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Research article

Structural changes in cell wall pectins during strawberry fruit development

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ABSTRACT

Strawberry (*Fragaria* × *anannasa* Duch.) is one of the most important soft fruit. Rapid loss of firmness occurs during the ripening process, resulting in a short shelf life and high economic losses. To get insight into the role of pectin matrix in the softening process, cell walls from strawberry fruit at two developmental stages, unripe-green and ripe-red, were extracted and sequentially fractionated with different solvents to obtain fractions enriched in a specific component. The yield of cell wall material as well as the per fresh weight contents of the different fractions decreased in ripe fruit. The largest reduction was observed in the pectic fractions extracted with a chelating agent (trans-1,2- diaminocyclohexane-N,N,N'N'-tetraacetic acid, CDTA fraction) and those covalently bound to the wall (extracted with Na_2CO_3). Uronic acid content of these two fractions also decreased significantly during ripening, but the amount of soluble pectins extracted with phenol:acetic acid:water (PAW) and water increased in ripe fruit. Fourier transform infrared spectroscopy of the different fractions showed that the degree of esterification decreased in CDTA pectins but increased in soluble fractions at ripen stage. The chromatographic analysis of pectin fractions by gel filtration revealed that CDTA, water and, mainly PAW polyuronides were depolymerised in ripe fruit. By contrast, the size of Na_2CO_3 pectins was not modified. The nanostructural characteristics of CDTA and Na_2CO_3 pectins were analysed by atomic force microscopy (AFM). Isolated pectic chains present in the CDTA fractions were significantly longer and more branched in samples from green fruit than those from red fruit. No differences in contour length were observed in Na_2CO_3 strands between samples of both stages. However, the percentage of branched chains decreased from 19.7% in unripe samples to 3.4% in ripe fruit. The number of pectin aggregates was higher in green fruit samples of both fractions. These results show that the nanostructural complexity of pectins present in CDTA and Na_2CO_3 fractions diminishes during fruit development, and this correlates with the solubilisation of pectins and the softening of the fruit.

1. Introduction

Ripening of fleshy fruit is a combination of physiological and biochemical processes that make edible fruit more attractive to seed dispersal organisms (Gapper et al., 2013). In soft fruit such as strawberry, the rapid loss of firm texture is one of the most noticeable changes during ripening, but it poses a major problem to strawberry growers due to the short postharvest life of this commodity. It is generally accepted that the modification of the physical and chemical features of parenchyma cell walls and the loss of intercellular adhesion resulting from the dissolution of the middle lamella are the major determining factors of fruit softening (Brummell, 2006; Goulao and Oliveira, 2008; Mercado et al., 2011). Nevertheless, the biochemical mechanisms underlying softening are not completely clear.

Strawberry softening is characterised by an increase in pectin

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solubilisation, i.e. an increase in the amount of pectins loosely bound to the cell wall, extracted with water but also with chelating agents such as CDTA in some cultivars (Huber, 1984; Koh and Melton, 2002; Posé et al., 2011). The increasing levels of soluble pectins is paralleled with a decrease in the amount of covalently bound pectins, those supposedly located in the primary cell wall, mainly extracted with sodium carbonate (Posé et al., 2011). Several hypotheses about the causes of pectin solubilisation during fruit ripening have been proposed (Paniagua et al., 2014). One of them relies on the loss of arabinan and galactan side chains from rhamnogalacturonan I (RGI) (Gross and Sams, 1984; Redgwell et al., 1997a), weakening the cell wall network. Moreover, the loss of neutral sugars might also increase the cell wall porosity, which could facilitate the access of pectinases to their substrates (Smith et al., 2002). In accordance with this, the silencing of a β-galactosidase gene reduced strawberry fruit softening and this was related to a lower pectin solubilisation and an increased content of galactose in cell walls (Paniagua et al., 2016). However, not all the types of fruit show a correlation between the loss of neutral sugar residues and pectin solubilisation (Redgwell et al., 1997a).

As an alternative hypothesis, depolymerisation of chelated and/or covalently bound pectins as result of the action of pectinases, e.g. polygalacturonase, pectin methyl esterase or pectate lyase, may facilitate the solubilisation of pectic components not previously present in the soluble fractions (Redgwell et al., 1997b; Rose et al., 1998; Vicente et al., 2007). In strawberry, the role of pectin depolymerisation in fruit softening is unclear. Some authors did not find changes in the average molecular weight of polyuronides during strawberry ripening (Huber, 1984; Redgwell et al., 1997a; Lee et al., 2011) while others observed a significant depolymerisation of bound pectins, depending on the cultivar studied (Rosli et al., 2004). Although there is not a general consensus about the importance of polyuronide depolymerisation, functional analyses of genes encoding pectin degrading enzymes suggest a key role of this process in the changes in texture taking place during strawberry development. Thus, the down-regulation of a pectate lyase or a polygalacturonase gene by antisense transformation increased strawberry fruit firmness at the red stage and improved post-harvest shelf life (Jiménez-Bermúdez et al., 2002; Quesada et al., 2009).

