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Note

Structural heterogeneity of wheat arabinoxylans revealed by Raman spectroscopy

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Abstract—A set of arabinoxylan samples differing in their arabinose composition and various samples of arabino-xylo-oligosaccharide samples were analysed by Raman spectroscopy. Specific signatures for arabinose substitution were found in several spectral regions, that is, 400–600, 800–950 and 1030–1100 cm^{-1}. A linear relationship was observed between the peak ratio 855/895 cm^{-1} of the second derivative spectra and the A/X ratio determined by chemical analysis. Moreover, spectral changes were observed in the 400–600 cm^{-1} region assigned to the coupled vibrations mode in the skeleton: while the intensity of the band at 570 cm^{-1} increased with the degree of substitution, that at 494 cm^{-1} decreased. Similarly, a linear relationship was observed between the peak intensity ratio 570/494 cm^{-1} calculated on the second derivative spectra and the composition data. Analysis of Raman spectra of arabino-xylo-oligosaccharides allowed to identify specific spectral features of disubstitution.

Keywords: Raman spectroscopy; Arabinoxylans; Wheat

Arabinoxylans (AX) are the main non-starch polysaccharide constituents found in wheat grain endosperm. These cell wall components amount to 2–4% of wheat flour and are involved to a great extent in second transformation processes such as breadmaking.[1,2]

Wheat AX include a linear backbone of β-(1→4)-linked D-xylopyranosyl units, which are either non-substituted (uXyl), monosubstituted at O-3 (mXyl) or disubstituted at O-3 and O-2 (dXyl) with α-L-arabinofuranosyl units.[1,3] Some of the arabinose residues are esterified at O-5 by phenolic acids, essentially ferulic acid. In wheat flour, about 25% of AX are water extractable (WE-AX). WE-AX exhibit large natural variations in their structure, which are depicted generally by their substitution ratio (A/X) with extreme values ranging from 0.31 to 1.06.[4] More precisely, the substitution pattern could be revealed by chemical analysis or NMR spectroscopy, giving relative proportions of uXyl (60–65%), mXyl (12–20%) and dXyl (15–30%). As the substitution ratio increases, the relative proportion of mXyl is stable whereas uXyl decreases and dXyl increases.[4]

Sugar analysis or NMR spectroscopy are the main techniques used to access AX structural heterogeneity. However, they need relatively high amounts of isolated and purified material and do not allow spatially resolved studies. Vibrational spectroscopy was shown to be sensitive to AX structural features[5,6] and more recently, specific assignment of the arabinose substitution was determined on infrared spectra.[7] Both infrared and Raman spectroscopies can give molecular information, but the latter allows a higher spatial resolution when coupled to a microscope. As the physical phenomena involved are different, Raman spectroscopy could be complementary to infrared spectroscopy, as previously shown on plant cell walls analysis.[8] In the particular case of wheat endosperm cell walls, phenolic constituents and polysaccharides could be analysed at the same

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time with Raman spectroscopy, whereas the low infrared absorbance of phenolic moieties does not lead to any analysable infrared signal. Raman spectroscopy was shown to be a powerful technique to analyse primary or secondary plant cell wall composition and organisation[8–12] and their main polysaccharides.[6,13] However, few analyses have been carried out on AX and their structural heterogeneity. Even though the presence of esterified ferulic acid or glucuronic acid residues was identified by specific Raman diffusion bands,[6,14] little is known about the effect of the substitution ratio (A/X) on Raman spectra.

The present paper is aimed at identifying Raman signatures of the arabinose AX substitution in using a set of isolated polysaccharides, characterized for their degree of arabinose substitution.[15] The suitability of Raman spectroscopy to quantify AX substitution was also investigated.

