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Interactions of pectins with cellulose during its synthesis in the absence of calcium

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ABSTRACT

Pectins are major polysaccharide components in plant cell walls and play various roles in plant development and growth. In order to understand how pectin associates with cellulose, different kinds of pectins varying in neutral sugar side-chain content and backbone homogalacturonan degree of esterification were used to study their interaction with cellulose during its synthesis by *Gluconacetobacter xylinus* (ATCC53524). Binding studies in the absence of calcium revealed that all types of pectin were able to bind to cellulose and confirmed that binding of pectin with a high content of neutral sugar side chains (~40%) was greater than that of homogalacturonan (less than 7% neutral sugars). ^{13}C NMR revealed that associated pectin molecules were neither rigid nor affected cellulose crystallinity; furthermore no apparent change in cellulose architecture was observed in scanning electron micrographs. The binding was reversible as a result of water washing and the de-binding behaviour was dominated by surface permeability rather than diffusion within the pellicle, as suggested by model fitting. Irreversible deformation of composites occurred as a result of mechanical compression accompanied by the release of pectins, and our results showed that both cellulose and pectin contributed to the load-bearing capacity of composites during compression.

1. Introduction

Cellulose is a major polysaccharide in the primary cell wall of plants, playing the main structural and load-carrying roles (Keegstra, 2010). It is composed of an unbranched (1, 4)-linked β-D-glucan chain with an apparent degree of polymerization (DP) between 2000 and 6000 in primary cell walls, which are tightly connected by hydrogen bonds and Van der Waals forces to form semi-crystalline microfibrils (Cosgrove, 2005; Waldron, Parker, & Smith, 2003). The cellulose chains of plant walls are synthesized by cellulose synthase complexes at the plasma membrane and packed into an ordered microfibril of indefinite length and uncertain cross sectional area and shape. The cellulose microfibril has distinct hydrophobic and hydrophilic surfaces which are thought to bind xyloglucan, xylan, and lignin with different affinities (Besombes & Mazeau, 2005; Cosgrove, 2014; Busse-Wicher et al., 2014; Zhao, Crespi, Kubicki, Cosgrove, & Zhong, 2014).

Besides cellulose and associated polysaccharides, the other abundant polysaccharide in many primary cell walls is pectin, which is characterized by relatively high extractability using acid or calcium chelators and a high content of galacturonic acid (GalA) (Harholt, Suttangkakul, & Scheller, 2010). The pectic polysaccharides, a highly complex and heterogeneous group, are mainly composed of a backbone of (1, 4)-linked α-D-GalA residues up to 200 units long that can be methyl esterified and acetylated (Mohnen, 2008; Scheller, Jensen, Sørensen, Harholt, & Geshi, 2007). Homogalacturonan (HG) is the major type of pectin in cell walls, accounting for approximately 65% of pectin (Mohnen, 2008). HG is partially methyl esterified at the C-6 carboxyl and may be O-acetylated at O-2 or O-3 (Harholt et al., 2010; Mohnen, 2008). Rhamnogalacturonan I (RG I), representing 20–35% of pectin, is considerably more complex in structure than HG. RG I consists of (1, 2)-α-L-Rha-(1, 4)-α-D-GalA motifs with up to 100 repeats (Naran, Chen, & Carpita, 2008). A large proportion of the rhamnose residues are substituted at O-4 by neutral sugar side chains including arabinan, galactan, and arabinogalactan I and II, among which arabinan and galactan are the most abundant (Harholt et al., 2010). RG-II, the most structurally complex component, makes up

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approximately 10% of pectin and plays a central role in wall architecture, although it is only present in minor amounts in most plant cell walls (Ishii, Matsunaga, & Hayashi, 2001; Pérez, Rodriguez-Carvajal, & Doco, 2003).

It is proposed that the interactions between cell wall polysaccharides is to a large extent responsible for the complexity of structure and dynamic rearrangements of cell walls, thus revealing these interactions is necessary to understand plant bioprocesses, deconstruct cell walls, construct novel biomaterials, and develop good quality characteristics of plant-based foods. Due to the complexity and dynamism of the cell wall structure, *in vitro* assemblies of cell wall polysaccharides have been widely used to investigate the interactions between cell wall polysaccharides. Based on *in vitro* models (Gu & Catchmark, 2014; Lopez et al., 2010), the network of xyloglucan/cellulose has been well studied, demonstrating that xylogucan is able to coat or tether the cellulose microfibrils through hydrogen bonds or van der Waals interactions, consistent with the network of xyloglucan/cellulose playing a load-bearing role in the primary cell wall (Carpita & Gibeaut, 1993; Hayashi, 1989; Hayashi, Marsden, & Delmer, 1987; Whitney, Brigham, Darke, Reid, & Gidley, 1995). However, the interaction of pectins with cellulose and the roles of pectins in cell walls are still controversial and incompletely understood. Several authors have reported that the pectic network is independent from the cellulose/xylogucan network (Cosgrove, 2000; Hayashi, 1989; Thompson, 2005), while Zykwinska et al. (2007) have demonstrated that pectins rich in neutral sugar side chains are able to bind to cellulose microfibrils, whereas pectins with limited neutral sugars were not able to bind to cellulose microfibrils under the *in vitro* conditions used; while other researchers found that pectins with low levels of neutral sugar side chains can be associated with cellulose microfibrils (Gu & Catchmark, 2012; Tokoh, Takabe, & Fujita, 2002). Taken together, it is hypothesised that pectins of different composition could have different types of associations with cellulose and play different roles in the dynamic cell wall depending on their position *in muro* (Mohnen, 2008).