Most studies dealing with the role of pectins in strawberry fruit texture analysed polyuronides by classical techniques such as size exclusion chromatography. Recently, Posé et al. (2015) used atomic force microscopy (AFM) to characterize the effect of pectate lyase and polygalaturonase genes silencing in pectin nanostructure. They found that down-regulation of both genes increased pectin chains length and their structural complexity, the magnitude of these changes being correlated with the increase in fruit firmness.

The main objective of this research was to analyse and compare the cell wall complexity in green and red strawberry fruit, focusing the structural changes that occurs in the chelated and covalently bound pectin fractions.

2. Materials and methods

2.1. Plant material

Strawberry (*Fragaria* × *ananassa* Duch. cv. 'Chandler') plants obtained by runner propagation were grown in 22 cm diameter pots containing a mixture of peat moss, sand and perlite (6:3:1). These plants were cultured in a greenhouse until fruiting, under natural photoperiod and temperature conditions. Fruit were collected from March to June. After harvest, fruit were immediately frozen in liquid nitrogen and stored at −25 °C until used. Two developmental stages were selected, green-unripe and red-ripe (Fig. 1A). Fruit from the green stage were about 1 cm in diameter displaying both green receptacle and green achenes. Ripe red fruit were characterized by its fully red receptacle and soft texture.

2.2. Cell wall extraction and fractionation

Achenes from frozen fruit were removed and the cell wall from fruit receptacles was extracted following the method of Redgwell et al. (1992), as modified by Santiago-Doménech et al. (2008). This procedure yielded cell wall material (CWM) and polymers soluble in PAW (phenol:acetic acid:water, 2:1:1, w:v:v), solvent used to inactivate enzymes (PAW fraction). The CWM (80 mg) was sequentially extracted following the procedure of Santiago-Doménech et al. (2008) to obtain fractions enriched in pectins (water, CDTA and sodium carbonate solubilised fractions) and two hemicellulose enriched fractions extracted with 1M KOH and 4M KOH. Pectin fractions to be analysed by AFM were stored until required at −20 °C as aqueous solutions, in order to avoid possible aggregation induced by freeze-drying.

The uronic acid (UA) content in PAW and cell wall fractions was measured by the carbazol method as reported by Filisetti-Cozzi and Carpita (1991), using GalA as standard. The total neutral sugar content was determined by the orcinol method (Rimington, 1931) using glucose as standard. For neutral sugars analysis of CDTA and Na_2CO_3 fractions, samples were hydrolysed with 72% (w/w) sulphuric acid. After neutralization with ammonia, carbohydrates were derivatised to alditol acetates and analysed by gas chromatography with flame ionization detection (Blakeney et al., 1983). GC analysis was carried out in triplicate.

2.3. Fourier transform infrared spectroscopy

Dried samples were used to capture the infrared spectra 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). The samples were processed as described by Posé et al. (2012) and the spectra were collected in the 4000–600 cm^{-1} range with a resolution of 4 cm^{-1} and averaged over 25 scans per sample.

2.4. Size exclusion chromatography

The gel filtration chromatography measurements were performed as described previously by Santiago-Doménech et al. (2008). Briefly, pectin fractions were loaded onto a 40 cm height × 10 mm internal diameter column filled with Sepharose (Sigma-Aldrich Química SA, Spain) CL6B for PAW and water fractions or CL2B for CDTA and sodium carbonate pectins. Gel medium was equilibrated with 0.2 M acetate buffer, pH 5, for PAW, water and CDTA fractions, or 0.1 M TRIS-HCl buffer, pH 8.4, for sodium carbonate samples. Samples, 6–8 mg, were dissolved in the corresponding equilibration buffer, loaded on the column and eluted at a 14 mL h^{-1} flow rate. Blue dextran standard (2000 kDa) and acetone were used for column calibration. Fractions (1 mL) were collected and assayed for uronic acids (Filisetti-Cozzi and Carpita, 1991).

2.5. Atomic force microscopy

The ionically and covalently-bound pectin fractions, corresponding to CDTA and sodium carbonate soluble polymers, respectively, were analysed by AFM as described by Posé et al. (2012). Briefly, samples were diluted in water to a concentration of 2–4 mg L^{-1}. Then, 3 μL were pipetted onto freshly-cleaved mica and dried over a heating block at 37 °C. The sample was then