Carbohydrate Polymers 86 (2011) 1657–1664

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Influence of calcium on pectin methylesterase behaviour in the presence of medium methylated pectins

Pauline Videcoq, Catherine Garnier, Paul Robert, Estelle Bonnin*

INRA, UR 1268, Biopolymères Interactions Assemblages, BP 71627, 44316 Nantes, France

ARTICLE INFO

Article history:
Received 8 April 2011
Received in revised form 13 May 2011
Accepted 30 June 2011
Available online 7 July 2011

Keywords:
Pectin
Rheological behaviour
Degree of methoxylation
Degree of blockiness
Demethylation
Enzymes

ABSTRACT

Pectins account for one of the major polysaccharides of plant cell walls and are commonly used in the food industry as gelling and stabilising agents. Pectin methylesterase (PME) can alter pectin structure and affect their gelling properties by de-esterifying galacturonic acid. Previously, we reported different behaviours of plant and fungal PMEs on high methylated pectins in solution or during the gelling process (Slavov et al., 2009). In the present study, the behaviour of the two PMEs is studied on a medium methylated pectin in solution, i.e. in the absence of calcium, or during the gelling process, i.e. in the presence of calcium. Only slight modifications were observed for *Aspergillus aculeatus* PME behaviour between the two conditions. On the opposite we show a role of calcium on the processivity of orange PME: the processivity decreases in the presence of calcium.

1. Introduction

Pectins are a complex group of structural plant polysaccharides, mainly located in primary cell walls of higher plants. They are involved in the control of cell wall properties and in many physiological processes as plant cell growth (Willats, McCartney, Mackie, & Knox, 2001; Wolf, Mouille, & Pelloux, 2009) or plant defence against phytopathogens (Lionetti et al., 2007; Messiaen & Van Cutsem, 1994; Ridley, O'Neill, & Mohnen, 2001). Next to biological interest, pectins are also used for their thickening and gelling properties in the industry. In food industry, pectins can be used as stabilizing agent in acidic milk beverage (Jensen, Rolin, & Ipsen, 2010), gelling agent in dairy dessert or fruit jam (Arltoft, Madsen, & Ipsen, 2008) or fat agent replacer (Min, Bae, Lee, Yoo, & Lee, 2010). In pharmaceutical industry, pectins have several utilizations and have been recently used in the treatment of breakthough cancer pain as transmucosal delivery system (Portenoy, Burton, Gabrail, & Taylor, 2010). Pectin structure is complex (Voragen, Coenen, Verhoef, & Schols, 2009). Commercial pectins are mainly constituted by homogalacturonan, a linear homopolymer of α(1–4) linked D-galacturonic acid, which can be esterified with methanol at C6 carboxyl group. The degree of methylation (DM), as the degree of polymerization, affects gelling properties of pectins. The DM subdivides pectins in two classes with different gelling abilities. Pectin with a high DM (high methoxylated (HM) pectin, DM > 50%) can form a gel in acidic conditions in the presence of high sugar concentration. In the opposite, gelation of low methoxyl pectin (low methoxylated (LM) pectin, DM < 50%) occurs at higher pH in the presence of divalent ions, such as calcium, which acts as a bridge between pairs of carboxyl groups of different pectin chains. The main industrial sources for pectin extraction are apple pomace and citrus peels, which provide HM pectin. LM pectin can be obtained after chemical de-esterification of HM pectins. However, this process often induces pectin depolymerisation, thus reducing gel forming ability of pectin (Fraeye, Duvetter, Doungla, Van Loey, & Hendrickx, 2010b).

In planta, pectins are integrated in plant cell walls and evolve during cell growth and development. They are secreted into the wall as highly methylesterified. They are further modified by endogenous pectin methylesterases (PME) that catalyse the enzymatic de-esterification of pectin. The modulation of PME action allows control of plant cell wall properties like local pH, porosity or stiffness (Denès, Baron, Renard, Péan, & Drilleau, 2000). PMEs can also be found in some plant cell wall degrading microorganisms like phytopathogenic fungi and bacteria. In this case PMEs demethylate the pectin network for facilitating bacterial or fungal invasion (Fries, Ihrig, Brocklehurst, Shevchik, & Pickersgill, 2007). PMEs offer demethylation without molecular weight loss of pectin (Hotchkiss et al., 2002). Moreover, whereas chemical demethylation leads to a random pattern of methylation, PMEs can lead to more ordered pattern depending on their origin. The mode of action of PMEs has been widely studied in pectin solution (Limberg

* Corresponding author. Tel.: +33 02 40 67 50 58; fax: +33 02 40 67 50 84.
E-mail address: estelle.bonnin@nantes.inra.fr (E. Bonnin).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.06.081

et al., 2000). Most fungal PMEs demethylate short sequences of GalA residues before attacking next chain. This action generates short sequences of free galacturonic acid with a random-like distribution. Plant PMEs demethylate in a more blockwise fashion, converting large parts of a chain before attacking next chain (Catoire, Pierron, Morvan, Hervé du Penhoat, & Goldberg, 1998). This action generates long sequences of free contiguous galacturonic acid with a blockwise distribution. A relationship between the strength of calcium pectin gel and the patterns of methylation generated after plant and fungal PME action has been evidenced (Fraeye et al., 2009; Luzio & Cameron, 2008). In the presence of blocks, stronger gels are formed. Moreover, Hotchkiss et al. (2002) demonstrated that a pectin de-esterified by a plant PME exhibits calcium sensitivity at a higher DM than a pectin chemically de-esterified.

Simultaneously, several authors reported about PME's activity on HM pectin in presence of calcium (O'Brien, Philp, & Morris, 2009; Slavov et al., 2009; Vincent & Williams, 2009). In these works, pectin gelation and pectin de-esterification by PME occur concomitantly and the context is closer to PME environment found *in planta*. O'Brien et al. (2009) reported that different calcium concentrations and temperature values influence rheological properties of the system in the presence of fungal PME. However, no change in the gelation time was noticed as a function of temperature (20–40 °C) even when the temperature approaches optimum temperature of the PME. This behaviour was attributed to competition between de-esterification and formation of Ca^{2+} induced "eeg-box" junctions. Vincent and Williams (2009) characterized the gelation mechanism in the presence of a plant PME using microrheology. They also investigated the pattern of demethylation by capillary electrophoresis (Vincent, Cucheval, Hemar, & Williams, 2009). They reported a reduction in the block length generated by PME in the presence of calcium. The demethylation pattern generated by plant PME in the presence or in the absence of calcium led to the formation of different networks in presence of calcium, a flexible and semi-flexible, respectively.

