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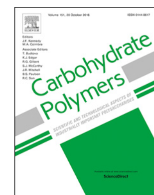

Characterisation of pectin-xylan complexes in tomato primary plant cell walls

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The primary plant cell wall is composed of a complex network of pectin, hemicellulose and cellulose. Potential interactions between these polysaccharides were studied for carrot, tomato and strawberry, with a focus on the role of pectin. The Chelating agent Unextractable Solids (ChUS), the residue after water- and EDTA extraction, was ball milled and subsequently water extracted. For tomato and strawberry, pectin and substantial amounts of hemicellulose were solubilised. Anion exchange chromatography (AEC) showed co-elution of pectin and acetylated glucuronoxylan in tomato, representing 18% of solubilised uronic acid and 48% of solubilised xylose by ball milling from ChUS. The existence of a covalently linked pectin-xylan complex was proposed since xylan co-precipitated with pectin under mild alkali conditions. It was proposed that pectin links with xylan through the RG-I region since degradation of HG did not alter AEC elution patterns for RG-I and xylan, suggesting RG-I – xylan interactions.

1. Introduction

The primary plant cell wall of fruits and vegetables is composed of the polysaccharides pectin, hemicellulose and cellulose. Pectin consists of galacturonic acid as the most prevailing building block, mostly present in homogalacturonan (HG) and rhamnogalacturonan I (RG-I) structural elements. The HG backbone is composed of galacturonic acid (GalA) residues and can be methyl-esterified and/or acetylated. The rhamnose residues in RG-I can be substituted with side chains, composed of arabinose and galactose (Voragen, Coenen, Verhoef & Schols, 2009).

Xyloglucan is the major hemicellulosic polysaccharide in primary plant cell walls of fruits and vegetables, and is composed of a cellulose-like backbone branched by xylosyl residues (O'Neill & York, 2003). The xylose units can be substituted by several other monosaccharides such as galactose, fucose and arabinose (Fry, 1989). Xylans have a backbone of xylose residues and depending on origin xylans can be substituted with GlcA, its 4-*O*-methylated derivative, arabinose and acetyl groups. In fruits and vegetables, glucuronoxylans are the most dominant xylans (Assor, Quemener, Vigouroux & Lahaye, 2013; Coimbra, Waldron & Selvendran, 1995). Mannans are categorised into gluco- and galactomannans, composed of a mixed glucose-mannose backbone or a mannose backbone substituted with galactose, respectively (Scheller & Ulvskov, 2010). Cellulose is composed of an unsubstituted linear backbone of glucose residues (Scheller & Ulvskov, 2010).

The load-bearing network of the cell wall was for a long time believed to be composed of cross-linked hemicellulose and cellulose, referred to as the tethered network model (Cosgrove, 2005). Increasing evidence suggests that pectin might interact with hemicellulose and/or cellulose and hereby has a larger load-bearing and cross-linking role than indicated before (Cosgrove, 2014; Höfte, Peaucelle & Braybrook, 2012). Amongst many interactions between pectin and (hemi)cellulose suggested, recent research in *Arabidopsis thaliana* suggested interactions between RG-I and xylan, indicating that xylans might act as covalent connection between pectin and the hemicellulose-cellulose network or between pectin and cell wall proteins (Ralet et al., 2016; Tan et al., 2013). Although the precise details of suggested cross-links between pectin and hemicellulose are in most cases not defined, RG-I is more often thought to be involved in cross-linking than HG (Mort, 2002; Popper & Fry, 2005).

In order to study potential cross-links between cell wall polysaccharides, targeted disruption of the cell wall architecture might be useful. In this study, planetary ball milling and subsequent water extraction were used as a tool to mildly release and solubilise pectin and hemicellulose from the insoluble cell wall matrix. Subsequently, the solubilised populations for carrot, tomato and strawberry cell walls were studied by anion exchange chromatography (AEC) in combination with targeted enzymatic digestion to reveal potential interactions between these polysaccharides.

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2. Materials and methods

2.1. Plant and polysaccharide materials

Carrots (*Daucus carota* cv. Romance) and strawberries (*Fragaria ananassa* cv. Elsanta) were purchased from a local vegetable store. Tomatoes (*Solanum lycopersicum* cv. H2401) were kindly donated by Heinz (Heinz, Nijmegen, The Netherlands).

Birch wood and beech wood xylans were from Sigma (St. Louis, USA), lemon pectin DM30 and DM70 from CP Kelco (Lille Skensved, Denmark), and soy and potato RG-I from Megazyme (Wicklow, Ireland).

2.2. Extraction of cell wall polysaccharides

Cell wall polysaccharides were extracted using the procedure as described before (Broxterman, Picouet & Schols, 2017; Houben, Jolie, Fraeye, Van Loey & Hendrickx, 2011).

Shortly, Alcohol Insoluble Solids (AIS) were extracted by blending 400 g carrots, tomatoes or strawberries in a 1:3 w/v ratio in 96% ethanol. Prior to blending, only for peeled tomatoes, microwave pretreatment was performed to inactivate pectinases (10 min, 900W). The suspension was filtered and the residue was washed with 70% ethanol until the filtrate gave a negative reaction in the phenol-sulfuric acid test (DuBois, Gilles, Hamilton, Rebers & Smith, 1956). The water soluble solids (WSS) and chelating agent soluble solids (ChSS) were subsequently extracted from AIS according to the references mentioned above. The residue after WSS and ChSS was extensively dialysed, first against potassium acetate, followed by distilled water. After freeze-drying the Chelating agent Unextractable Solids (ChUS) were obtained. This fraction was used to identify potential interactions between pectin, hemicellulose and cellulose.