Raman spectra of reference arabinoxylans

Raman spectra of non-feruloylated (nF series) and feruloylated (F series) AX are shown in Figure 1a and b, respectively, and their composition in Table 1. Specific diffusion bands were observed at 896, 985, 1278–1462 and at 1091–1123 cm^{-1}. They were typically assigned to AX.[6,9,14] While the 1400–1300 cm^{-1} spectral region could be assigned to CH and OH bending, COC, CO and CC stretching were observed in the 1000–1200 cm^{-1} range, that is, the polysaccharide fingerprint region. The presence of β-(1→4) glycosidic linkages of the xylan backbone was identified by the Raman band at 896 cm^{-1}. Below 700 cm^{-1}, specific bands were observed at 570, 532 and 494 cm^{-1} as already shown with AX[14] and hemicellulosic material.[10] They were usually assigned to vibrations arising from coupled modes of heavy atoms, C–C and C–O stretching.[10,13] Beside the polysaccharidic bands, specific Raman diffusion bands were observed at 1598 and 1628 cm^{-1} when examining feruloylated AX. This doublet was related to the stretching vibration of the phenolic ring and could be linked to the presence of ferulic acid esters.[9] The diffusion band at 1664 cm^{-1} and the sharp one at 1003 cm^{-1} were observed for the F70 fraction. They raised from amide I and ring modes of aromatic amino acids,[6] respectively, revealing a higher amount of proteins due to the presence of arabinogalactan-proteins in the F70 fraction (Table 1).

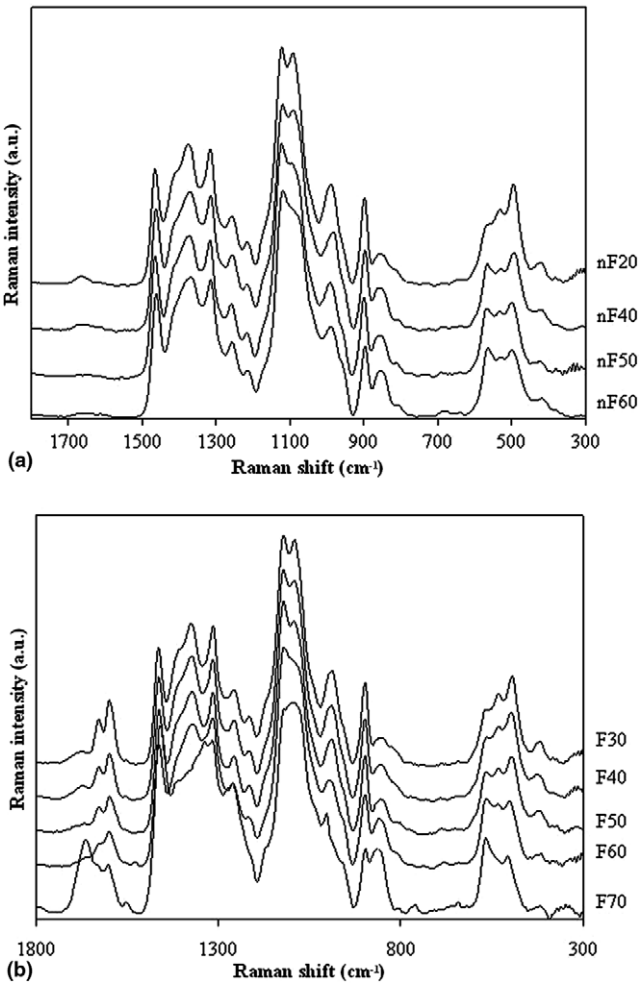

Figure 1. Raman spectra of AX with various arabinose to xylose ratios. (All the spectra were baseline corrected and normalised on the intensity measured between 1024 and 1151 cm^{-1}, and offset for clarity.) (a) Non-feruloylated AX. (b) Feruloylated AX.