In a previous work, we reported how *Aspergillus aculeatus* (Aa-PME) and Orange (O-PME) demethylate HM pectins in the presence or in the absence of calcium (Slavov et al., 2009). We observed an effect of calcium on the mode of action of the enzymes. Aa-PME acted in a more blockwise manner and O-PME acted in a more random manner than in the absence of calcium. The differences have been linked to the gelation behaviour of pectins in the presence of calcium and Aa-PME or O-PME. One hypothesis was that during the demethylation process, gelation occurs which can generate physical constraints for PME and modify its behaviour. In the present work, a medium methylated pectin (MM pectin) of 46% DM was used to study PME's behaviour in a pre-gelled system where it is possible to take into account its physical constraints. We report now the biochemical characterisation of the system with the aim of studying the influence of the media on PME's behaviour in a physical point of view. In this way, the modifications in PME's behaviour can be investigated and connected to the gelation behaviour

2. Materials and methods

2.1. Materials

The citrus MM pectin was kindly provided by Cargill Texturizing solutions (lot n°13830, Baupte, France). It has a DM of 46% and a galacturonic acid content of 77.3%. Pectin solutions were prepared at 25 mg ml^{-1} by dissolving the pectin powder in 50 mmol l^{-1} MES (2-[N-morpholino] ethane-sulphonic acid) buffer at pH 6 during 2 h at 4 °C under magnetic stirring. The pH was thereafter adjusted to 6 using 0.1 mol l^{-1} NaOH and the pectin solutions 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 successive porosity ranging from 8 μm to 0.8 μm (Millipore). The concentration was determined by drying 2 ml of solution in an oven at 110 °C overnight and measuring the dry weight.

The pectin methylesterases used were from orange (O-PME, 461 U mg^{-1}, lot 087K7435, P4500, Sigma, L'Isle d'Abeau, France) and *A. aculeatus* (Aa-PME, Uniprot Q12535), the later one being kindly provided by Novozyme A/S (Copenhagen, Denmark). The enzymes were solubilised at 5 mg ml^{-1} in 10 mmol l^{-1} MES buffer (pH 6) and dialysed overnight at 4 °C against the same buffer. Activities of PMEs were 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). Before enzymatic incubation, enzymes were diluted as necessary with 10 mmol l^{-1} MES buffer. The enzymes used in the present work have different pH optima: O-PME has its pH optimum at 9 (Savary, Hotchkiss, & Cameron, 2002) while Aa-PME optimum pH is 4.5 (Duvetter et al., 2006). In order to compare their action without changing the experimental conditions, an intermediate pH value of 6 was chosen.

The endo-polygalacturonase (*An*PGII, E.C. 3.2.1.15, Uniprot P26214, Novozymes) was from *Aspergillus niger* and was purified in the laboratory as described elsewhere (Sakamoto, Bonnin, Quemener, & Thibault, 2002).

2.2. Methods

2.2.1. Determination of PME kinetic parameters

The kinetic parameters were determined by performing PME activity assays at different MM pectin concentrations from 1 to 20 mg ml^{-1}. Activity assays were conducted at 30 °C and pH 6. The Michaelis constant (K_m) and the maximum velocity (V_{max}) were determined using Lineweaver–Burk double reciprocal plot in which the reciprocal of the initial rates of PME activity were plotted against the reciprocals of the pectin concentration. Assays were performed in duplicate.

2.2.2. Kinetics

Pectin and calcium chloride solutions were preheated at 50 °C during 2 min. Then $CaCl_2$ solution was added drop by drop to pectin under strong magnetic stirring. The final concentrations were 15 mg ml^{-1} for pectin and 5 mmol l^{-1} for calcium. The mixture was held at 50 °C under stirring for 1 min. Then O-PME or Aa-PME solution was added to have a final enzyme concentration of 0.4 nkat ml^{-1}. The final mixture was split in 150 μl-samples which were incubated at 30 °C. PME activity was stopped by heating the samples at 70 °C (10 min) every hour during 6 h and then at 10 and 24 h. After heating, the samples were diluted in 50 mmol l^{-1} MES pH 6 at 2 mg ml^{-1} for DM and DBabs determination.

2.2.3. Degree of methylation (DM)

DM was determined using infrared spectroscopy. Infrared spectra were recorded between 1800 and 1535 cm^{-1} at 4 cm^{-1} intervals using a Brucker spectrometer (Vector 22). Pectin films were prepared in duplicate drying 150 μl of 2 mg ml^{-1} pectin–water on BaF_2 window. In the selected spectral range, the absorptions bands at 1740 and 1620 cm^{-1} are assigned to C=O stretching vibrations of ester and carboxylate, respectively. An equation of prediction was developed by applying a Principal Components Regression to infrared data of pectins (Danisco Ingredients, Braband, Denmark) which DM varied (DM: 11, 19, 28, 46, 71). The equation of prediction was characterised by a coefficient of determination r^2 0.9914 and a standard error of calibration sec of 2.18%.

2.2.4. Measurement of the absolute degree of blockiness (DBabs)

After enzymatic fingerprinting of the pectins with AnPGII (Slavov et al., 2009), the absolute degree of blockiness

was calculated from the amount of non-methylesterified monomer, dimer and trimer released by AnPGII and was expressed as a percentage of total GalA (Guillotin et al., 2005).

Pectins were diluted at 0.1% in 50 mmol l^{-1} acetate buffer pH 4. Then 0.21 nkat AnPGII was added and incubation was carried out at 40 °C for 72 h. Further enzyme (0.21 nkat) was added at 24 and 48 h. Since the preferred substrate of endoPGII is polygalacturonic acid, the more blocky structure the pectin has, the more degradation products are obtained. The degradation products were measured by high performance anion exchange chromatography (HPAEC) at pH 13. HPAEC was performed using a column Carbopac PA-1 (2 × 50 mm, Dionex) at 25 °C and was eluted at 0.250 ml min^{-1} with a gradient of sodium acetate. The oligosaccharides were detected by a pulsed amperometric detector. The GalA content of pectins was colorimetrically determined by the automated m-hydroxybiphenyl method (Thibault, 1979). The processivity expresses the multiple attack on a single chain that means the capacity of the enzyme to stay on the pectin chain for many catalytic events. Consequently it generates blocks of free galacturonic acids that are evaluated by DBabs.

2.2.5. Rheological measurements

Dynamic rheological measurements were performed at 30 °C using a stress-controlled rheometer (AR2000, TA instrument) equipped with a Peltier temperature controller and with a cone-plane device (40 mm diameter, 2° angle, gap 98 μm). The visco-elastic properties of pectin gels were characterised by measuring the storage (G') and loss moduli (G'') over time during 48 h at a frequency of 1 rad s^{-1}. Then mechanical spectra were obtained by measuring G' and G'' over the frequency range 0.01–100 rad s^{-1}. G' and G'' values are given with a standard error of 50 Pa. All experiments were performed with low strain amplitude of 1%. It was checked that the stress applied was well within the linearity domain of visco-elasticity. To prevent evaporation of the solvent, the free surface of the sample was always covered with a thin layer of low-viscosity paraffin oil. Pectin and $CaCl_2$ solutions were preheated at 50 °C during 2 min. $CaCl_2$ solution was then added drop by drop under strong magnetic stirring. The mixture (final pectin concentration: 15 mg ml^{-1}, final calcium concentration: 5 mmol l^{-1}) was held at 50 °C under stirring for 1 min before PME addition (Aa-PME or O-PME, 72 μl, final concentration: 0.4 nkat ml^{-1}). The mixture was transferred to the plate of the rheometer preheated at 50 °C.

