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Gelation of high methoxy pectin in the presence of pectin methylesterases and calcium

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ABSTRACT

High methoxy pectin was submitted to various amounts of a fungal pectin methylesterase (PME) from *Aspergillus aculeatus* and of a plant PME from orange in the presence of calcium. The systems were characterized by rheological means during the gelation process. By the way of *in situ* demethoxylation with low amount of orange PME, it was possible to gel pectin from the beginning of the reaction although its high degree of methylation around 70. To understand this unusual properties, the behaviour of the two enzymes was investigated in pectic gels and in solution through the analysis of content and distribution of the remaining methyl esters. In the gel, the degree of methylation decreased slowly with orange PME and rapidly with *Aspergillus* PME. The degree of methylation and degree of blockiness after treatment with each PME in solution or in gels were slightly different. Possible explanations for this are evolving visco-elastic properties, including gel formation or influence of calcium on the enzyme–substrate complex.

1. Introduction

Pectin is an acidic heteropolysaccharide frequently found in primary plant cell walls and having main application as gelling agent in food industry (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). Despite its structural complexity and diversity, three major building subunits are generally recognized – homogalacturonan, rhamnogalacturonan I (RG I) and rhamnogalacturonan II (RG II). Homogalacturonan consists of a long linear "smooth" domain of α-(1 → 4)-galacturonic acid (GalA). RG I has a backbone built by repeating disaccharide unit [→4)-α-D-Gal*p*A-(1 → 2)-α-L-Rha*p*-(1→] bearing neutral sugars side-chains (arabinan, (arabino)galactan) linked to the rhamnose residues. RG II has a backbone similar to homogalacturonan and carries very complex side chains. The carboxyl groups of the GalA units are usually methylesterified and the molar ratio of methoxyl groups per 100 GalA is defined as the degree of methoxylation. This allows to define Low Methoxy (LM) pectin and High Methoxy (HM) pectin when the DM is lower and higher than 50, respectively. Thus, DM is an important parameter influencing the process and mechanism of association of pectins.

One of the main characteristic of LM pectin is certainly its capacity to bind calcium ions and to be cross-linked through these ions when consecutive free carboxyl groups are present in sufficient number. These properties have been extensively studied and the "egg-box" model has been proposed to describe a typical structure associating pectin molecules and calcium ions, the chain forming a series of electronically negative cavities into which the calcium ions can fit (Voragen et al., 1995).

Such calcium-mediated structures have been claimed to be present in the cell walls, leading to a pectin network. The extraction of pectins by calcium chelating agents (Selvendran & O'Neill, 1987) as well as the recognition of such structures by monoclonal antibodies (Liners, Thibault, & Van Cutsem, 1992) are often presented as proofs for this hypothesis, although the pectins present in these extracts are generally highly methylated and sometimes acetylated, ruling out the probability of long free GalA sequences (Renard & Thibault, 1993). The distribution of the unesterified GalA moiety in the pectin macromolecule is more and more recognized as having a critical role in the calcium associations since it affects the reactivity of pectin with cations (Ralet, Dronnet, Buchholt, & Thibault, 2001). Indeed block wise distribution of free carboxylic groups favors the calcium binding much more than a random one. The use of chemicals and enzymes for methanol removing may lead to different esterification patterns. When studied in solution, it is well known that alkaline de-esterification or fungal pectin methylesterases (PME, E.C. 3.1.1.11) cause random distribution of the free GalA whereas plant PMEs are known to lead to blocky structure. The mode of action of the PMEs and its impact on the

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properties of the de-esterified pectins has been the subject of great number of investigations (Grasdalen, Andersen, & Larsen, 1996; Markovic, Machova, & Slezarik, 1983; Catoire, Pierron, Morvan, Hervé du Penhoat, & Goldberg, 1998; Denès, Baron, Renard, Péan, & Drilleau, 2000; Limberg et al., 2000; van Alebeek, van Scherpenzeel, Beldman, Schols, & Voragen, 2003).

The aim of the present work is the first investigation of the behaviour of plant and fungal PMEs when incubated with HM pectin in the presence of calcium. In these conditions demethylation and gelation can occur simultaneously, allowing to determine how the de-esterification process affects the gelation kinetics and if the gelation affects the mode of action of the enzymes. This work gives information on how the calcium-mediated association of pectins could arise in the cell wall, on the PME behaviour, as well as on the possibility to use PMEs for obtaining food systems with physico-chemical properties evolving with time.

2. Materials and methods

2.1. Materials

The two lime pectins used were kindly provided by Cargill Texturizing Solution (Redon, France). Their composition is presented in Table 1.

The pectin methylesterases used were from orange (*O*–PME, Sigma P5400, L'Isle d'Abbeau, France) and from *Aspergillus aculeatus* (*Aa*–PME, UniProt Q12535, Novozymes A/S, Copenhagen, Denmark). The enzymes were solubilized at 5 mg/ml in 2-[*N*-Morpholino] Ethane–Sulphonic acid (MES) buffer (10 mM, pH 6) and dialyzed overnight at 4 °C against the same buffer. Before enzymatic incubation the enzymes were diluted as necessary with the same buffer. The absence of pectin acetylesterase and polygalacturonase was checked in both enzymes, by the determination of the remaining degree of acetylation and viscosity measurement (Ralet et al., 2005), respectively.