All fractions were milled for 30 s in a Retsch Cryomill MM440 at a frequency of 20 Hz to obtain homogeneous material (Retsch GmbH, Haan, Germany). Extractions were carried out in duplicate, unless stated otherwise.

2.3. Planetary ball milling of ChUS

ChUS was milled in a PM100 planetary ball mill (Retsch, Haan, Germany), particle sizes wre determined by laser diffraction (Mastersizer 3000; Malvern, Worcestershire, UK). A 12 ml stainless steel jar containing 40 φ5 mm stainless steel balls was used at a frequency of 600 rpm. After every 15 min of milling a pause of 10 min was set to prevent overheating. 500 mg of ChUS was milled per batch with net milling times of 15, 45 or 90 min. In the remainder of the manuscript, milling refers to planetary ball milling, and not to the short homogenisation milling step described in section 2.2.

2.4. Extraction of water soluble cell wall polysaccharides after planetary ball milling

To study the effect of milling on the extractability of polysaccharides, 30 ml water was added to 150 mg milled and non-milled ChUS, and extraction was done for 1 h at 40 °C with continuous head-over-tail rotation. Extractions were carried out in duplicate. Subsequently samples were centrifuged (15 min, 30.000 × *g*) and 30 ml water was added once more to the residue, centrifuged (15 min, 30.000 × *g*) and supernatants were collected and freeze-dried. Supernatants were referred to as SN. Samples were coded according to substrate and milling time, e.g. C45 for carrot ChUS milled for 45 min. T refers to tomato, and S refers to strawberry.

2.5. Sugar composition of the extracts

To determine the uronic acid content of the extracted fractions, the uronic acid content was determined by the automated colorimetric m-hydroxydiphenyl method (Blumenkrantz & Asboe-hansen, 1973). The neutral carbohydrate composition was analysed after pretreatment with 72% (w/w) H_2SO_4 (1 h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 (3 h, 100 °C). Neutral monosaccharides were analysed after dilution (20x) using an ICS5000 High Performance Anion Exchange Chromatography system with Pulsed Amperometric detection (ICS5000 ED) (Dionex Corporation, Sunnyvale, CA, USA), equipped with a CarboPac PA-1 column (250 mm × 2 mm i.d.) and a CarboPac PA guard column (25 mm × 2 mm i.d.).

10 μl of sample was injected and eluted at a flow rate of 0.4 ml/min using a combination of three mobile phases: A) 0.1 M NaOH, B) 1 M NaOAc in 0.1 M NaOH and C) H_2O. The gradient used was: 0–35 min isocratic 100% C; 35.1–50 min linearly from 100% A to 40% B; 50.1–55 min isocratic 100% B; 55.1–63 min isocratic 100%A; 63.1–78 min isocratic 100% C. Post column addition of 0.5 M NaOH at 0.1 ml/min was performed between 0–35 min and 63–78 min. Determination of the sugar composition by methanolysis using 3N HCl in dry MeOH (16 h, 80 °C) followed by 2N TFA hydrolysis (1 h, 121 °C), was used to distinguish between glucuronic acid and galacturonic acid in T45 SN (De Ruiter, Schols, Voragen & Rombouts, 1992). Analysis of the monosaccharide composition was performed in duplicate.

Although it is recognised that UA:Rha is the most common ratio for determination of HG:RG-I backbone ratios, rhamnose levels are often low and lead to less accurate HG:RG-I ratios. The HG to RG-I ratio in the extracts was therefore calculated by $\frac{\text{UA}}{\text{Ara + Gal}}$ in mol%, leading to a HG to RG-I ratio including RG-I side chains, assuming that Ara and Gal originated from RG-I. The ratio of hemicellulose to pectin was calculated by $\frac{\text{Man + Xyl}}{\text{UA}}$ in mol%.

2.6. Determination of methyl-esterification and acetylation

Samples were saponified at ≈ 3 mg/ml in 0.25 M NaOH (3 h, 4 °C) to determine the degree of methyl-esterification (DM) in duplicate using a colorimetric method as previously described (Klavons & Bennett, 1986). The same saponification procedure was used to determine the degree of acetylation (DA), by measuring released acetic acid by a Megazyme acetic acid kit (Megazyme, Wicklow, Ireland). The DM and DA were calculated as the moles of methyl esters and acetyl groups per 100 mol of GalA, respectively.

2.7. Starch digestion

The presence of starch in AIS, WSS, ChSS and ChUS was analysed in duplicate by using the Megazyme total starch assay procedure for resistant starch (Megazyme, Wicklow, Ireland). After digestion of ~10 mg sample with amylase and amyloglucosidase, samples were filtered using a 3 kDa filter (Millipore centrifugal filter units, Merck, Billerica, Massachusetts, United States), to remove glucose originating from starch, and freeze-dried. Subsequently the sugar composition after H_2SO_4 hydrolysis was measured as indicated in 2.5.

2.8. High performance size exclusion chromatography (HPSEC)

Extracted polysaccharide populations were analysed for their molecular weight distribution using an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) coupled to a Shodex RI-101 detector (Showa Denko K.K., Tokyo, Japan). A set of TSK-Gel super AW columns 4000, 3000, 2000 (6 mm × 150 mm) preceded by a TSK-Gel super AW guard column (6 mm ID × 40 mm) (Tosoh Bioscience, Tokyo, Japan) was used in series. The column temperature was set to 55 °C. Samples (5 mg/ml) were injected (10 μl) and eluted with 0.2 M $NaNO_3$ at a flow rate of 0.6 ml/min. Pectin standards from 10 to 100 kDa were used to estimate the molecular weight distribution (Voragen, Schols, De Vries & Pilnik, 1982).

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