova NMR spectrometer operating

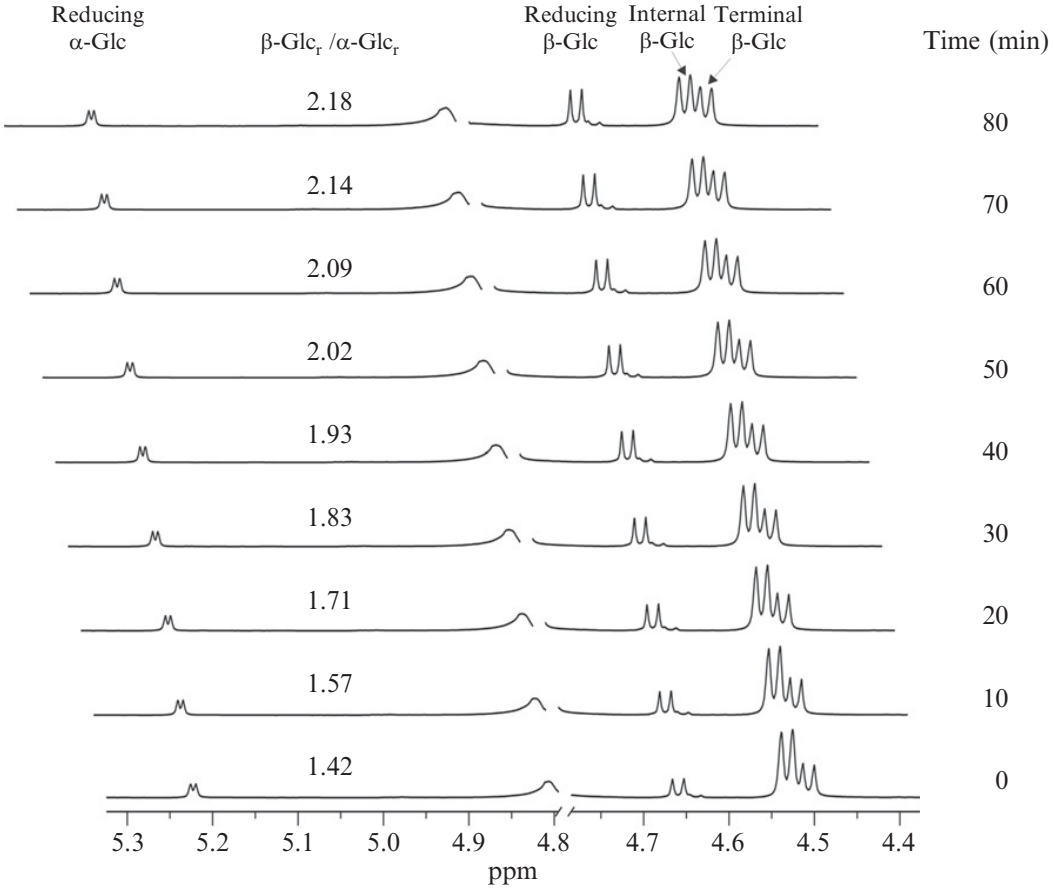

Figure 7.2 Real-time ^{1}H NMR spectra of cellopentaose hydrolysis with a GH12 β-(1 → 4)-endoglucanase from *Aspergillus niger* (Megazyme) at 25 °C. Ratio of the integrals of anomeric signals of reducing β-Glc*p* (β-Glc$_r$) and reducing α-Glc*p* (α-Glc$_r$) residues indicates that the hydrolysis product has a β-Glc*p* residue at the reducing end, demonstrating that the enzyme cleaves the glycosidic linkage by a retaining mechanism.

at 600 MHz and equipped with a 3 mm cold probe) and set the desired sample temperature for the experiment (this will be the temperature for the enzymatic reaction).

4. Allow sufficient time for the sample temperature and the substrate reducing end α/β anomeric ratio to reach equilibrium. Tune the probe and then lock and shim the instrument. Set the conditions for an array experiment including the time interval between collection of spectra. It is recommendable to use an experiment with a water suppression technique (e.g., presaturation) to record the 1D spectra.
5. Remove the sample from the instrument and add the enzyme to the oligosaccharide solution. The enzyme should be in the same buffer as the sample in order to maintain the initial anomeric ratio (α/β) at the reducing end of the oligosaccharide. Immediately return the tube to the instrument, check and adjust the shimming if necessary and start the array experiment. A series of ^{1}H NMR spectra summing 16 scans each are recorded at defined time intervals during the enzymatic reaction.
6. Data are processed using commercially available software (e.g., MNova, Universidad de Santiago de Compostela, Spain). Chemical shifts are calibrated relative to a diagnostic resonance previously identified in the spectrum of the sample.