The endo-polygalacturonase (*An*PGII, E.C. 3.2.1.15, UniProt P26214, provided by Novozymes) was from *Aspergillus niger* and was purified in the laboratory as described elsewhere (Sakamoto, Bonnin, & Thibault, 2003).

2.2. Methods

2.2.1. Pectin characterization

The GalA content of the pectins was colorimetrically determined by the automated *m*-hydroxybiphenyl method (Thibault, 1979).

Individual neutral sugars were determined after hydrolysis (2.5 h in 2 M trifluoro-acetic acid, 120 °C), derivatization in alditol acetates and analysis by gas–liquid chromatography on a BP-225 fused-silica capillary column (SGE, Courtaboeuf, France; 25 m × 0.32 mm i.d.) mounted on a Perkin-Elmer autosystem gas chromatograph (Courtaboeuf, France) (Blakeney, Harris, Henry, & Stone, 1983).

DM and degree of acetylation (DAc) were determined by quantification of methanol and acetic acid released by alkaline de-esterification (0.5 M NaOH) for 1 h at 4 °C in presence of $CuSO_4$. For their determination reverse phase HPLC was carried out with a C18 Superspher column (Merck) using 4 mM H_2SO_4 as solvent at a flow rate of 0.7 ml/min at 25 °C (Levigne, Thomas, Ralet, Quéméner, & Thibault, 2002). Isopropanol was used as internal standard and DM and DAc were calculated as the molar ratio of methanol and acetic acid to 100 GalA, respectively.

Protein content determination was performed by the Bradford test using bovine serum albumin (Sigma, A-3912) as a standard (Bradford, 1976).

High-performance size exclusion chromatography (HPSEC) was performed at room temperature on two columns in series (Shodex OH-Pack SB-804 HQ and OH-Pack SB-805 HQ) eluted at 0.7 ml/min with 50 mM sodium nitrate containing 0.02% sodium azide. The column effluent was monitored using a differential refractometer (Erma 7512, Japan), an online multi-angle laser light-scattering (MALLS) detector (Mini Dawn, Wyatt Technology Corp., Santa Barbara, CA). The weight-average molar mass (M_w) was calculated with Astra 1.4 software using a refractive index $dn/dc = 0.146$ g/ml.

Each determination was made in duplicate (sugar content, molar mass) or in triplicate (DM, DAc, protein content).

2.2.2. Incubation with PMEs

The enzymes used in the present work have different pH optima: *O*–PME has its pH optimum at 7 (Kim, Teng, & Wicker, 2005) while *Aa*–PME optimum pH 4.5 (Duvetter et al., 2006). In order to compare their action without changing the conditions, an intermediate pH 6 was chosen for both enzymes.

Pectin were dissolved at 2% in 50 mM MES buffer pH 6 by stirring overnight at 4 °C. Activities of PMEs were colorimetrically determined using *N*-methylbenzothiazolinone-2-hydrazone (MBTH, Sigma M8006-1G) and alcohol oxydase (E.C. 1.1.3.13, Sigma A2404) for oxidation of the released methanol (Anthon & Barrett, 2004). Activity measurements were carried out in triplicate.

Enzymatic de-esterification of pectin was done by the following procedure: 2% pectin and 6 mM $CaCl_2$ solutions in 50 mM MES buffer (pH 6) preheated to 50 °C were mixed slowly volume to volume to reach final solutions of 1% pectin and 3 mM $CaCl_2$. The PME solution at the required activity was added and mixture was incubated at 30 °C for the specified time. Visual gelation of the system was observed by tilting the tubes at regular interval of time and the visual gel point was defined once the mixture did not flow. Enzymatic reaction was stopped by boiling 5 min and equal volume of 2.5% EDTA was added. Mixture was dialyzed 4 h against 2.5% EDTA, then extensively dialyzed against deionized water. The time course of demethoxylation was followed twice. The pH was controlled along the de-esterification and was found to stay around 6 (± 0.1). In the experiments without calcium, the $CaCl_2$ solution was replaced with MES buffer.

The enzymatic fingerprinting using *An*PGII was carried out by the following procedure: pectins were dissolved at 1 mg/ml by stirring overnight at 4 °C in 50 mM acetate buffer pH 4. 0.21 nkat *An*PGII 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. Degradation products were analyzed by HPAEC at pH 13 as described elsewhere (Ralet et al., 2005).

2.2.3. Rheological measurements

To follow the rheological properties of the system during the coupled phenomena of demethylation and calcium interactions, the reaction mixture was prepared as described above and 1.5 ml

Table 1
Initial pectins characteristics: content in sugars and protein, degree of substitution and molar mass.