Table 1
Specific activity of O-PME and Aa-PME in the presence or the absence of 5 mmol L^{-1} calcium.

Origin of PME	Specific activity (nkat mg^{-1} powder)	
	Without calcium	In the presence of calcium
Aa-PME	37.7 ± 2.5	126.6 ± 2.3
O-PME	31.9 ± 2.7	42.3 ± 4.8

3. Results

3.1. Determination of kinetic parameters of Aa-PME and O-PME

As the pH and temperature chosen for these experiments were not Aa-PME and O-PME optima, specific activity and kinetic parameters were investigated in the conditions applied in this study.

The specific activity was determined in the presence and the absence of calcium (Table 1). As already observed by Slavov et al. (2009) on HM pectin, Aa-PME specific activity was higher in the presence than in the absence of calcium. In the case of O-PME a slight increase in the specific activity was noticed when adding calcium.

K_m and V_{max} of O-PME and Aa-PME were measured (Table 2). The Michaelis constant (K_m) allows quantifying the affinity between the enzyme and its substrate, whereas V_{max} is the maximum velocity of the enzyme. These two parameters evolve conversely. An efficient enzyme can be defined by a low K_m in association with a high V_{max}. In comparison with kinetic parameters found by Christgau et al. (1996) without calcium, our parameters reflected a lower affinity for pectic chains and a lower maximum rate of catalysis of Aa-PME. In the presence of calcium, K_m and V_{max} decreased compared to the values characterising the same system without calcium. Thus, calcium ions enhanced the affinity of Aa-PME for pectins and decreased the maximum velocity.

For O-PME, the K_m found was 10 fold higher and V_{max} was slightly higher than the values in the literature, showing how these values depend on reaction parameters such as temperature, salt concentration, pH of the reaction medium and pectin structure. In the presence of 5 mmol l^{-1} calcium, an increase of K_m and V_{max} was noticed. This means that calcium ions decreased O-PME affinity for pectic chains but increased its demethylation velocity.

3.2. Characterisation of the demethylation rate of Aa-PME and O-PME in the presence or not of calcium

The DM of MM pectin incubated with PMEs was monitored during 24 h in the presence or not of 5 mmol l^{-1} of calcium. With

Table 2
Kinetic parameters of O-PME and Aa-PME.

References	Conditions	K_m (mg ml^{-1})	V_{max} (μmol min^{-1} ml^{-1} mg^{-1})
Citrus sinensis (Valencia) in this work	DM = 46% pH 6; 30 °C 50 mmol l^{-1} MES	8	15.6
Citrus sinensis (Valencia) in this work	DM = 46% pH 6; 30 °C 50 mmol l^{-1} MES 5 mmol L^{-1} $CaCl_2$	19.8	68.6
Citrus sinensis (Navel) (Versteeg, Rombouts, Spaansen, Pilnik, 1980)	DM = 62% pH 7; 30 °C 150 mmol l^{-1}	0.083	
Citrus sinensis (Valencia) (Hou, Jeong, Walker, Wei, Marshall, 1997)	DM = 64% pH 7; 22 °C 100 mmol l^{-1} NaCl	1.1	0.53
Aspergillus aculeatus in this work	DM = 46% pH 6; 30 °C	92.5	65.7
Aspergillus aculeatus in this work	DM = 46% pH 6; 30 °C 5 mmol l^{-1} $CaCl_2$	3.53	297
Aspergillus aculeatus (Christgau et al., 1996)	DM = 75% pH 4.6; 30 °C	27	

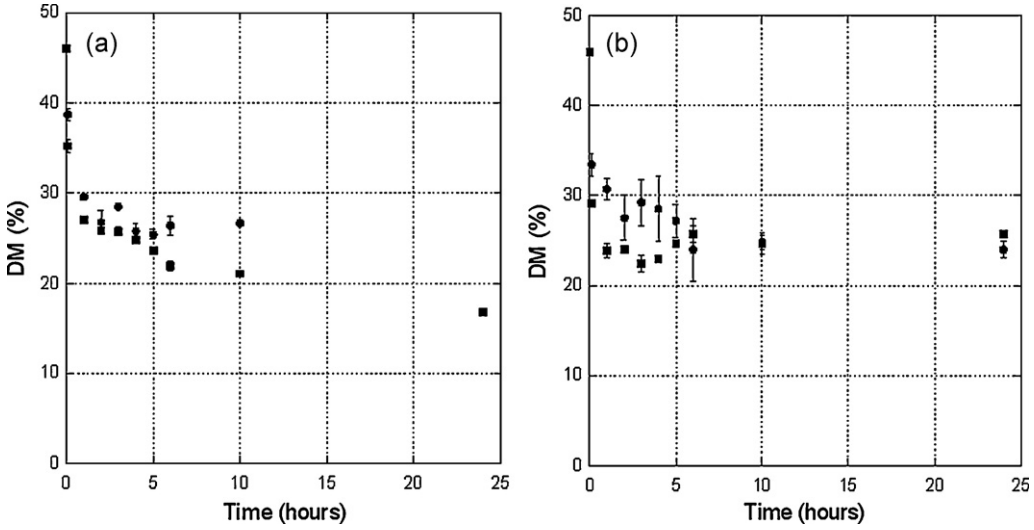

Fig. 1. Evolution of DM during Aa-PME action (a) and O-PME action (b) on MM pectin (15 mg ml^{-1}) with (squares) or without (circles) 5 mmol l^{-1} calcium.

Aa-PME (Fig. 1a), in the absence of calcium, the DM dropped down in the first hour from 46% to 27%, and then decreased until 24 h to reach a final DM of 16%. With O-PME (Fig. 1b), the same DM drop was observed in the beginning of the incubation. Afterward the decrease stopped around 6 h at a DM of 24% that remained rather constant until 24 h. Indeed, Aa-PME can de-esterify MM pectin more extensively than O-PME. In the presence of calcium, for both PMEs, a slight increase in the demethylation rate was observed (Fig. 1a and b). This increase during the first hour of the kinetics did not affect the final respective DM, as similar values with and without calcium were found after 24 h of incubation.

