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Methylesterase behaviour is related to polysaccharide organisation in model systems mimicking cell walls

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ABSTRACT

Pectin gels and pectin–cellulose binary gels were used as cell wall-mimicking systems to investigate the diffusion ability of a fungal pectin methylesterase. Increasing content of cellulose in the gel appears to result: (i) in longer demethylated blocks thus favouring AaPME processivity, and (ii) in accelerated enzyme kinetics. To better understand this unexpected behaviour, a method was set up to investigate the gel porosity as a function of the cellulose content by following the passive diffusion of three pullulans having different hydrodynamic volumes. Like the enzyme, the pullulans diffused more efficiently in the gels containing the highest proportions of cellulose. Altogether, these results suggest that the gel settled differently during formation according to the respective proportions of the two polysaccharides. With cellulose present, a fraction of pectin would form close interactions with the microfibrils resulting in a larger volume accessible to diffusing molecules. This volume would be related to the cellulose concentration.

1. Introduction

The plant cell wall is composed of several polymers including cellulose as a load-bearing component, embedded in a matrix of polysaccharides (pectin, hemicelluloses) and proteins. Pectin is the most complex cell wall polysaccharide, composed of as many as 17 monosaccharides and characterised by a high galacturonic acid (GalA) content. Pectin structure can be split into different domains; the most common are the homogalacturonan (HG) or 'smooth' region, and the rhamnogalacturonan I (RGI) or "hairy" region (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). HG consists of a (1,4)-α-linked D-GalA backbone with a degree of polymerisation (dp) of 100–200 residues (Thibault, Renard, Axelos, Roger, & Crépeau, 1993; Zhan, Janssen, & Mort, 1998; Bonnin, Dolo, Le Goff, & Thibault, 2002). In the RGI backbone domains, (1,4)-α-linked D-GalA units are interrupted by (1,2)-linked rhamnopyranosyl residues (Rha). RGI may carry side chains, typically α(1,5)-linked arabinans or β (1,4)-linked galactans, primarily attached to the C-4 of the Rha residues (Albersheim, 1978). The GalA carboxyl group may carry a methyl ester, whereas the hydroxyl groups on C-2 or C-3 may be O-acetylated. The degree of methylation (DM) and degree of acetylation are defined as the percentage of GalA esterified with methanol and acetic acid, respectively. *In planta* as well as *ex planta*, pectin interacts with calcium to form a physical gel set up *via* electrostatic interactions. Since 8–15 consecutive non-methyl esterified GalA units are required to form a stable calcium-mediated junction zone (Chen & Mort, 1996; Benen, Kester, & Visser, 1999), the length of the non-methyl esterified stretches is a key parameter for predicting the gelation ability of pectin (Thibault & Rinaudo, 1985a; Ralet, Dronnet, Buchholt, & Thibault, 2001; Ström et al., 2007; Löfgren, Guillotin, Evenbratt, Schols, & Hermansson, 2005; Slavov et al., 2009). Structural parameters based on enzymatic fingerprinting of pectin have been defined to investigate the length of the non-methyl esterified stretches. The degree of blockiness (DB) expresses the amount of non-esterified GalA-oligosaccharides of dp 1, 2 and 3 liberated by an endopolygalacturonase as a percentage of the amount of non-esterified GalA (Daas, Meyer-Hansen, Schols, De Ruiter, & Voragen, 1999). The absolute degree of blockiness (DB_{abs}) expresses the amount of non-esterified GalA-oligosaccharides of dp 1, 2 and 3 liberated by an endopolygalacturonase as a percentage of the total amount of GalA (Guillotin et al., 2005). Moreover, the DB and DB_{abs} of the highly methyl esterified stretches were defined as two new parameters from the analysis of the degradation products generated by the action of a purified pectin lyase (Ralet et al., 2012). All these structural parameters are affected by random or

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blockwise deesterification (Ralet et al., 2012). By combining the information obtained by endopolygalacturonase and pectin lyase, a clear differentiation was possible over a large range of DM values between randomly deesterified HG, HG exhibiting long and scarce deesterified stretches, and HG exhibiting short and numerous deesterified stretches. These two enzymes recently applied to sugar beet pectin revealed the distribution of both acetyl and methyl-esters (Remoroza, Bristerman, Gruppen, & Schols, 2014).

In planta, pectin is secreted into the wall in a highly esterified state and evolves during cell growth and development. The DM is reported to decrease during cell life (Stewart, Iannetta, & Davies, 2001; Draye & Van Cutsem, 2008). The DM decrease (i) allows control of cell wall properties like local pH and porosity (Denès, Baron, Renard, Péan, & Drilleau, 2000), (ii) is associated with a loss of cellular adhesion and (iii) influences plant development and stress response (Pelloux, Rusterucci, & Mallerowicz, 2007). The DM decrease results from the action of a range of enzymes including pectin methylesterases. PMEs, which belong to the CE8 family of the Carbohydrate Esterases (www.cazy.org), de-esterify GalA carrying methyl ester groups and thus can modify pectin gelation ability. When a sufficient number of contiguous free GalA residues is generated by enzyme action, new junction zones can be established between pectin chains in the presence of calcium. PMEs are present in fungi as well as in plant kingdoms, and are reported to lead to more or less ordered patterns of methyl esterification depending on their origin, as investigated by the determination of the DB or DB_{abs} of the treated pectin. Fungal PMEs demethylate short sequences of GalA residues on one chain before attacking the next one, thus generating short sequences of free GalA with a random-type distribution (Thibault & Rinaudo, 1985b; Daas et al., 1999; Limberg et al., 2000). On the other hand plant PMEs demethylate in a processive mode, thus releasing long free stretches (Catoire, Pierron, Morvan, Hervé du Penhoat, & Goldberg, 1998; Denès et al., 2000; Ralet et al., 2001; Savary, Hotchkiss, & Cameron, 2002; Cameron, Luzio, Goodner, & Williams, 2008).

