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A copolymer analysis approach to estimate the neutral sugar distribution of sugar beet pectin using size exclusion chromatography

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ABSTRACT

Partially degraded sugar beet (*Beta vulgaris*) pectins were characterised in terms of galacturonic acid, neutral sugar and ferulic acids contents. It was shown that the total neutral sugar content is correlated with the ferulic acid content. One pectin (C) was further characterised by size exclusion chromatography coupled to refractive index and UV detectors (SEC-RI-UV). This gave the opportunity to estimate how the ferulic acid and neutral sugar contents changed with hydrodynamic radius. Pectin C was found to be heterogeneous in composition with neutral sugar-rich fractions of both high and low hydrodynamic radii. A neutral sugar-poor fraction was found at intermediate hydrodynamic radii.

1. Introduction

Pectins are a complex family of heteropolysaccharides that constitute a large proportion of the primary cell walls of dicotyledons and play important roles in growth, development and senescence (Ridley, O'Neil, & Mohnen, 2001; Willats, McCartney, Mackie, & Knox, 2001). Pectic polysaccharides are copolymers of the two anionic polysaccharides homogalacturonan (HG) and type I rhamnogalacturonan (RG-I) often described in simplified terms as the "smooth" and "hairy" regions respectively (Williams, Cucheval, Ström, & Ralet, 2009). The HG region is composed of (1 → 4) linked α-D-Gal*p*A residues that can be partially methylated at *C*-6 (Pilnik & Voragen, 1970) and possibly partially acetyl-esterified at O-2 and/or *O*-3 (Rombouts & Thibault, 1986). The degree of methylation (DM) and the degree of acetylation (DAc) are defined as the number of moles of methanol or acetic acid per 100 moles of GalA. The degree of methylation in native pectins is generally in the order of DM ≈ 70–80; whereas degree of acetylation is generally much lower, *e.g.* DAc ≈ 35 for sugar beet pectins (Rombouts & Thibault, 1986). It has been reported that GalA residues in the RG-I region are partially acetylated (Ishii, 1997; Perrone et al., 2002) but not methylated (Komalavilas & Mort, 1989; Perrone et al., 2002).

In the case of the *Amaranthaceae* family which includes sugar beet (*Beta vulgaris*) and spinach (*Spinacia oleracea*) pectins the neutral side chain sugars (arabinose and galactose) are substituted with ferulic acid (Fry, 1982; Ishii & Tobita, 1993; Levigne, Ralet, Quéméner, Pollet, et al., 2004; Ralet, Thibault, Faulds, & Williamson, 1994; Rombouts & Thibault, 1986). Arabinose and galactose are substituted with ferulic acid at ratios of 1 in 56 and 1 in 16, respectively (Ralet et al., 1994). Ferulic acid groups are predominantly ester-linked to O-2 of arabinose residues of α-(1 → 5)-linked arabinan side chains and to O-6 of galactose residues of the β-(1 → 4)-linked type I galactan side chains (Colquhoun, Ralet, Thibault, Faulds, & Williamson, 1994; Ishii & Tobita, 1993). Minor amounts of ferulic acid have also been shown to be linked to O-5 of Ara residues of the arabinan side chains, and therefore have a peripheral location on the "hairy" region (Levigne, Ralet, Quéméner, Pollet, et al., 2004). Further evidence indicates that pectin chains can be dimerised via diferulic bridges (Levigne, Ralet, Quéméner, Pollet, et al., 2004; Levigne, Ralet, Quéméner, & Thibault, 2004; Ralet, André-Leroux, Quéméner, & Thibault, 2005). There are a number of different ways in which ferulic acid can dimerise the most common being: 5–5′; 8–O–4′; 8–5′ cyclic and 8–5′ non-cyclic dimers (Micard, Grabber, Ralph, Renard, & Thibault, 1997). Ferulic acid trimers and tetramers may also play an important role in the cross-linking of plat cell wall polysaccharides (Fry, Willis, & Paterson, 2000).

In this paper we will demonstrate the use of size exclusion chromatography coupled to refractive index (RI) and UV detectors (SEC-RI-UV) to determine the neutral sugar content of pectin fractions.