3.3. Characterisation of the demethylation pattern by Aa-PME and O-PME in the presence or not of calcium

The initial DBabs of MM pectin was 16% ± 0.41. It was further monitored during 24 h on MM pectin incubated with PMEs in the presence or not of 5 mmol l^{-1} of calcium (Fig. 2). The higher the DBabs is, the most blocky the methyl distribution is.

For both PMEs and both conditions, the DBabs increased rather quickly until 6 h then the increasing rate slowed down. This deceleration was well marked for Aa-PME kinetics.

As expected, O-PME acted in a more blocky way than Aa-PME in the absence of calcium. This difference between the two enzymes was lowered in the presence of calcium. Slight differences of the methyl distribution were found between the pattern of demethylation of Aa-PME in the presence (DBabs = 42.9%) or in the absence of calcium (DBabs = 37.8%) after 6 h (Fig. 2a). Aa-PME tended to act in a more blocky way in the presence of calcium. On the opposite, with O-PME, the methyl distribution that was expected to be more random, exhibited a higher DBabs in the absence of calcium (Fig. 2b). After 6 h, the DBabs reached 48.1% and 35.4% in the absence or presence of calcium, respectively. This gap was kept constant after 24 h, where the DBabs was 63.3% and 51.1%, without calcium and with calcium, respectively. After 24 h action in the presence of calcium, the DBabs induced by O-PME and Aa-PME were close to each other. Then, in the presence of calcium, O-PME hydrolyses methylesters in a more random way and generates shorter blocks than in the absence of calcium.

3.4. Comparison of rheological behaviour of calcium–pectin systems resulting from the action of O-PME and Aa-PME in situ

MM pectin (final concentration, 15 mg ml^{-1}) was mixed with calcium (final concentration, 5 mmol l^{-1}) at 50 °C. Before pouring

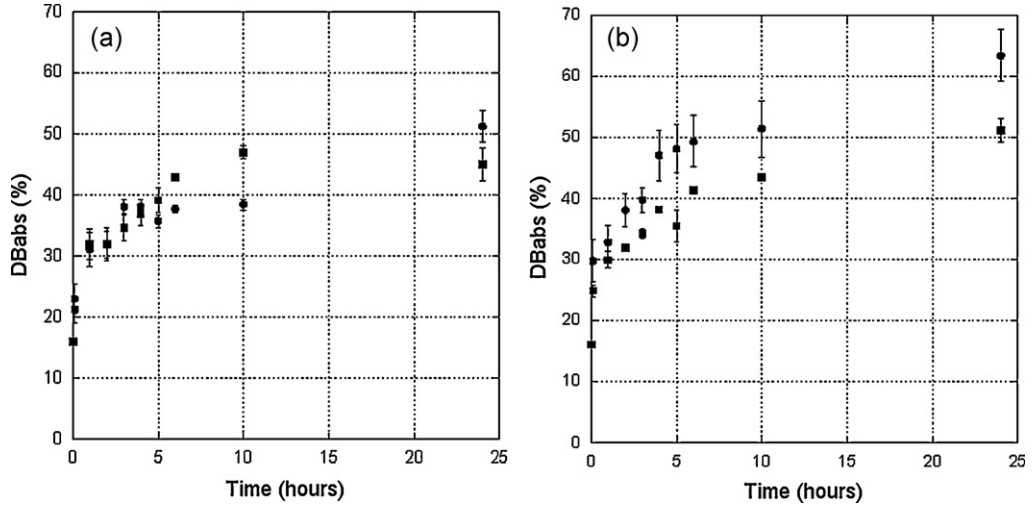

Fig. 2. Evolution of DBabs during Aa-PME action (a) and O-PME action (b) on MM pectin (15 mg ml^{-1}) with (squares) or without (circles) 5 mmol l^{-1} calcium.

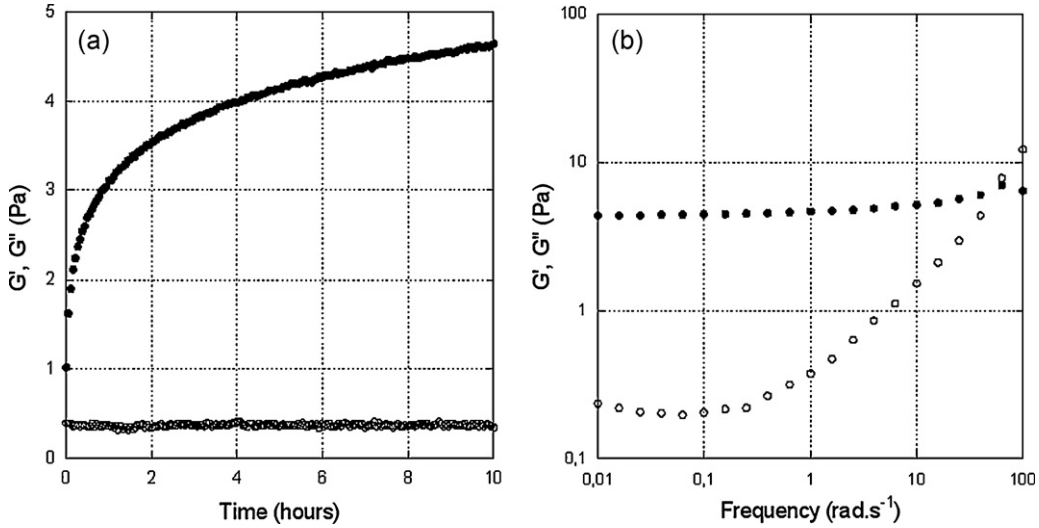

Fig. 3. Kinetics (a) and mechanical spectra (b) for MM pectin (15 mg ml^{-1}) in the presence of 5 mmol l^{-1} calcium.

the mixture on the rheometer plate, PME was added. The evolution of rheological parameters was followed by measuring G' and G'' as a function of time at 1 rad s^{-1}.

In the absence of PME, the storage modulus G' of MM pectin gel increased slowly with time until a value of 4.6 Pa (Fig. 3a). Then the mechanical spectra displayed the typical characteristics of a weak physical gel and exhibited a storage modulus of 4.6 Pa at 0.01 rad s^{-1} (Fig. 3b). G' was independent of frequency at least at low frequency and was over G''.

In the presence of both O-PME and calcium, G' increased rapidly as a function of time. Gelation kinetics was characteristic of those of LM pectin (Fig. 4a). After 48 h, the equilibrium was reached with a modulus of 3078 Pa. Then, the mechanical spectra exhibited a G' nearly independent of frequency on all frequency range and largely over G''. These parameters characterised a strong gel behaviour with a G' of 2964 Pa at 0.01 rad s^{-1} (Fig. 4b).