In the example in Fig. 7.3, the cellopentaose substrate was generated from Avicel by phosphoric acid hydrolysis (Liebert *et al.*, 2008) and purified using a Bio-Gel P2 as described in Section 3.3.1.

4.6. Electrospray ionization mass spectrometry

Electrospray ionization with multiple-stage MS (ESI-MS^n) is a useful technique to determine the glycosyl sequences and branching pattern of oligosaccharides. These structural features may be difficult to determine by other analytic techniques (e.g., NMR) due to the redundancy of the characteristic signals as well as higher amounts of sample required. One advantage of ESI-MS^n is its ability to selectively fragment a molecular ion to generate fragment ions that can be further fragmented. This process can be repeated many times, with every generation giving structural information about the parent ion (see Fig. 7.3). To resolve ambiguities during the identification of the fragments, the analysis is usually performed on per-*O*-methylated oligosaccharides or, preferably, per-*O*-methylated oligoglycosyl alditols. Treatment with a reducing agent such as $NaBH_4$ prior to methylation converts the reducing end residue to an alditol, allowing the so-called A, B, and C fragments, which contain the nonreducing end to be distinguished from X, Y, and Z fragments, which contain the alditol (Domon and Costello, 1988; Mazumder and York, 2010). Methylation of the free hydroxyl groups increases sensitivity and, more