Table 1. Biochemical characterisation of wheat AX fractions

Fraction name	A/X (molar ratio)	Ara (% w/w)	Xyl (% w/w)	Gal (% w/w)	Protein (% w/w)	FA ($mg \cdot g^{-1}$ AX)
nF20	0.40	23.5	58.7	0	nd	0
nF40	0.61	30.8	50.7	0	nd	0
nF50	0.67	31.8	46.9	0.5	nd	0
nF60	0.79	38.7	47.3	0.6	nd	0
F30	0.37	22.3	60.2	0.2	2.7	2
F40	0.46	24.6	53.6	0.2	3.5	1.9
F50	0.57	31.8	56.6	0.2	3.1	1
F60	0.75	37.7	50.0	0.6	2.7	0.8
F70	0.85	28.6	21.9	14.1	8.8	0.7

Ara: arabinose, Xyl: xylose, Gal: galactose, FA: ferulic acid, nd: non determined.

In order to investigate the main changes in spectra related to arabinose substitution, 2D-correlation analysis was carried out on spectral ranges specific of polysaccharides. The main differences according to the substitution ratio (A/X) were observed between (i) 400–600 cm^{-1}, (ii) 800–950 cm^{-1}, and, with less intensity, (iii) 1030–1100 cm^{-1}. Specific 2D-correlation analyses were then applied to the 400–600 and 800–1000 cm^{-1} regions (Fig. 2). In the synchronous map, two auto-peaks were observed along the diagonal at 494 and 855–865 cm^{-1}, confirming that the major changes according to the increase of substitution ratio occurred at these wavenumbers. When the A/X ratio increased, the intensity at 494 cm^{-1} decreased while that at 855–865 cm^{-1} increased. Slight variations occurred also at 532 cm^{-1} and around 980 cm^{-1}. Positive synchronous correlation was observed between 532 and 494 cm^{-1}, 855 and 820 cm^{-1}, 855 and 898 cm^{-1} (Fig. 2a). The asynchronous map revealed intensity changes at 570, 440–460, 890, 910 and 982 cm^{-1}, which were not often easily straight was detected by the spectra in relation with A/X ratio. The Raman diffusion intensity observed at 570 cm^{-1} increased with A/X ratio. The diffusion band at 982 cm^{-1} decreased when A/X ratio increased. Infrared absorption at 984 cm^{-1} was also shown to decrease when the A/X ratio increased.[7] A common infrared and Raman band, sensitive to the arabinose substitution, was then observed at about 982–984 cm^{-1}.

Raman spectra of xylo-oligosaccharides

Well-characterized xylo-oligosaccharides mono- and di-substituted by arabinose were analysed by Raman spectroscopy (Fig. 3). The Raman diffusion spectra of xylo-oligosaccharides showed similar diffusion bands as the polymers, implying that only four xyloses as a backbone give rise to diffusion bands comparable to polymers. The diffusion band at 855 cm^{-1} was absent for xylotetraose samples whereas its intensity increased in the arabino-xylo-oligosaccharides A1X4, A2X4, A3X5, in ascending order. This diffusion band could therefore be specific of arabinose. Strong differences were depicted in the 400–600 cm^{-1} region where the main diffusion bands were observed at about 490, 536 and 573 cm^{-1}. While the A1X4 oligosaccharide exhibited a strong diffusion band at 490 cm^{-1}, the A2X4 had a maximum at 573 cm^{-1}. Both 490 and 573 cm^{-1} peaks were observed in the A3X5 spectrum where mono- and disubstituted xylose residues were encountered. Therefore, the Raman diffusion band at 573 cm^{-1} could be a signature of dXyl. This finding was corroborated in the AX sample series where the increase of the 570 cm^{-1} band with A/X ratio could be correlated to the increase of dXyl measured by NMR spectroscopy.[4] However, an accurate assignment of the uXyl or mXyl could not be performed using the observed decrease of intensity at 494 cm^{-1} because