In the presence of calcium, Aa-PME showed a different behaviour. Its action on pectin led to a slow gelation kinetics (Fig. 5a). G' increased slowly, in the first 10 h and the slope increased afterwards. Then, the equilibrium took a long time to be reached. After 48 h, the mechanical spectrum characterised a strong gel with a storage modulus of 2762 Pa at 0.01 rad s^{-1} (Fig. 5b).

The rheological characterisation of these mixtures showed differences in the kinetics of action of the two enzymes: after 24 h, Aa-PME-formed gel exhibited a G' of 1559 Pa whereas O-PME-formed gel reached a G' of 2699 Pa. On the contrary after 48 h, both systems behaved as strong gels with rather similar final moduli, 2929 Pa for Aa-PME and 3049 Pa for O-PME.

However, the relative importance of viscous and elastic contributions to gel behaviour was slightly different between the two systems, but very different from the one obtained without enzymes. This was indicated by calculating the ratio of G'' over G', named the loss tangent ($\tan\delta$). A higher viscous component was observed in the Aa-PME gel ($\tan\delta = 0.02$ at 0.01 rad s^{-1}) than in the O-PME gel ($\tan\delta = 0.01$ at 0.01 rad s^{-1}). The gel formed after 24 h in the presence of O-PME was more elastic, where as the DM and DBabs were higher than the one obtained with Aa-PME.

4. Discussion

4.1. Influence of calcium on PME activity

In our experimental conditions, an increase of specific activity was observed for both enzymes in the presence of $CaCl_2$. The increase is well marked for Aa-PME and slighter for O-PME.

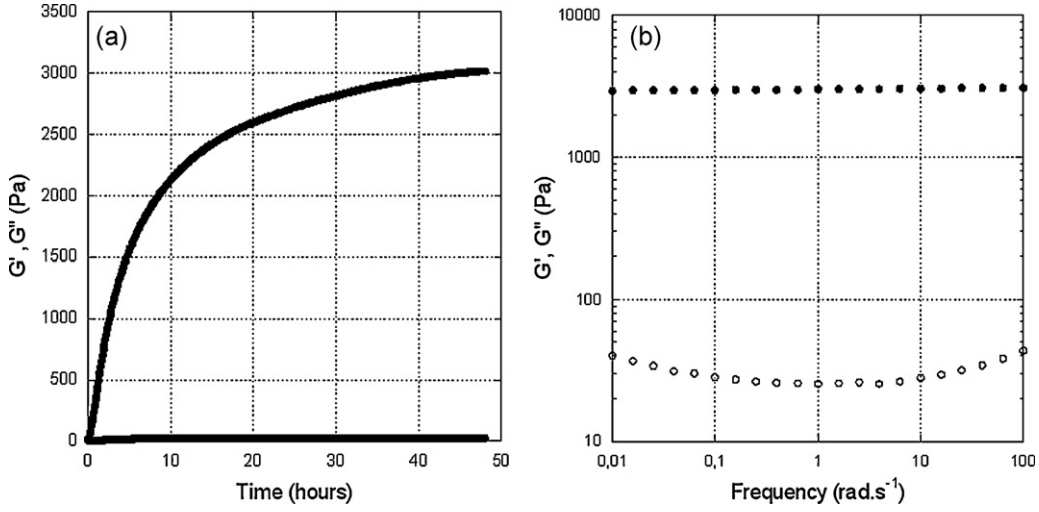

Fig. 4. Kinetics (a) and mechanical spectra (b) for MM pectin (15 mg ml^{-1}) incubated with O-PME (0.4 nkat ml^{-1}) in the presence of 5 mmol L^{-1} calcium.

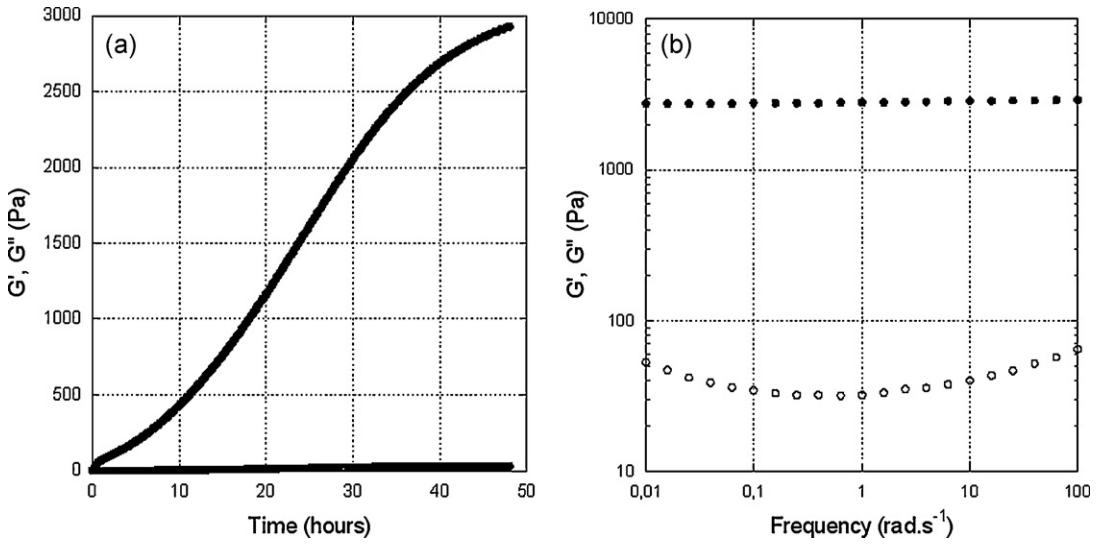

Fig. 5. Kinetics (a) and mechanical spectra (b) for MM pectin (15 mg ml^{-1}) incubated with Aa-PME (0.4 nkat ml^{-1}) in the presence of 5 mmol l^{-1} calcium.

The activity of plant and fungal PME is known to be enhanced by metal ions. Some plant PMEs referred to as "salt dependant" are unable to hydrolyse methylester in the absence of salt (Cameron et al., 2003). The commercial PME from orange peel has been classified as a thermolabile salt independent (type I) PME (Savary et al., 2002). However, in the presence of NaCl (1.2% w/v), the optimum pH shifted to a neutral range (about 6) instead of 9 in the absence of NaCl (Savary et al., 2002). Moreover, low concentration of calcium ions increases the rate of demethylation of O-PME (Charnay, Nari, & Noat, 1992). Experiments in the presence of methylene blue have been performed to investigate this activation on a PME from soybean (Nari, Noat, & Ricard, 1991). The methylene blue can mimic the interaction between calcium ions and free galacturonic acid. In the range of calcium ion concentration where an enhancement of the de-esterification is observed, methylene blue has the same effect, suggesting that the interaction between ions and pectin is predominant to favour the activity. Moreover, it has been shown that polygalacturonic acid behaves as a competitive inhibitor of the reaction by trapping PME on blocks of free carboxyl groups (Fayyaz, Asbi, Ghazali, Man, & Jinap, 1995; Maldonado, Strasser de Saad, & Callieri, 1994; Nari et al., 1991). The activation observed in the presence of cations may be due to the interactions between free galacturonic acids and cations that can release the PME from free galacturonic acid. However, at high concentration, calcium ions act as a competitive inhibitor, decreasing the enzyme affinity without changing the catalysis rate (Charnay et al., 1992). Indeed, a free carboxylate group adjacent to the ester bond is required to initiate plant PME activity. Massiot, Perron, Baron, and Drilleau (1997) reported that O-PME could not act on totally methylesterified apple pectin. So at high calcium concentration, these required free carboxylate groups could be masked by calcium ions that inhibit enzyme reaction.