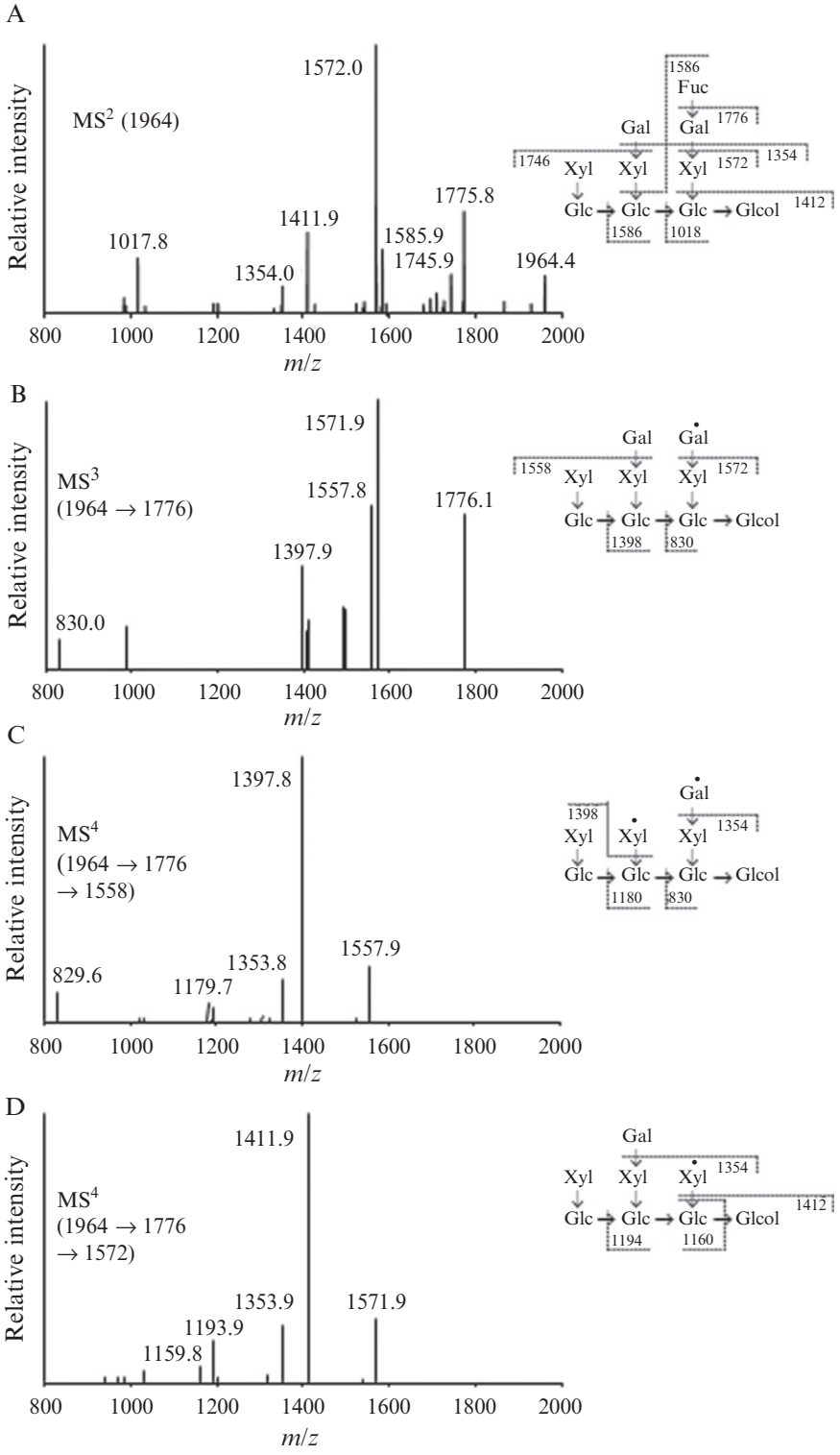

Figure 7.3 ESI-MS^n spectra of a per-*O*-methylated xyloglucan oligoglycosyl alditol (XLFGol) generated from Arabidopsis xyloglucan. All labeled peaks, except the ion at *m/z* 1160 in panel D, correspond to sodiated Y-type ions. The pseudomolecular ion at *m/z* 1964 was selected from the full scan spectrum (not shown) for collision-induced dissociation (CID) fragmentation. The resulting MS^2 mass spectrum (A) shows extensive

importantly, helps to identify the hydroxyl groups involved in glycosic linkages, since fragmentation at the glycosidic bond exposes nonmethylated hydroxyl group analogously to acid hydrolysis of methylated oligosaccharides.

1. Dissolve the per-*O*-methylated oligosaccharides or oligoglycosyl alditols in methanol and mix with 50% aqueous acetonitrile containing 0.1% TFA to give a final concentration of ~1 ng/μl.
2. Introduce the sample into the MS source through a fused silica capillary (150 μm i.d. × 60 cm; Thermo Finnigan, USA) at flow rate of 3 μl/min using a syringe pump.
3. Record ESI mass spectra (e.g., with a Thermo Scientific LTQ XL ion trap mass spectrometer). The electrospray source is operated at a voltage of 5.0 kV and a capillary temperature of 275 °C. To obtain MS^n spectra, the collision energy is adjusted to obtain optimal fragmentation.

ACKNOWLEDGMENTS

This work was supported in part by US Department of Energy Grant DE-FG02-96ER20220, US Department of Energy-funded Center for Plant and Microbial Complex Carbohydrates (DE-FG02-93ER20097), and National Science Foundation Grant DBI-0421683. This research was also supported by the BioEnergy Science Center. The BioEnergy Science Center is a US Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science.
The *P. pastoris* clone (AN1542.2) carrying the gene encoding oligoxyloglucan reducing end-specific xyloglucanobiohydrolase was obtained from the Fungal Genetics Stock Center (Kansas City, Missouri, USA; http://www.fgsc.net/).

fragmentation, to generate a sodiated Y-ion (XLLG, *m/z* 1776) with a single hydroxyl group exposed by cleavage of the glycosidic bond that linked the fucosyl residue to the galactosyl residue. Such "scars," which are indicated as dots (•), provide sequence information not available when native oligosaccharides are analyzed. The diagnostic ion at *m/z* 1018 (FG with one scar, panel A) establishes the location of the fucosylated side chain. The ion at *m/z* 1586 can arise from two distinct fragmentation pathways, forming either LFG with one scar or XGFG with one scar. The ion at *m/z* 1776 was then selected from the MS^2 spectrum and fragmented to generate the MS^3 spectrum (B), which included signals corresponding to fragment ions at *m/z* 1558 (XXLG with two scars) and *m/z* 1572 (XLXG with one scar). These two ions were individually selected for further fragmentation. The resulting MS^4 (C and D) included additional diagnostic signals. The ion at *m/z* 1160 (D) arises from two MS^4 fragmentation events, which resulted in the loss of one side chain (Y-type fragmentation) and the alditol residue (B-type fragmentation). Together, these data unambiguously show that the fucosyl residue is attached to the galactosyl residue closest to the alditol end.

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