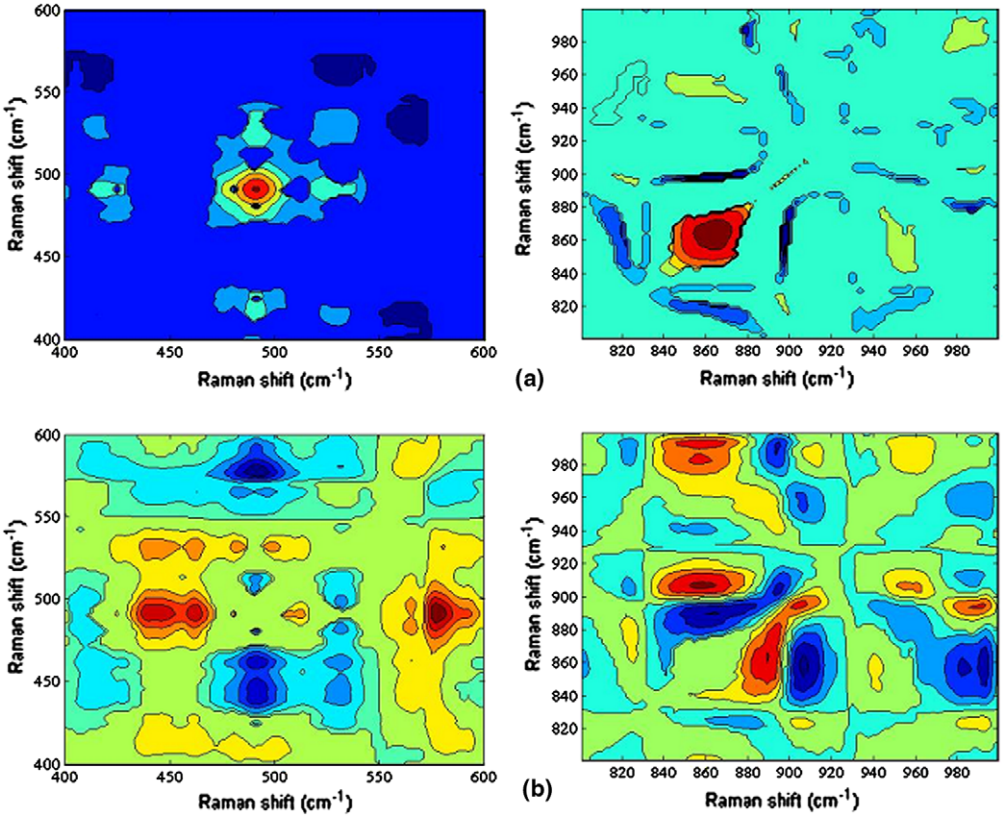

Figure 2. 2D correlation analysis of Raman spectra according to the A/X ratio. (a) Synchronous maps. (b) Asynchronous maps.

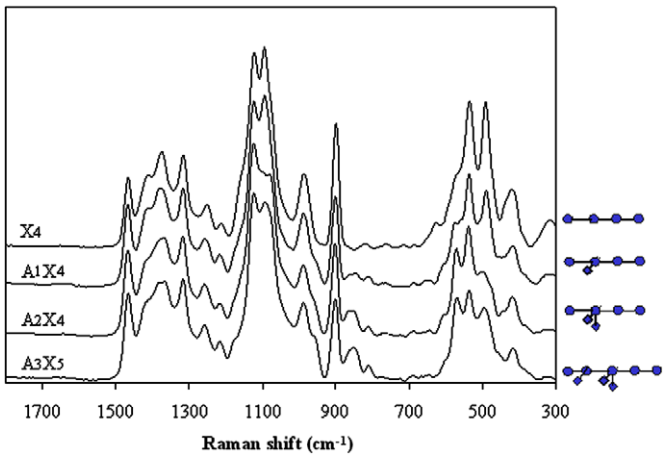

Figure 3. Raman spectra of xylo-oligosaccharides.

the relative proportion of mXyl was stable when the A/X ratio increased.[4]

Quantification of the substitution ratio

Based on the strong correlations observed between the arabinose substitution and the intensities of bands at 855 and 494–570 cm^{-1}, a quantitative analysis of A/X ratio by Raman spectroscopy was carried out. In both cases, better quantitative results were obtained by using the second derivative spectra, which showed well-resolved peaks.