This inhibition may also exist for Aa-PME because the presence of one or two free galacturonic acids is preferred at the active-site of *A. niger* PME (van Alebeek, van Scherpenzeel, Beldman, Schols, & Voragen, 2003). Indeed, *A. niger* PME is highly similar to Aa-PME, so their behaviour may be close (Christgau et al., 1996). However, the possible action of *A. niger* PME on totally methylesterified galaturonic acid zone from apple pectins (Massiot, Perron, Baron, & Drilleau, 1997) show that a free galacturonic acid is not required for the enzyme to bind to its substrate. An increase of 150% of Aa-PME activity on HM pectin was already observed by Christgau et al. (1996) in the presence of 10 mmol l^{-1} of calcium.

Then, in our system, an interaction divalent cations – pectin might enhance the activity of Aa-PME and O-PME by screening negative charges from galacturonic acid. But this theory alone does not explain the role of cations on PME activity. If divalent cations enhance activity only by screening negative charges, the same activation should have been observed with sufficient amount of monovalent cations (Leiting & Wicker, 1997).

4.2. Modification in the demethylation pattern

By comparing the pattern of demethylation of pectin de-esterified in the presence or not of calcium, we observed that O-PME generates smaller blocks on MM pectin with than without calcium.

A reduction in the length of blocks generated by O-PME action in the presence of calcium was already observed by capillary electrophoresis on HM pectin (Vincent et al., 2009). The same authors have linked these results to the microstructure of pectin gels investigated by microrheology (Vincent & Williams, 2009). Two kinds of gels have been obtained, one network done of semi-flexible polymers, the other one composed of flexible polymers. The flexible one is obtained by de-esterification of HM pectin by a plant PME in the presence of calcium whereas the semi-flexible one is obtained by calcium addition after enzymatic de-esterification with the same enzyme. The length of the generated blocks was short in the flexible network. Each binding calcium pectin zone had only the minimum length for ensuring stability at a pre-requisite temperature (Vincent & Williams, 2009). From the results, it can be hypothesized that the presence of calcium ions could generate a steric hindrance for the interaction between PME and pectin. A junction zone is formed each time enough contiguous free galacturonic acids are liberated by O-PME's action. These junction zones can create steric hindrance, and trigger O-PME uncoupling. This theory concords with the decrease of O-PME affinity for pectins in presence of calcium reported here and the competitive inhibitor role of calcium reported by Charnay et al. (1992).

The decrease of the block length has also been observed when O-PME acts in an acidic medium suggesting a modification in the O-PME mode of action according to pH (Cameron, Luzio, Goodner, & Williams, 2008). This observation has also been reported on apple PME (Denès et al., 2000).

In the present study, the chosen pH is 6. pH value seems to have no effect on Aa-PME processivity as no differences have been found between the demethoxylation pattern at pH 4.5 and 8 (Duvetter

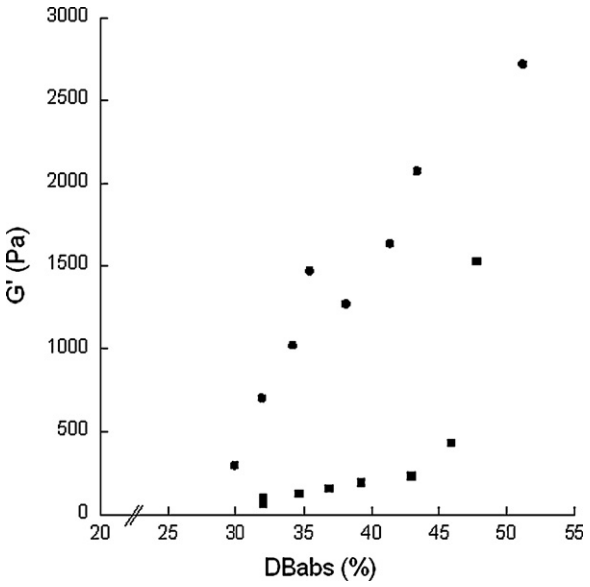

Fig. 6. The storage modulus (G') as a function of DBabs for the kinetics of Aa-PME (squares) and O-PME (circles) in the presence of 5 mmol l^{-1} calcium.

et al., 2006). On the opposite, it could lead to an intermediate degree of processivity for O-PME.

Aa-PME led to longer blocks on HM pectin in the presence than in the absence of calcium at pH 6 (Slavov et al., 2009). Indeed, Aa-PME mode of action is giving rise to short blocks of de-esterified residues distributed all over the chain (Fraeye et al., 2007). The block size can be increased by several successive binding as suggested for plant PME (Vincent & Williams, 2009) or by inducing several catalytic events along the chain. Thus, this hypothesis agrees with the increase of Aa-PME affinity for pectin in the presence of calcium.

The influence of calcium on Aa-PME demethylation pattern was not observed on MM pectin. This difference might be due to the lower DM of the pectin used in the present work. The appearance of longer blocks depends on the initial degree of methylation. Christensen, Nielsen, Kreiberg, Rasmussen, and Mikkelsen (1998) measured the K_m of an orange PME (*Citrus sinensis* Navelina) in the presence of pectin with different DMs (Christensen et al., 1998). They reported an increase in the affinity concomitantly with an increase of DM. Here, the MM pectin used has a relatively low DM, which allows a weak previous gelation in the presence of calcium. It means that pectin with a DM of 46 already have blocks of free galacturonic acid long enough to allow junction formation thanks to calcium ionic linkages, as shown by the initial value of DBabs of 16%. The probability of having contiguous free galacturonic acid is higher with DM 46% than with DM 71%. Moreover, in the presence of calcium, part of the starting points for PME demethylation might be unavailable for the enzyme as they are involved in ionic interactions with calcium. In the case of HM pectin, less galacturonic acid are involved in such interaction and thus available for enzyme action. Hence, the modification of PME behaviour might be a consequence of several factors: a modification of the electrostatic potential, a steric hindrance provoked by the formation of junction zone and destabilizing the PME, less accessibility to the starting sites required for demethylation.