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2. Experimental

2.1. Materials and methods

2.1.1. Acid extraction of sugar beet pulp (pectin C)

The alcohol insoluble residue (AIR) (5 g) from sugar beet pulp was obtained as described in Levigne, Ralet, and Thibault (2002). In brief sugar beet roots were immersed in 3 l of boiling ethanol (96%) and the slurry was filtered through G3 sintered glass and the insoluble material was repeatedly suspended in 70% ethanol until the filtrate gave a negative reaction to the phenol–sulphuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The resultant AIR was then dispersed in 150 ml HCl at pH 1.0 and heated for 30 min at 95 °C with agitation. Under these conditions a proportion of the arabinofuranose residues will have been hydrolysed from the main pectin chain and consequently pectin C is a partially degraded sugar beet pectin (Morris, Ralet, Bonnin, Thibault, & Harding, 2010). The residue was separated through G3 sintered glass. The supernatant was adjusted to pH 4.5 with 2 M NaOH, concentrated under vacuum at 40 °C, extensively dialysed against distilled water and freeze–dried at condenser temperature of −55 °C and a pressure of 4 mbar (400 Pa) for 48 h. The resultant pectin (yield 350 mg/g) will be referred to as pectin C.

2.1.2. Chemical characterisation of pectin C

Galacturonic acid and neutral sugar (expressed arbitrarily as arabinose) contents were determined in triplicate by the automated m-hydroxbiphenyl (Thibault, 1979) and orcinol methods (Tollier & Robin, 1979), respectively, the latter being corrected for interfering galacturonic acid.

Individual neutral sugars were obtained by hydrolysis with 2 M trifluoroacetic acid at 121 °C for 2 h and converted to their corresponding alditol acetates (Blakeney, Harris, Henry, & Stone, 1983). These alditol acetates were subsequently analysed by gas chromatography using myo-inositol (0.5 mg) as an internal standard on a DB-225 fused-silica capillary (30 m × 0.32 mm i.d.) column (J&W Scientific, Courtaboeuf, France) mounted in a DI 200 chromatograph (Delsi Nermag Instruments, Argenteuil, France) with hydrogen as the carrier gas at a constant temperature of 220 °C.

Phenolic acids were determined by HPLC after saponification and extraction. Pectin C (10.4 mg) was dissolved in 1 ml of 2 M NaOH and saponified under argon for 30 min at 35 °C in the dark. The internal standard (*o*-coumaric acid) was added, prior to neutralisation with 2 M HCl and the extraction of the phenolic compounds into ether. The ether phase was evaporated and the residue dissolved in 50/50 water/methanol. 20 μl of this material was injected onto a Purospher C18 column (Merck, Darmstadt, Germany) and a gradient elution was performed using acetonitrile (*A*) and pH 4.6 sodium acetate buffer (B) at 60 ml/h and 30 °C: (0 min, *A* = 15%; 6 min, *A* = 15%; 26 min, *A* = 35%; 26.5 min, *A* = 60%; 30.5 min, *A* = 60%; 31 min, *A* = 15%; 35 min, *A* = 15%). The eluent was detected at 320 nm. Response factors were determined relative to *o*-coumaric acid (ferulic acid = 0.57 and for the diferulic acids 5–5′ = 0.50; 8–O–4′ = 1.04; 8–5′ cyclic dimer = 1.09 and 8–5′ non-cyclic dimer = 0.90).

2.1.3. Physical characterisation of pectin C

High performance size exclusion chromatography (HPSEC) was performed at room temperature on a system consisting of a Shodex OH SB-G guard column (Showa Denko, Tokyo, Japan) followed by in series (Shodex OH-Pak SB-805 HQ and Shodex OH-Pak SB-804 HQ) eluted with 50 mM sodium nitrate buffer containing 0.02% sodium azide as an antibacterial agent at a flow rate of 42 ml/h. The eluent was detected on-line by:

1. SpectroMonitor 3000 variable wavelength UV detector at 325 nm (LDC/Milton Roy, Paris, France)
2. MiniDawn light scattering (LS) detector (Wyatt, Santa Barbara, U.S.A.)
3. T-50A differential pressure viscometer (DPV) (Viscotek, Huston, U.S.A.)
4. ERC 7515A differential refractometer (RI) (Sopares, Gentilly, France)

