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Probing the structural details of xylan degradation by real-time NMR spectroscopy

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ABSTRACT

The biodegradation of abundantly available cell wall polysaccharides has recently received much attention, not least because cell wall polysaccharides are substrates for the human gut microbiota and for environmentally sustainable processes of biomass conversion to value-added compounds. A major fraction of cereal cell wall polysaccharides consists of arabinoxylans. Arabinoxylan and its degradation products are therefore present in a variety of agro-industrial residues and products. Here, we undertook to track the structural details of wheat arabinoxylan degradation with high resolution NMR spectroscopy. More than 15 carbohydrate residues were distinguished in the substrate and more than 20 residues in partially degraded samples without any sample cleanup. The resolution of a plethora of structural motifs *in situ* permits the readout of persisting structures in degradation processes and in products. Reaction progress was visualized for the biodegradation of arabinoxylan by different crude microbial enzyme preparations. The direct observation of structural details in complex mixtures containing arabinoxylan fragments is significant, as such structural details reportedly modulate the health-promoting functions of arabinoxylan fragments.

1. Introduction

The cell wall supports the plant body plan by forming a fiber-glass-like composite of various macromolecules, specifically cellulose, hemicelluloses and pectin polysaccharides alongside lignin, a polymer of aromatic alcohols (Cosgrove, 2000). The biological function of plant cell wall polysaccharides is the construction of a strong and flexible structure, the formation of a barrier to pathogens and the storage of metabolic energy that can be remobilized in some tissues, for instance in the cereal grains (Burton, Gidley, & Fincher, 2010; Cosgrove, 2000; Scheller & Ulvskov, 2010). Recently, plant cell wall degradation has received much additional attention due to the role of cell walls as the main source of dietary fiber and as renewable biomass sources (Burton et al., 2010). Due to their wide abundance, polysaccharides of plant cell walls are of economic interest as the starting materials for various products and processes.

Unlike cellulose, hemicelluloses and pectin form irregular branched structures with a limited tendency to aggregate (Burton et al., 2010). Due to this irregularity, the structural analysis of hemicellulose (xylan being the main hemicelluose component) and pectin structure is both challenging and labor-intensive. This analysis usually proceeds *via* chemical or enzymatic fragmentation, subsequent purification of fragments and deduction of chemical structure in the purified fragments (Bauer, Vasu, Persson, Mort, & Somerville, 2006). In this work-stream, the preparative fractionation of oligosaccharides has remained a limiting step.

Beyond the structural analysis of polymers, detailed insights into polysaccharide degradation pathways are of additional relevance as they help in the controlled production of oligosaccharides with desired qualities. As an example, cereal-derived arabinoxylan oligosaccharides act as prebiotics by supporting the growth of bifidobacteria and lactobacilli in the human gut (Broekaert et al., 2011). The prebiotic function is modulated by structural differences in arabinoxylan oligosaccharides, where different lengths and arabinose contents result in different prebiotic effects (Van Craeyveld et al., 2008). In addition to being prebiotics and intermediates in cell wall degradation, cell wall derived oligosaccharides are of biological interest as they have been implicated in growth regulation and signaling pathways (Burton et al., 2010).

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Here, we show that ^{1}H, ^{13}C and ^{1}H–^{13}C NMR spectroscopy provide structural detail for tracking the degradation of cell wall polysaccharides in crude mixtures. In the substrate wheat arabinoxylan, 15 different structural units are resolved and identified as their chemical structures. More than 20 (arabino-)xylooligosaccharide units, most of them near cleavage sites, are directly assigned in oligosaccharide mixtures produced by fungal enzyme preparations. The carbohydrate units can be distinguished using only anomeric signals as the structural reporters (Vliegenthart, Dorland, & Halbeek, 1983), while disregarding other NMR spectral signals. Especially the greater ^{13}C chemical shift dispersion permits the distinction of unsubstituted, mono- and disubstituted xylan units in different structural motifs. Enzyme action on the different structural motifs and limiting enzyme activities in biomass deconstruction can then be directly read out from oligosaccharide structures (Komiyama et al., 2009) and from time-resolved observations of their transformations. Persisting structural motifs of resultant arabinoxylan oligosaccharides can be distinguished in crude carbohydrate mixtures. The approach is used to characterize arabinoxylan degradation with microbial enzyme preparations from *Trichoderma reesei*, *Humicola insolens*, *Aspergillus aculeatus*, *Aspergillus niger*, *Talaromyces emersonii* and *Bacillus subtilis*. Such direct NMR observations have a role to play in facilitating industrial and scientific work streams concerned with the use and understanding of plant cell wall degradation.

2. Materials and methods

2.1. Enzyme preparations

Commercial enzyme preparations from microbial sources were used without further modification or purification. These preparations originated from *T. reesei* (Laminex; Danisco DuPont, Madison, WI, USA), *H. insolens* (Ultraflo L; Novozymes, Bagsværd, Denmark), *A. aculeatus* (Viscozyme; Novozymes, Bagsværd, Denmark), *A. niger* (Finizym; Novozymes, Bagsværd, Denmark), *T. emersonii* (Beerzym BG; Erbslöh, Geisenheim, Germany) and *B. subtilis* (Neutrase; Novozymes, Bagsværd, Denmark).

