Carbohydrate Polymers 88 (2012) 882–890

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Structural characterization of cell wall pectin fractions in ripe strawberry fruits using AFM

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ARTICLE INFO

Article history:
Received 14 November 2011
Received in revised form 8 January 2012
Accepted 9 January 2012
Available online 24 January 2012

Keywords:
Fragaria × *ananassa*
Fruit ripening
Fruit softening
Pectins
Primary cell wall
Atomic force microscopy

ABSTRACT

Cell wall disassembly during fruit ripening is the main process leading to fruit softening. In strawberry fruit (*Fragaria* × *ananassa*, Duch.), functional analysis with transgenic plants have related the loss of firm texture to the metabolism of pectins. To gain insights into the role of pectins in strawberry softening we have analyzed physicochemical features of ionic and covalently bound pectins, isolated from ripe fruits, by Fourier transform infrared spectroscopy (FTIR), size exclusion chromatography (SEC) and atomic force microscopy (AFM). Cell wall material was isolated and chemically fractionated by sequential extraction with cyclohexane-trans-l,2-diamine tetraacetate (CDTA) and sodium carbonate (Na_2CO_3) in order to extract ionic and covalently bound pectins, respectively. Uronic acids (UA) were detected as the main component in CDTA samples, whilst a significantly higher content of neutral sugar was observed in the Na_2CO_3 samples, representing 33% of total sugars vs. 12% in the CDTA fraction. The spectral profile in the FTIR region 1200–900 cm^{-1} was similar in the case of both pectin fractions, although some minor changes in the band intensities suggest an enrichment of the carbonate fraction in rhamnogalacturonan-I pectin domains. SEC analysis showed that the average molecular weight of the CDTA pectin was higher than that of the carbonate extract. AFM histograms of polymer length were well approximated by a Log normal distribution function for both pectin fractions. The CDTA and Na_2CO_3 polymer length distributions were statistically different with mean values of ~87 and 65 nm, respectively. AFM studies revealed the presence of sidechains and multiple branching, previously attributed to branching of the galacturonic acid backbone. Both fractions were about 9% branched, but the Na_2CO_3 samples showed a higher percentage of multi-branched polymers. These results demonstrate that AFM is an excellent tool revealing new information on pectin structure which, when combined with classical techniques such as FTIR or SEC, provides a deeper characterization of fruit pectins.

1. Introduction

Pectin is considered as one of the most complex natural plant biopolymers (Vincken et al., 2003; Voragen, Coenen, Verhoef, & Schols, 2009) although individual components are well characterized, the way they are interconnected structurally within the cell wall and in extracts is still not well understood. These polysaccharides are a major component of the primary cell wall of non-graminaceous plants, accounting for up to 60% of the cell wall mass in many fruits (Redgwell et al., 1997a). Pectin metabolism during fruit ripening is one of the key processes leading to a reduction in fruit firmness. Extracted pectins are also widely used as gelling agents and stabilisers in processed food products (Willats et al., 2006). Thus, a comprehension of the relationship between pectin structure and texture is essential for both plant and food scientists. Previous work by the authors has been focused on the role of cell wall disassembly in strawberry fruit softening. By means of a functional approach, we have shown how the changes in different pectin fractions are related to softening during strawberry ripening (Quesada et al., 2009; Santiago-Doménech et al., 2008).

The main component of pectin is D-galacturonic acid (GalA) and one of the major pectic component polysaccharides is homogalacturonan (HG), an essentially linear polymer consisting of (1→4) linked α-D GalA units with some of the carboxyl groups methyl-esterified or acetylated. The polygalacturonic (PGA) chain is interrupted by a pectic domain named Rhamnogalacturonan I (RGI) which is enriched in rhamnose (Rha) residues. RGI consists of

Abbreviations: AFM, atomic force microscopy; CDTA, cyclohexane-trans-l,2-diamine tetraacetate; FTIR, Fourier transform infrared spectroscopy; GalA, galacturonic acid; HG, homogalacturonan; L_N, number-average contour length; L_W, weight-average contour length; PDI, polydispersity index; PGA, polygalacturonic acid; RGI, rhamnogalacturonan I; RGII, rhamnogalacturonan II; SEC, size exclusion chromatography; UA, uronic acids.

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doi:10.1016/j.carbpol.2012.01.029

the repeating disaccharide [→4)-α-D-GalA-(1→2)-α-L-Rha-(1→] backbone decorated with neutral sugar sidechains. The composition of the RGI regions depends on plant source and extraction but may contain galactan, arabinan and/or arabinogalactan sidechains attached to the RGI backbone via Rha residues, forming highly branched (hairy) regions (Mohnen et al., 1996; Willats, McCartney, Mackie, & Knox, 2001). However, Vincken et al. (2003) have recently proposed an alternative structure to the above contigous structure in which the RGI form the backbone with HG regions attached as long side chains. The third type of pectic domain is Rhamnogalacturonan II (RG II), which is less abundant but very highly conserved in the plant kingdom, with a HG backbone and very complex side chains attached to the GalA residues (Willats et al., 2006).