The refractive index increment, dn/dc was taken to be 0.146 ml/g (Chapman, Morris, Selvendran, & O'Neill, 1987; Levigne et al., 2002; Morris, Foster, & Harding, 2000, 2000, 2008, 2010). The injected mass of ferulic acid (g) and % of ferulic acid were calculated using the following equations:

$$FA\ (\mathrm{g}) = \frac{Absorbance_{325\,\mathrm{nm}}}{3950} \quad (1)$$

$$FA\,(\%) = 100 \times \frac{FA\,(\mathrm{g})}{Total\,(\mathrm{g})} \quad (2)$$

3. Results and discussion

3.1. Chemical characterisation of pectin C

The composition of sugar beet pectin C shown in Table 1 is consistent with previous findings for sugar beet pectins (Levigne et al., 2002; Morris et al., 2010). It has been suggested previously that there is a correlation between total neutral sugar content (or arabinose + galactose content) and ferulic acid content (Levigne et al., 2002). In order to further explore this hypothesis seven other sugar beet pectins (A, E, G, CP1, SP1, SP4 and SP6) previously described in Levigne et al. (2002) were also characterised in terms of sugar composition and ferulic acid content. The resultant plot (includes the mean value from Levigne et al. (2002) and this study). Additionally values for samples containing 0% or 100% neutral sugars, HG and arabinan fractions respectively (Morris et al., 2010) are shown in Fig. 1A and B from which we can therefore estimate the total neutral sugar (%) and arabinose + galactose contents (%) from the ferulic acid content (%) using the following equations:

$$\text{Total neutral sugar}\,(\text{mol}\%) = 50.2\,FA(\%) + 5.4 \quad (3)$$

$$\text{Ara} + \text{Gal}\ (\text{mol}\%) = 53.8\,FA\,(\%) - 2.7 \quad (4)$$

The rhamnose and galacturonic acid contents are then calculated by difference.

N.B. In this case total neutral sugars do not include minor sugars such as xylose and glucose as they are present in very small amounts in this case.

3.2. Physical characterisation of pectin C

Acid extracted pectin is heterogeneous with respect to molar mass, intrinsic viscosity and composition (Morris et al., 2010; Oosterveld, Beldman, Schols, & Voragen, 1996) (Fig. 2). The use of UV absorbance (325 nm) allows the visualisation of those populations of pectic molecules, which are substituted with ferulic acid, *i.e.* the arabinan, galactan or arabinogalactan side chains present on the RG-I region of sugar beet pectin (Morris et al., 2010; Oosterveld et al., 1996).

UV detection also allows a global estimate of the total % of ferulic acid in pectin C, which was found to 0.60%, and is in excellent agreement with the value of 0.59% (Table 1) found from C18 HPLC after saponification and organic extraction and with the value found previously of 0.60% (Levigne et al., 2002). However we can also use the response from the UV and RI detectors to estimate the ferulic acid content (Fig. 3A) and hence the neutral sugar content (Fig. 3B)

Table 1
"Average" chemical composition of sugar beet pectin C.

	Sugar composition, mol%					Ferulic acid, %	Ferulic acid dimers, %
	GalA	Rha	Ara	Gal	Total neutral sugars		
Analytical measurement	70 ± 1	9 ± 1	6 ± 1	15 ± 1	30 ± 3	0.59 ± 0.03	0.06 ± 0.01
SEC-RI-UV	64 ± 6	7 ± 2	–	–	36 ± 6	0.60 ± 0.03	–