The intensity ratio 855/898 cm^{-1} was assessed on the second derivative spectra of all the AX samples. The 898 cm^{-1} peak, assigned to the β-(1→4) glycosidic linkage, was used as a specific band for the xylan backbone. A linear relationship ($R^2 = 0.97$) was obtained between Raman intensity ratio and A/X ratio measured by biochemical analysis (Fig. 4a). The standard error of prediction of the A/X ratio was equal to 0.03. Similar data treatment applied to xylo-oligosaccharides spectra revealed that the relationship obtained for polymers was still valid (Fig. 4a). The 855 cm^{-1} Raman diffusion band seemed to be a signature of the total amount of arabinose in AX.

On the other hand, the ratio between peak intensities at 570 and 494 cm^{-1} was assessed on the second derivative spectra of all the AX samples. A linear relationship ($R^2 = 0.97$) was obtained between the 570/494 cm^{-1} ratio and the degree of substitution of AX measured by biochemical analysis (Fig. 4b). The standard error of prediction of the A/X ratio was equal to 0.03. In this case, the ratio assessed for the xylo-oligosaccharides did not fit with the linear relationship. The modification of the intensity of these diffusion bands was not directly linked to the amount of arabinose in AX. These changes could therefore be related to an indirect effect of the arabinose substitution on the coupled vibration modes of the xylan backbone.

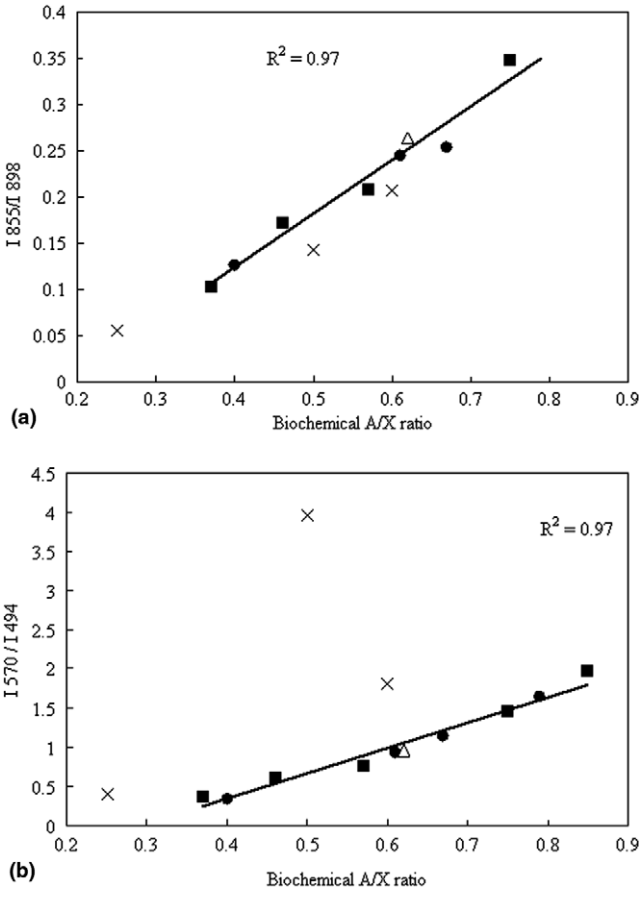

Figure 4. Relationships between Raman spectral features of AX and their substitution ratio (biochemical data). (A) Ratio of the intensities at 855/898 cm^{-1} measured on second derivative spectra multiplied by −1. (B) Ratio of the intensities at 570/494 measured on second derivative spectra multiplied by −1. ● Non-feruloylated AX, ■ feruloylated AX, × arabino-xylo-oligosaccharides, △ initial non-feruloylated AX before any ethanolic fractionation.