As stated previously, pectins are extremely complex and their composition and structure vary with source species, tissues, environmental conditions and developmental stages. Cell wall disassembly during fruit ripening is a paradigmatic example of fast changes in different structural components, including pectic polymers, which result in a different fruit texture. In the case of strawberry fruits, this process is characterized by a high solubilisation of pectins (Figueroa et al., 2010; Redgwell et al., 1997a; Santiago-Doménech et al., 2008), a slight depolymerization of covalently bound pectins (Redgwell et al., 1997a; Rose, Hadfield, Labavitch, & Bennett, 1998; Rosli, Civello, & Martinez, 2004; Santiago-Doménech et al., 2008), a moderate loss of galactose and arabinose (Redgwell, Fischer, Kendal, & MacRae, 1997b), as well as a reduction in the hemicellulosic content (Huber, 1984; Lee & Kim, 2011; Rosli et al., 2004). In general, ionic and covalently bound pectins, those extracted with chelating or alkaline reagents, respectively, are the cell wall pectin fractions that suffer the most dramatic changes during fruit softening (Brummell, 2006; Mercado, Pliego-Alfaro, & Quesada, 2011). In strawberry, these pectins fractions have been identified as a key component of fruit texture, since the down-regulation of a pectate lyase (Jiménez-Bermúdez et al., 2002; Santiago-Doménech et al., 2008) or a polygalacturonase gene (Quesada et al., 2009) reduced both fruit softening and the above mentioned ripening-related changes in cell wall pectins. However, the quantitative and qualitative biochemical changes observed were not easily related to the change in texture which is physical in nature. A more detailed analysis of the pectic polysaccharides molecules present in these fractions is required to achieve a better understanding of the inter-relationship between pectin structure and fruit texture during strawberry softening. In this context, the atomic force microscope (AFM), first described by Binnig, Gerber, Stoll, Albrecht, and Quate (1987), is a powerful technique that allows characterization of complex and heterogeneous samples at the molecular level. AFM has successfully provided three-dimensional images of the surface topography of biological molecules such as DNA (Strick, Allemand, Bensimon, Bensimon, & Croquette, 1996), proteins (Gunning et al., 1996), and polysaccharides (Kirby, Gunning, Waldron, Morris, & Ng, 1996b; Morris, Gromer, Kirby, Bongaerts, & Gunning, 2011; Morris et al., 1997; Ovodov, 2009; Round, Rigby, MacDougall, & Morris, 2010), including pectins (Kirby, Gunning, Morris, & Ridout, 1995). AFM can be used to study materials with just minor sample preparation and provides a valuable tool for studying polysaccharide conformation in conditions that more closely mimic their natural environment. AFM imaging allows information to be obtained on the contour length distribution and the branching of polysaccharides complementing values obtained by size exclusion chromatography (SEC). SEC provides physical characterization of molecules based on the average properties of a whole group of pectin molecules with similar hydrodynamic properties, whereas the AFM imaging gives information on individual isolated pectin molecules. Thus at present it is useful to supplement conventional SEC and FTIR analysis with new molecular values from AFM to elucidate a clearer picture of pectin structure.

The main goal of the present study was to use AFM conditions to visualize and characterize different ionic and covalently bound pectic fractions from strawberry cell wall samples. This has shed new light on the molecular heterogeneity and branching of strawberry pectin polymer extracts. The AFM results were complemented with measurements of sugar composition, ATR–FTIR and SEC analysis.

2. Materials and methods

2.1. Polysaccharide extraction

The method used for the isolation of the cell wall material (CWM) from ripe strawberry fruits (*Fragaria* × *ananassa*, Duch., cv. Chandler) was based on the protocol of Redgwell, Melton, and Brasch (1992) with modifications, as described previously by Santiago-Doménech et al. (2008). The cell wall preparations were sequentially extracted as described previously (Santiago-Doménech et al., 2008) to obtain CDTA (ionically bound) and Na_2CO_3 (covalently bound) soluble fractions for further analysis. Extensive dialysis was used to obtain pectin fractions of high purity free from salts and fractionation reagents. Both the CDTA and carbonate pectin fractions were stored until required as aqueous solutions at −20 °C, in order to avoid possible aggregation induced during freeze-drying. Aliquots of 1 ml were lyophilised for FTIR and SEC analysis. Extraction and fractionation process were performed in triplicate.

Neutral sugar content was determined by the orcinol method (Rimington, 1931; Tillmans & Philippi, 1929) using glucose as standard. UA was determined by the carbazole method (Filisetti-Cozzi & Carpita, 1991) with slight modifications using GalA as standard. Absorbance data were corrected as described by Montreuil, Spik, Fournet, and Tollier (1997) in order to eliminate interferences from neutral sugars, and UA, in the carbazole and orcinol methods, respectively.

2.2. Fourier transform infrared spectroscopy

Infrared spectra were obtained with an Attenuated Total Reflectance (ATR) accessory (MIRacle ATR, PIKE Technologies, USA) coupled to a Fourier transform infrared (FTIR) spectrometer (FT/IR-4100, JASCO, Spain) as described previously (Heredia-Guerrero, San-Miguel, Sansom, Heredia, & Benítez, 2010). Briefly, the freeze-dried samples were mounted on top of the ATR crystal and compressed gently with a sample clamp whilst monitoring the spectral absorbance. All spectra were recorded in the 4000–600 cm^{-1} range with a resolution of 4 cm^{-1} and averaged over 25 scans. ATR effect and atmospheric contributions from carbon dioxide and water vapour were corrected by means of the Spectra Manager v.2 software (JASCO, Spain) over the full spectral range.

2.3. Size exclusion chromatography

A manually poured column (c10/40, GE healthcare) of Sepharose CL-2B (Sigma–Aldrich Química SA, Spain) was used to fractionate the polymers present in the CDTA and Na_2CO_3 fractions within the molecular mass range 100–20 × 10^3 kDa (for dextrans/pullulans). Gel media were equilibrated with 0.2 M acetate buffer, pH 5, for CDTA samples and Tris–HCl 0.05 M, pH 8.5, for Na_2CO_3 samples. Cell wall fractions (3–5 mg) were dissolved in 1 ml of the same buffers used for gel equilibration. The loading volume was 250 μl and the elution flow rate was 10 ml h^{-1}. Fractions (1 ml) were collected and assayed for UA and neutral sugar contents as previously