4.3. Relationship between gel properties and pectin structures

The systems formed by Aa-PME or O-PME in the presence of MM pectin and calcium exhibited differences in their evolution into gels. Rheological measurements showed differences in the gel strength after 24 h. O-PME-formed gel exhibited a G' value 1.7 fold higher than Aa-PME's one whereas the pectin DM is higher than the one obtained for pectin after action of Aa-PME. This phenomenon, already observed with HM pectin (Slavov et al., 2009) can be explained by the differences in DBabs values. Indeed, DBabs seems to better correlate with elastic modulus than the DM does (Fraeye et al., 2010a; Ström et al., 2007). For 24 h of demethylation, the DBabs reached with O-PME is higher than the one obtained with Aa-PME. The delayed gelation kinetics is observed when Aa-PME acts on MM pectin in the presence of calcium and not with O-PME. Even with this delayed gelation, rearrangements occur in the network with Aa-PME and lead to close final characteristics of the gel. Moreover, a linear relation exists between DBabs and the gel strength when O-PME acts on MM pectin in the presence of calcium (Fig. 6). On the contrary, in the presence of Aa-PME, this relation can be divided in two parts: a linear relation at the beginning of the kinetics with a different slope than the one obtained with O-PME, and another part where the values tend to join O-PME's ones. These observations suggest the appearance of a change in Aa-PME kinetics, which can modify the evolution of the gel strength.

5. Conclusion

O-PME in the presence of calcium exhibited less processivity on MM pectins than on HM pectin. On the opposite, the effect of calcium on the pattern of demethylation of MM pectin with Aa-PME was reduced. The presence of calcium modulates O-PME activity on MM pectins but may be not the only origin for the modifications observed in PME's behaviour. As already suggested, gel formation can generate physical constraint for PME influencing its diffusion and inducing changes in its activity. The influence of the environment state on the diffusion of PME will be analysed to investigate this issue.

Acknowledgments

We thank Marie-Jeanne Crépeau and Sylvie Durand for skilful technical assistance and Bernard Quémener for all HPAEC experiments.

References

Anthon, G. E., & Barrett, D. M. (2004). Comparison of three colorimetric reagents in the determination of methanol with alcohol oxidase. Application to the assay of pectin methylesterase. *Journal of Agricultural and Food Chemistry*, *52*(12), 3749–3753.

Arltoft, D., Madsen, F., & Ipsen, R. (2008). Relating the microstructure of pectin and carrageenan in dairy desserts to rheological and sensory characteristics. *Food Hydrocolloids*, *22*(4), 660–673.

Cameron, R. G., Luzio, G. A., Goodner, K., & Williams, M. A. K. (2008). Demethylation of a model homogalacturonan with a salt-independent pectin methylesterase from citrus. I. Effect of pH on demethylated block size, block number and enzyme mode of action. *Carbohydrate Polymers*, *71*(2), 287–299.

Cameron, R. G., Savary, B. J., Hotchkiss, A. T., Fishman, M. L., Chau, H. K., Baker, R. A., et al. (2003). Separation and characterization of a salt-dependent pectin methylesterase from *Citrus sinensis* Var. Valencia fruit tissue. *Journal of Agricultural and Food Chemistry*, *51*(7), 2070–2075.

Catoire, L., Pierron, M., Morvan, C., Hervé du Penhoat, C., & Goldberg, R. (1998). Investigation of the action patterns of pectinmethylesterase isoforms through kinetic analyses and NMR spectroscopy. *Journal of Biological Chemistry*, *273*(50), 33150–33156.

Charnay, D., Nari, J., & Noat, G. (1992). Regulation of plant cell-wall pectin methyl esterase by polyamines "Interactions with the effects of metal ions". *European Journal of Biochemistry*, *205*(2), 711–714.

Christensen, T. M. I. E., Nielsen, J. E., Kreiberg, J. D., Rasmussen, P., & Mikkelsen, D., Jr. (1998). Pectin methyl esterase from orange fruit: Characterization and localization by in-situ hybridization and immunohistochemistry. *Planta*, *206*(4), 493–503.

Christgau, S., Kofod, L. V., Halkier, T., Andersen, L. N., Dörreich, K., Hockauf, M., et al. (1996). Pectin methyl esterase from *Aspergillus aculeatus*: Expression cloning in yeast and characterisation of the recombinant enzyme. *Biochemical Journal*, *319*, 705–712.

Denès, J.-M., Baron, A., Renard, C. M. G. C., Péan, C., & Drilleau, J.-F. (2000). Different action patterns for apple pectin methylesterase at pH 7.0 and 4.5. *Carbohydrate Research*, *327*(4), 385–393.

Duvetter, T., Fraeye, I., Sila, D. N., Verlent, I., Smout, C., Hendrickx, M., et al. (2006). Mode of de-esterification of alkaline and acidic pectin methyl esterases at different pH conditions. *Journal of Agricultural and Food Chemistry*, *54*(20), 7825–7831.

Fayyaz, A., Asbi, B. A., Ghazali, H. M., Man, Y. B. C., & Jinap, S. (1995). Kinetics of papaya pectinesterase. *Food Chemistry*, *53*(2), 129–135.

Fraeye, I., Colle, I., Vandevenne, E., Duvetter, T., Van Buggenhout, S., Moldenaers, P., et al. (2010). Influence of pectin structure on texture of pectin–calcium gels. *Innovative Food Science & Emerging Technologies*, *11*(2), 401–409.

Fraeye, I., Doungla, E., Duvetter, T., Moldenaers, P., Van Loey, A., & Hendrickx, M. (2009). Influence of intrinsic and extrinsic factors on rheology of pectin–calcium gels. *Food Hydrocolloids*, *23*(8), 2069–2077.

Fraeye, I., Duvetter, T., Doungla, E., Van Loey, A., & Hendrickx, M. (2010). Fine-tuning the properties of pectin–calcium gels by control of pectin fine structure, gel composition and environmental conditions. *Trends in Food Science & Technology*, *21*(5), 219–228.

Fraeye, I., Duvetter, T., Verlent, I., Ndaka Sila, D., Hendrickx, M., & Van Loey, A. (2007). Comparison of enzymatic de-esterification of strawberry and apple pectin at elevated pressure by fungal pectinmethylesterase. *Innovative Food Science & Emerging Technologies*, *8*(1), 93–101.

Fries, M., Ihrig, J., Brocklehurst, K., Shevchik, V. E., & Pickersgill, R. W. (2007). Molecular basis of the activity of the phytopathogen pectin methylesterase. *The EMBO Journal*, *26*(17), 3879–3887.

Guillotin, S. E., Bakx, E. J., Boulenguer, P., Mazoyer, J., Schols, H. A., & Voragen, A. G. J. (2005). Populations having different GalA blocks characteristics are present in commercial pectins which are chemically similar but have different functionalities. *Carbohydrate Polymers*, *60*(3), 391–398.