Regardless of the spectral region analysed, no interference with the feruloylation degree ([FA] < 2 μg mg^{-1} AX) was noticed on these linear relationships. Although ferulic acid revealed low diffusion bands at 812, 571 and 535 cm^{-1}, the low concentrations of ferulic acid in the AX reference samples precluded any disturbance.

Similar data treatments were applied to the starting purified AX, before any ethanolic fractionation. This sample exhibited an average A/X ratio of 0.62 but was in fact composed of polydisperse AX molecules with varying individual A/X ratio. Regardless of the calculated ratio, this sample fitted well with the determined linear relationships (Fig. 4a and b). The Raman signal was then sensitive to the average A/X value of a population of molecules.

In conclusion, analysis of well-characterized AX samples allowed to detect specific Raman spectral features relating to the arabinose substitution. Several spectral regions could be used to study the structural heterogeneity of AX. While the 855 cm^{-1} Raman band seemed to be characteristic of arabinose, the modification of the Raman diffusion intensities in the 400–600 cm^{-1} region was assigned to modifications of the coupled vibration modes in the xylan backbone with arabinose substitution. Using the 855/898 and 570/494 cm^{-1} ratios, the quantitative determination of A/X ratios was effective for AX sample with A/X values ranging from 0.3 to 0.9. These promising results must be validated with more complex mixtures of cell wall polysaccharides (including e.g., β-glucans or arabinogalactans) so that the method could be further applied to the in situ analysis of wheat plant cell walls.

1. Experimental

1.1. Polysaccharides and oligosaccharides

Water-extractable AX samples with different arabinose-to-xylose ratios were further fractionated by size exclusion chromatography from graded ethanol fractionation (saturation level of ethanol: 20%, 30%, 40%, 50%, 60% and 70%) of feruloylated or de-esterified WE-AX.[4,15] The substitution ratio (A/X) was measured by sugar analysis and varied between 0.37 and 0.85 as indicated in Table 1.

Xylotetraose (X4) was purchased from Megazyme (Bray, Ireland). Arabinose-substituted xylo-oligosaccharides were prepared from WE-AX by using an endoxylanase from *Trichoderma viride* (M1—Megazyme—Bray, Ireland) as previously described.[16] Three arabino-xylo-oligosaccharides were used: (i) a xylotetraose mono-substituted by arabinose at O-3 on the third xylose residue (A1X4), (ii) a xylotetraose di-substituted by arabinose at O-2 and O-3 on the third xylose residue (A2X4), (iii) a xylopentaose mono-substituted by arabinose at O-3 on the second xylose residue and di-substituted by arabinose at O-2 and O-3 on the third xylose residue (A3X5).

1.2. Raman spectroscopy

Raman spectra were recorded between 95 and 3500 cm^{-1} Raman shift using a confocal Raman microspectrometer Almega (Thermo-Electron) with the following configuration: excitation laser He–Ne $\lambda_0 = 633$ nm, grating 500 grooves/mm, pinhole 25 μm, objective ×50. The collection time was about 10 min. At least five spectra were recorded for each sample. The spectral resolution varied between 5 and 9 cm^{-1} according to the spectral region.

All spectra pre-treatments were performed with Omnic v6.2 and TQ Analyst v6.2 softwares (Thermo-Electron). Processing included: (i) a multipoint linear baseline correction, (ii) a normalisation according to the area of the AX doublet (1024–1151 cm^{-1}), (iii) the calculation of mean spectra for each sample. In the case of the quantification study, second derivative spectra were calculated from the raw data with the Norris algorithm (segment length: 5, gap between segments: 5). Before peak height analysis, the spectra were multiplied by −1.

1.3. 2D-correlation analysis

Spectra ranked with an increasing substitution ratio were analysed by 2D-correlation spectroscopy, by analogy with time-dependant experiments.[17] Dynamic spectra were obtained by subtracting the spectrum from the sample with the lowest A/X ratio and synchronous correlations were then calculated. Asynchronous correlations were computed by taking advantage of the discrete Hilbert–Noda transformation.[18]

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