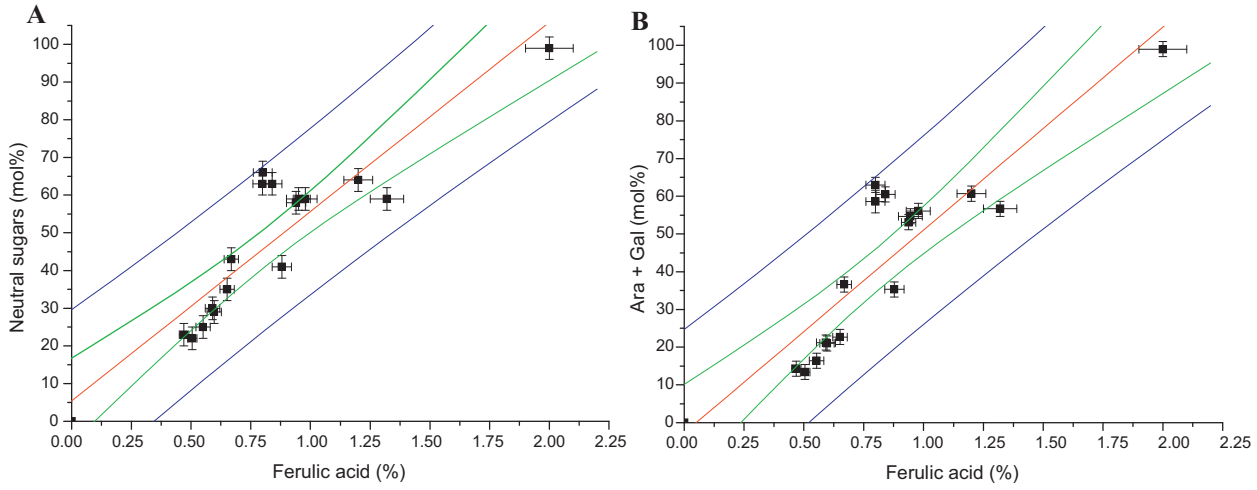

Fig. 1. Relationship between total ferulic acid content and total neutral sugar (A) and arabinose and galactose (B) contents. Best fit to the data ( ); 95% confidence limits ( ) and 95% prediction limits ( ). Values shown are the mean ± 1 standard deviation from this study, Levigne et al. (2002) and Morris et al. (2010). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(from Eq. (3)) at each individual slice (1348 data points) across the elution profile as has been reported for glycoconjugate and copolymer analysis (Chen, 2011; Kendrick, Kerwin, Chang, & Philo, 2001; Trathnigg, 2000). Similarly we can use Eq. (4) to calculate the arabinose + galactose content.

As we can see from Fig. 3A and B there are distinct populations of pectic molecules; the components eluting at the lowest and highest elution volumes, 11–13 and 16–19 ml, respectively are rich in both ferulic acid and neutral sugars. We believe that the higher molar mass (or more precisely higher hydrodynamic radius) neutral sugar/ferulic acid rich component may be the result of dimerisation of pectin "monomers" via diferulic bridges (Ralet, André-Leroux, Quéméner, & Thibault, 2005). Diferulic acids are present in this pectin sample (Table 1) and have been shown to bridge arabinose residues of Driselase® degraded sugar beet pectin (Levigne, Ralet, Quéméner, & Thibault, 2004) and to have peripheral locations on pectin hairy regions (Levigne, Ralet, Quéméner, Pollet, et al., 2004).

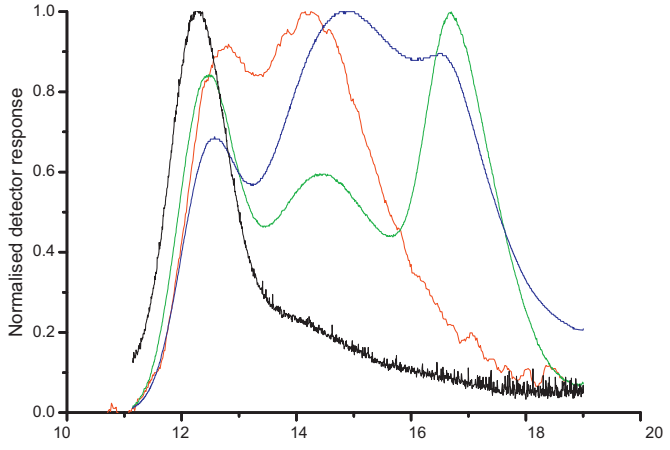

Fig. 2. Normalised multi-detector HPSEC chromatogram for pectin C: LS 90° ( ), RI ( ), UV 325 nm ( ) and DPV ( ). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.3. Conclusions

Acid extracted sugar beet pectin was shown to be heterogeneous in terms of composition. In this case there are 3 distinct populations:

(i) Rich in neutral sugar side chains and large hydrodynamic radius
(ii) Rich in galacturonic acid and intermediate hydrodynamic radius
(iii) Rich in neutral side chains and low hydrodynamic radius