Hotchkiss, A. T., Savary, B. J., Cameron, R. G., Chau, H. K., Brouillette, J., Luzio, G. A., et al. (2002). Enzymatic modification of pectin to increase its calcium sensitivity while preserving its molecular weight. *Journal of Agricultural and Food Chemistry*, *50*(10), 2931–2937.

Hou, W. N., Jeong, Y., Walker, B. L., Wei, C.-I., & Marshall, M. R. (1997). Isolation and characterization of pectinesterase from Valencia Orange. *Journal of Food Biochemistry*, *21*(3), 309–333.

Jensen, S., Rolin, C., & Ipsen, R. (2010). Stabilisation of acidified skimmed milk with HM pectin. *Food Hydrocolloids*, *24*(4), 291–299.

Leiting, V. A., & Wicker, L. (1997). Inorganic cations and polyamines moderate pectinesterase activity. *Journal of Food Science*, *62*(2), 253–255.

Limberg, G., Körner, R., Buchholt, H. C., Christensen, T. M. I. E., Roepstorff, P., & Mikkelsen, J. D. (2000). Analysis of different de-esterification mechanisms for pectin by enzymatic fingerprinting using endopectin lyase and endopolygalacturonase II from *A. niger*. *Carbohydrate Research*, *327*(3), 293–307.

Lionetti, V., Raiola, A., Camardella, L., Giovane, A., Obel, N., Pauly, M., et al. (2007). Overexpression of pectin methylesterase inhibitors in Arabidopsis restricts fungal infection by botrytis cinerea. *Plant Physiology*, *143*(4), 1871–1880.

Luzio, G. A., & Cameron, R. G. (2008). Demethylation of a model homogalacturonan with the salt-independent pectin methylesterase from citrus. Part II. Structure–function analysis. *Carbohydrate Polymers*, *71*(2), 300–309.

Maldonado, M. C., Strasser de Saad, A. M., & Callieri, D. (1994). Purification and characterization of pectin esterase produced by a strain of *Aspergillus niger*. *Current Microbiology*, *28*(4), 193–196.

Massiot, P., Perron, V., Baron, A., & Drilleau, J.-F. (1997). Release of methanol and depolymerization of highly methyl esterified apple pectin with an endopolygalacturonase from *Aspergillus niger* and pectin methylesterases from *A. niger* or from orange. *Lebensmittel-Wissenschaft und-Technologie*, *30*(7), 697–702.

Messiaen, J., & Van Cutsem, P. (1994). Pectic signal transduction in carrot cells: Membrane, cytosolic and nuclear responses induced by oligogalacturonides. *Plant and Cell Physiology*, *35*(4), 677–689.

Min, B., Bae, I. Y., Lee, H. G., Yoo, S.-H., & Lee, S. (2010). Utilization of pectin-enriched materials from apple pomace as a fat replacer in a model food system. *Bioresource Technology*, *101*(14), 5414–5418.

Nari, J., Noat, G., & Ricard, J. (1991). Pectin methylesterase, metal ions and plant cell-wall extension. Hydrolysis of pectin by plant cell wall pectin methylesterase. *Biochemical Journal*, *279*, 343–350.

O'Brien, A. B., Philp, K., & Morris, E. R. (2009). Gelation of high-methoxy pectin by enzymic de-esterification in the presence of calcium ions: A preliminary evaluation. *Carbohydrate Research*,

Portenoy, R. K., Burton, A. W., Gabrail, N., & Taylor, D. (2010). A multicenter, placebo-controlled, double-blind, multiple-crossover study of fentanyl pectin nasal spray (FPNS) in the treatment of breakthrough cancer pain. *Pain*, *151*(3), 617–624.

Ridley, B. L., O'Neill, M. A., & Mohnen, D. (2001). Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry*, *57*(6), 929–967.

Sakamoto, T., Bonnin, E., Quemener, B., & Thibault, J.-F. (2002). Purification and characterisation of two exo-polygalacturonases from *Aspergillus niger* able to degrade xylogalacturonan and acetylated homogalacturonan. *Biochimica et Biophysica Acta (BBA) – General Subjects*, *1572*(1), 10–18.

Savary, B. J., Hotchkiss, A. T., & Cameron, R. G. (2002). Characterization of a salt-independent pectin methylesterase purified from Valencia orange peel. *Journal of Agricultural and Food Chemistry*, *50*(12), 3553–3558.

Slavov, A., Garnier, C., Crépeau, M.-J., Durand, S., Thibault, J.-F., & Bonnin, E. (2009). Gelation of high methoxy pectin in the presence of pectin methylesterases and calcium. *Carbohydrate Polymers*, *77*(4), 876–884.

Ström, A., Ribelles, P., Lundin, L., Norton, I., Morris, E. R., & Williams, M. A. K. (2007). Influence of pectin fine structure on the mechanical properties of calcium–pectin and acid–pectin gels. *Biomacromolecules*, *8*(9), 2668–2674.

Thibault, J.-F. (1979). Automatisation du dosage des substances pectiques par la méthode au méta-hydroxydiphenyl. *Lebensmittel-Wissenschaft und-Technologie*, *12*, 247–251.

van Alebeek, G.-J. W. M., van Scherpenzeel, K., Beldman, G., Schols, H. A., & Voragen, A. G. J. (2003). Partially esterified oligogalacturonides are the preferred substrates for pectin methylesterase of *Aspergillus niger*. *Biochemical Journal*, *372*(1), 211–218.

Versteeg, C., Rombouts, F. M., Spaansen, C. H., & Pilnik, W. (1980). Thermostability and orange juice cloud destabilizing properties of multiple pectinesterases from orange. *Journal of Food Science*, *45*(4), 969–971.

Vincent, R., Cucheval, A., Hemar, Y., & Williams, M. (2009). Bio-inspired network optimization in soft materials: Insights from the plant cell wall. *The European Physical Journal E: Soft Matter and Biological Physics*, 79–87. Springer Berlin/Heidelberg.

Vincent, R. R., & Williams, M. A. K. (2009). Microrheological investigations give insights into the microstructure and functionality of pectin gels. *Carbohydrate Research*, *344*(14), 1863–1871.

Voragen, A., Coenen, G.-J., Verhoef, R., & Schols, H. (2009). Pectin, a versatile polysaccharide present in plant cell walls. *Structural Chemistry*, *20*(2), 263–275.

Willats, W. G. T., McCartney, L., Mackie, W., & Knox, J. P. (2001). Pectin: cell biology and prospects for functional analysis. *Plant Molecular Biology*, *47*(1), 9–27.

Wolf, S., Mouille, G., & Pelloux, J. (2009). Homogalacturonan methyl-esterification and plant development. *Molecular Plant*, *2*(5), 851–860.