The biochemical properties and modes of action of these enzymes are most often studied in solution *i.e.* in homogenous medium and in very dilute conditions. These conditions are very far from those met in the cell wall, where the substrate is insolubilised by cross-linking with itself or with other polymers, and where the enzyme has to reach its substrate by diffusing through the solid matrix. How the enzyme diffuses and acts in the cell wall is not known. One approach to improve our understanding of enzyme mechanism within the cell wall is to use model systems mimicking the cell wall network. Previous studies on pectin–calcium gels showed that enzyme acts differently in solution and in gel. These behaviour differences were partly due to the presence of calcium. With calcium present in the medium *Aspergillus aculeatus* PME acted in a more blockwise manner while orange PME acted in a more random manner (Slavov et al., 2009). A reduction of block length generated by plant PME was demonstrated by capillary electrophoresis (Vincent, Cucheval, Hemar, & Wiiliams, 2009) as well as by rheological analysis (Slavov et al., 2009). Pectin de-esterification and pectin gelation occur concomitantly, inducing a competition between de-esterification and formation of Ca junctions (O'Brien, Philp, & Morris, 2009) that could explain this reduced processivity. The presence of calcium would generate physical constraints for PME, influencing its catalytic behaviour (Videcoq, Garnier, Robert, & Bonnin, 2011) and hampering its mobility (Videcoq, Steenkeste, Bonnin, & Garnier, 2013). However, pectin gel is different from cell wall structure, which consists of a fundamental framework of cellulose microfibrills maintained together by cross-linking hemicelluloses, and embedded in a pectin network (Somerville et al., 2004). To simulate the cell wall more closely, it is necessary to include the other cell wall polysaccharides in the gel. The controlled reconstruction of cell wall through *in vitro* sequential assembly of cell wall polysaccharides has already revealed some of their physicochemical properties (Agoda-Tandjawa et al., 2010; Cerclier, Cousin, Bizot, Moreau, & Cathala, 2010; Valentin et al., 2010; Winter et al., 2010). Mixing cellulose and low methoxy pectin in the presence of calcium led to a composite and interpenetrated gel where cellulose and pectin are however localised in different phases (Agoda-Tandjawa, Durand, Gaillard, Garnier, & Doublier, 2012).

In the present work, cellulose was added in various concentrations to pectin to build a new environment closer to the actual cell wall. PME behaviour was investigated either in precast gel systems or during the gelation process. To link our observations with the gel implementation, a method was developed to evaluate gel porosity, based on the use of pullulan as a molecular probe, which gave new insights into the role of the two polysaccharides in establishing gel porosity.

Table 1
Composition of citrus MM pectin and sugar beet cellulose (% dry weight).

	Pectin	Cellulose*
Sugar		
Rha	1.80	0
Ara	0.65	0.50
Xyl	0.11	3.93
Man	0.00	1.75
Gal	5.37	0.72
Glc	1.18	80.33
GalA	85.07	1.20
Protein	nd	1.35
Ash	nd	3.61

nd: not determined.
* From Agoda-Tandjawa et al. (2010).

2. Materials and methods

2.1. Materials

The citrus MM pectin was kindly provided by Cargill Texturizing Solutions (lot no. 12030618, Baupte, France). It has a GalA content of 85.07% (Table 1), a DM of 44% and a DB_{abs} of 11.3% (see below for the determination methods). Pectin solutions were prepared at 25 mg/mL by dissolving the pectin powder in 50 mmol/L MES (2-[*N*-morpholino] ethane–sulphonic acid) buffer at pH 6 during 2 h at 4 °C under magnetic stirring. The pH was re-adjusted to 6 using 0.1 mol/L NaOH and the pectin solutions were stored overnight at 4 °C. To remove large aggregates and impurities, pectin solution was centrifuged at 17,000 × *g* for 30 min and the supernatant was filtered on nitrocellulose filters with porosity of 8 μm and 3 μm successively (Millipore, St Quentin en Yvelines, France). The content of pectin was controlled in this supernatant before using it for further experiments.

Cellulose microfibrils were extracted from sugar beet pulp as described in Agoda-Tandjawa et al. (2010). The cellulose composition is shown in the Table 1. Defrosted cellulose was sonicated twice for 4 min at 30 °C in a Branson 200 ultrasonic cleaner, and then concentrated (from 1% w/w to 4.6% w/w) by osmotic compression against dextran solution (18% w/w, M_w = 100,000 g/mol).

The pectin methylesterase from *A. aculeatus* (AaPME, EC 3.1.1.11, Uniprot Q12535) was kindly provided by Novozyme A/S (Copenhagen, Denmark). It was solubilised at 5 mg/mL in 10 mmol/L MES buffer pH 6 and dialysed overnight at 4 °C against the same buffer. PME activity was determined by the colorimetric method using *N*-methylbenzothiazolinone-2-hydrazone (MBTH, Sigma M8006-1G) and alcohol oxidase (E.C.1.1.3.13, Sigma A2404) for oxidation of the released methanol (Anthon & Barrett, 2004). The stock solution had an activity of 516 nkat/mL. Before use, Aa-PME was diluted as necessary with 10 mmol/L MES buffer.