We have previously shown that isolated galactan and arabinan sidechains and, to a lesser extent, pectin containing neutral sugar sidechains are able to bind to cellulose during its synthesis by *Gluconacetobacter xylinus* (ATCC 53524) in the absence of calcium (Lin, Patricia Lopez-Sanchez, & Gidley, 2015). This behaviour is qualitatively different to that of pectins in the presence of calcium where a weak pectin/Ca^{2+} gel results in an inter-penetrating network of cellulose and pectin (Willats, Steele-King, Marcus, & Knox, 1999) with markedly different mechanical properties compared to cellulose alone or cellulose/xyloglucan (Chanliaud, Burrows, Jeronimidis, & Gidley, 2002). In order to increase our understanding of the compositional factors which control cellulose/pectin interactions, in this work the interactions between cellulose and pectins with different contents of neutral sugar side chains and degrees of esterification have been investigated during cellulose synthesis in the absence of calcium. The structural and mechanical implications are also reported.

2. Materials and methods

2.1. Composite preparation

G. xylinus (strain ATCC 53524) was cultured at 30 °C under static conditions in Hestrin-Schramm (HS) medium (pH 5.0) containing 0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 0.338% (w/v) Na_2HPO_4, 0.115% (w/v) citric acid, 2% (w/v) glucose and 0.5% (w/v) pectin (Mikkelsen & Gidley, 2011). After 96 h incubation, pectin/cellulose pellicles were harvested and gently shaken to remove residual media and bacterial cells. Then the pellicles were put in sodium azide solution 0.02% (w/v) and stored at 4 °C for further analysis.

Commercial citrus pectin CU (Herbstreith and Fox HG, Neuenbürg/Württ., Germany) and apple pectin AU (Herbstreith and Fox HG, Neuenbürg/Württ., Germany) with a degree of esterification (DE) of 33 and 30 respectively and containing 38% and 43% neutral sugar side chains respectively (Table 1) were used. These pectins will be denoted as C33 and A30 respectively. Pectins HG 3-1-A and 3-1-C were kindly supplied by CP Kelco (CP Kelco, Denmark). They and had a degree of esterification of 69 and 33 respectively and were homogalacturonan (less than 7% neutral sugars as specified by the supplier). They were denoted as HG69 and HG33 respectively.

2.2. Component analysis of cellulose composites

Monosaccharide analysis using GC-MS was used to quantify the neutral sugars present in the composites; the method of Pettolino, Walsh, Fincher, and Bacic (2012) was used with some modifications. Composite compositions were calculated from individual sugar contents on the basis of dry weights. Freeze dried samples (1–5 mg) were hydrolysed with 200 μL 12 M H_2SO_4 at 35 °C for 1 h, diluted to 2 M using 3.5 mL water and incubated for a further 3 h at 120 °C. The sample was cooled, then neutralised using approximately 550 μL of NH_4OH and centrifuged at 2000 rpm for 10 min. An aliquot of 100 μL was collected and 5 μg of internal standard (myo inositol) added and then dried with nitrogen. The sample was reduced using 200 μL of 20 mg/mL sodium borodeuteride in DMSO at 40 °C for 90 min. The reductant was destroyed using 20 μL of acetic acid then acetylated by adding 25 μL 1-methylimidazol followed by 250 μL of acetic anhydride. The sample was allowed to stand for 10 min, 2 mL of water was added followed by 1 mL dichloromethane (DCM) to extract the alditol acetates, the sample was mixed, centrifuged to aid separation and the DCM phase was then washed twice with 2 mL of water. The DCM was then dried under a stream of nitrogen and reconstituted into 100 μL of DCM, 1 μL of which was analysed by GC-MS using a high polarity BPX70 column.

The uronic acid was determined using the Filisetti-Cozzi method (Filisetti-Cozzi & Carpita, 1991) with some modifications. Calibration standards were prepared using 1 mg/mL D-glucuronic acid in 0.4 mL water; sample tubes were prepared by addition of 0.4 mL water, and kept on ice. Approximately 2 mg of sample was weighed and hydrolysed in 200 μL 75 mM sodium tetraborate in H_2SO_4, then set aside for 20 min. To both standard and sample tubes 40 μL of 4 M sulfamic acid-potassium sulfamate (pH 1.6) was added. To standard and sample tubes were then added 2.400 mL and 2.398 mL 75 mM sodium tetraborate in H_2SO_4 respectively. All tubes were vortexed and a 20 μL aliquot of the previously hydrolysed sample was then added to sample tubes. Tubes were then incubated at 100 °C for 20 min, cooled on ice and 80 μL of 0.15% m-hydroxybiphenyl in 0.5% sodium hydroxide was added. All tubes were placed in a vacuum oven at room temperature at 85 kPa for 5 min. Colour change was then measured at 525 nm.

Table 1
Monosaccharide composition of pectins and pectin composites (%).

Samples	Ara	Xyl	Man	Rha	Gal	Glu	GalA	Pectin content
Pectin C33	10.9	1.8	1.0	3.1	26.8	0.0	56.4	100
Pectin A30	2.8	8.5	1.9	1.9	40.7	0.0	44.2	100
HG69	2.8	n.a.	0.0	1.0	3.6	0.3	92.3	100
HG33	2.8	n.a.	0.0	1.0	3.7	0.2	92.3	100
Pectin C33/cellulose	0.5	0.0	1.2	0.0	1.4	92.1	4.8	8.5
Pectin A30/cellulose	0.2	0.0	2.0	0.0	3.3	85.7	8.8	15.4
HG69/cellulose	n.d.	n.d.	n.d.	n.d.	n.d.	94.1	5.9	5.9
HG33/cellulose	n.d	n.d.	n.d.	n.d.	n.d.	92.4	7.6	7.6