2.2. Chemicals and samples

Wheat arabinoxylan from Megazyme (Bray, Ireland) was used without further purification or modification. The arabinoxylan was dissolved under gentle heating to a concentration of 0.5% (w/v) in 600 μl of sodium acetate buffer (100 mM, pH 5.3). The buffer was produced as aqueous buffer prior to condensation and redissolution in D_2O (Cambridge Isotope Laboratories, Andover, MA, USA). 2 ml of commercial lager beer were condensed and resuspended in 600 μl D_2O in order to assess the spectral resolution of arabinoxylan fragments from other components of agro-industrial products.

2.3. NMR assays of arabinoxylan degradation

^{1}H NMR spectroscopic assays were conducted at 313 K on an 800 MHz Bruker (Fällanden, Switzerland) Avance spectrometer equipped with a TCI cryoprobe and an 18.7 T magnet (Oxford Magnet Technology, Oxford, UK). A ^{1}H time series of arabinoxylan degradation was acquired by recording 16,384 complex data points during an acquisition time of 1.6 s with a recycle delay of 2 s between the accumulation of 16 transients. In order to assess rapid initial steps of degradation, enzyme preparations were diluted in 100 μl buffer. This solution was taken up into the end of a transfer line attached to a syringe. The transfer line was placed above 500 μl of an arabinoxylan solution in a 5 mm NMR sample tube and the sample was inserted into the NMR magnet and ^{1}H NMR spectroscopic assays were conducted at 313 K on a 600 MHz Bruker DRX spectrometer equipped with a TCI cryoprobe and a 14.1 T magnet. A time series of 8000 ^{1}H NMR spectra was recorded as a pseudo-2D spectrum sampling 2048 complex data points during an acquisition time of 0.85 s, employing a recycle delay of 1 s and the accumulation of 4 transients. Once the pseudo-2D experiment had been started, the enzyme solution was manually injected *via* the transfer line into the sample.

2.4. NMR assignments of arabinoxylan-derived oligosaccharide mixtures

NMR assignments of structural motifs were conducted for intact arabinoxylan and for arabinoxylan oligosaccahrides obtained by partial degradation of arabinoxylan with an *A. aculeatus* preparation (Viscozyme) prior to quenching the degradation by heating to 100 °C for 5 min. Assignment spectra included DQF-COSY (2048 × 512 complex data points with acquisition times of 287 and 72 ms, respectively), NOESY with a mixing time of 200 ms (2048 × 256 complex data points with acquisition times of 307 and 38 ms, respectively), TOCSY with 10 kHz spin lock field during 60 ms (2048 × 512 complex data points sampling 307 and 76 ms, respectively), multiplicity edited ^{1}H–^{13}C HSQC (1024 × 512 complex data points with acquisition times of 153 and 18 ms), ^{1}H–^{13}C HSQC TOCSY (1024 × 256 complex data points sampling 153 and 10 ms), and ^{1}H–^{13}C HMBC (2048 × 256 complex data points with acquisition times of 300 and 6 ms). Some of the ^{1}H assignments could be independently validated by comparison to published assignments of purified arabinoxylan fragments or of intact arabinoxylan at high temperature (340 K) (Gruppen et al., 1992; Hoffmann, Leeflang, de Barse, Kamerling, & Vliegenthart, 1991; Hoffmann, Kamerling, & Vliegenthart, 1992; Kormelink et al., 1993). Spectra were referenced to the αH1 signal of the glucopyranosyl anomer of residual glucose in the Viscozyme preparation at ^{1}H = 5.223 ppm and ^{13}C = 92.990 ppm (Petersen, Hindsgaul, & Meier, 2014). Resultant sequential assignments are summarized in the supplemental Table 1S.

2.5. Data processing and analysis

All spectra were processed with Bruker Topspin 2.1 software using extensive zero filling in all dimensions. The presence and relative abundance of structural motifs were directly assessed from ^{1}H and ^{1}H–^{13}C spectra. The ^{1}H spectrum of arabinofuranosyl-residues in arabinoxylan was reconstructed from assignments of chemical shifts in five different structural motifs containing the arabinofuranosyl-residues. The experimental spectrum of wheat arabnioxylan can be well described as a sum of component spectra as

$$L(\delta) = \sum_{j=1}^{5} \sum_{i} a_j \frac{1}{1 + x_i}$$

where $x_i = [(\delta - \delta_i)/w]^2$. $L(\delta)$ is the Lorentzian function with amplitudes a_j for the five different component structural motifs having i different arabinose anomeric signals at spectral positions δ_i with a line width of $2w$.

3. Results

3.1. Assignment of strucural motifs in intact wheat arabinoxylan at ambient temperature

Arabinoxylan is a hemicellulose with β-1,4-linked D-xylopyranosyl backbone and frequent arabinofuranosyl residue