It is proposed that the fraction of side chain rich pectins at high molar mass (or more precisely hydrodynamic radii) may be the result of diferulic acid bridging between pectin molecules and more specifically between the RG-I regions of pectin molecules. It has been shown previously (Levigne, Ralet, Quéméner, & Thibault, 2004; Ralet, André-Leroux, Quéméner, & Thibault, 2005) that ferulic acid residues do form links between arabinan and galactan side chains of the pectin RG-I regions. The presence of diferulic acid dimers may help to explain the observation (Oosterveld, Beldman, & Voragen, 2002) that the weight average molar mass of sugar beet pectin hydrolysed by arabinan degrading enzymes decreases more than would be expected. In this case populations (ii) and (iii) may be the result of the enzymatic or chemical breakdown of population (i). The population with the lowest molar mass (iii) is also likely to contain "free" neutral sugar side chains which are most likely due to the hydrolysis of arabinofuranose residues during acid extraction (Morris et al., 2010). However, it is also probable that pectins occur naturally with different degrees of neutral sugar substitution

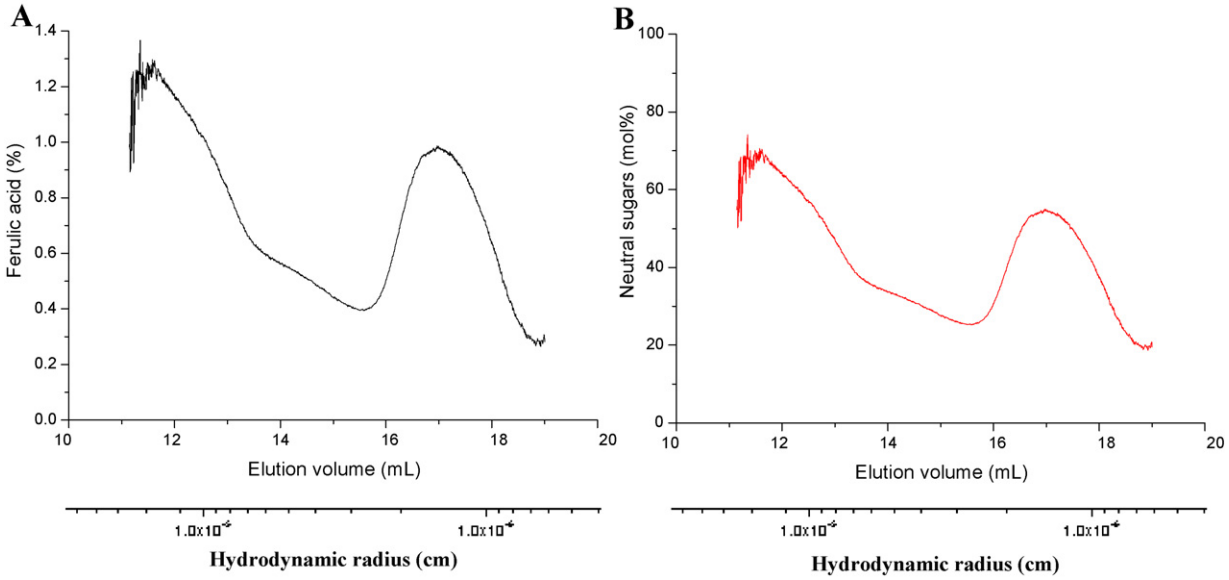

Fig. 3. The change in ferulic acid (A) and neutral sugar (B) contents with elution volume (or hydrodynamic radius) for sugar beet pectin C.

in vivo which enables them to perform different functions within the cell wall.

We have for the first time to our knowledge demonstrated using on-line RI and UV detection how the neutral sugar composition of a pectin molecule varies during elution. It is clear from Fig. 3A and B that even without taking into consideration the question of dimerisation via ferulic acid bridges there are clearly (at least 3) populations of pectins with very different proportions of ferulic acid and hence therefore GalA:neutral sugar (or HG:RG-I) ratios. Therefore as a consequence we should be aware that any measurements on a non-fractionated material will only give the "number-average" content of ferulic acid (or neutral sugars) and may not necessarily be representative of the distribution of ferulic acid present in the pectin molecules. This has repercussions for the molar masses, intrinsic viscosities and conformations of pectins (which we will discuss in a further paper) and more importantly their functional properties in commercial applications and *in vivo*.

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