	GalA (mg/g)	Gal (mg/g)	Rha (mg/g)	Ara (mg/g)	Xyl (mg/g)	Glu (mg/g)	Protein (mg/g)	DM	DAc	M_w (kDa)
P46	818.0	31.9	12.1	18.3	1.2	8.8	38.0	46.0	2.4	252
P71	882.0	83.3	18.7	7.6	2.5	13.6	13.0	71.0	1.4	226

Table 2
Enzyme activities towards P71 and P46 in the presence or in the absence of calcium (nkat/mg powder).

Enzyme/Pectin	Presence of 3 mM Ca	Absence of Ca	Ratio Ca/no Ca
Aa–PME/P71	195.2	167.6	1.16
Aa–PME/P46	99.0	74.7	1.33
O–PME/P71	124.1	67.1	1.85
O–PME/P46	142.3	99.9	1.42

was transferred onto the rheometer plate preheated at 50 °C. A control was realized in the presence of heat-inactivated enzyme to ensure that it does not influence the properties. The properties of a system prepared with pre-deesterified pectin were also studied in the same conditions.

Rheological measurements were performed at 30 °C using a controlled-stress rheometer (AR2000, TA instruments) equipped with a Peltier temperature controller and with a cone-plane device (20 mm diameter, 4° angle, gap between cone and plane 113 μm). The sample was covered with oil to prevent from water evaporation. 0.02% sodium azide was added to prevent from microbial contamination. The visco-elastic properties of pectin gels were characterized by measuring the storage (G′) and loss moduli (G”) during 24 h (or more if necessary) at a frequency of 1 rad/s and at a strain amplitude of 1%, followed by a frequency sweep test at the same deformation rate. It was checked that the strain applied was well within the linearity domain of visco-elasticity. All the rheological measurements were performed in duplicate.

3. Results

3.1. Enzyme activity

The influence of DM and calcium on enzyme activities was determined with P71 and P46 in MES buffer pH 6 (Table 2). With P71 that will be used for further experiments, *Aa*–PME activity was slightly higher than that of *O*–PME. On the contrary, *O*–PME showed the highest activity with P46. Both enzymes showed higher activity in the presence of Ca, which is in accordance with the literature (Christgau et al., 1996; Micheli, 2001). The increase was more pronounced for *O*–PME with both pectins.

Testing the activity on both P71 and P46 shows the effect of the substrate DM on the activity. Thus, *O*–PME showed higher activity on P46 than on P71. This effect was more pronounced in absence of Ca, and could be due to plant PME requirement for a free carboxyl group unit in the neighbourhood of a methoxylated unit to start demethoxylation (Solms & Deuel, 1955). On the contrary *Aa*–PME showed almost two times higher activity on P71 than on P46 in the presence as well as in the absence of Ca. A similar DM dependence effect was already observed with fungal enzyme (Christgau et al., 1996) and could be related to preference of enzyme to substrates offering higher probability to form enzyme–substrate complex, depending on DM and pattern of distribution of free carboxylic groups.

Substrate effect on PME activity was more pronounced in absence of Ca. The presence of Ca ions may affect the enzyme activity by forming ionic complexes with demethylated pectins, thus facilitating either the formation of enzyme–substrate complex or the removing the reaction products.

3.2. Kinetics of demethoxylation

Kinetic of de-esterification of P71 was followed for 24 h in the presence of calcium using the two enzymes at different activities (Fig. 1). Incubation of pectin with *Aa*–PME led to a very fast decrease of DM compared to *O*–PME. After 24 h incubation, the DM was below 40 whatever the *Aa*–PME activity used. A gel was visible only with the highest activities (7.11 and 3.56 nkat) after 165 and 320 min, respectively and was accompanied by a DM lower than 10.

O–PME decreased the DM moderately and the final DM was still above 50 after 24 h incubation with 0.08 and 0.24 nkat. Such values were much higher than expected with respect to the activity added in the system. On the contrary to what happens with *Aa*–PME, visual gelation was observed during *O*–PME treatment even for very low level of activity and after very short time (25 min at 1.19 nkat, 120 min at 0.24 nkat), corresponding to very high DM values (70 and 67, respectively), that are unusual values to form gel in the presence of calcium ions.

To obtain more information about the dynamics of gelation, the system evolution was investigated by time sweep oscillatory measurements and the rheological behaviour was tentatively related to DM changes.

Fig. 2 shows the evolution of storage and loss moduli as a function of demethoxylation time with both PMEs. Although DM decrease of samples treated by *Aa*–PME was fast and final DM values were all under 35, G′ increased slowly and reached equilibrium only for the highest activities of the *Aa*–PME, leading to final G′ values around 800 Pa with 7.11 nkat. For activities of 1.19 and 0.24 nkat, systems continued to evolve and no equilibrium was reached even after 24 h, with very low G′ values around 8 and 4 Pa, respectively. For the lowest activity (0.08 nkat), the system reached equilibrium relatively fast but with very low values of G′ (lower than 1 Pa). All these values are unusually and surprisingly

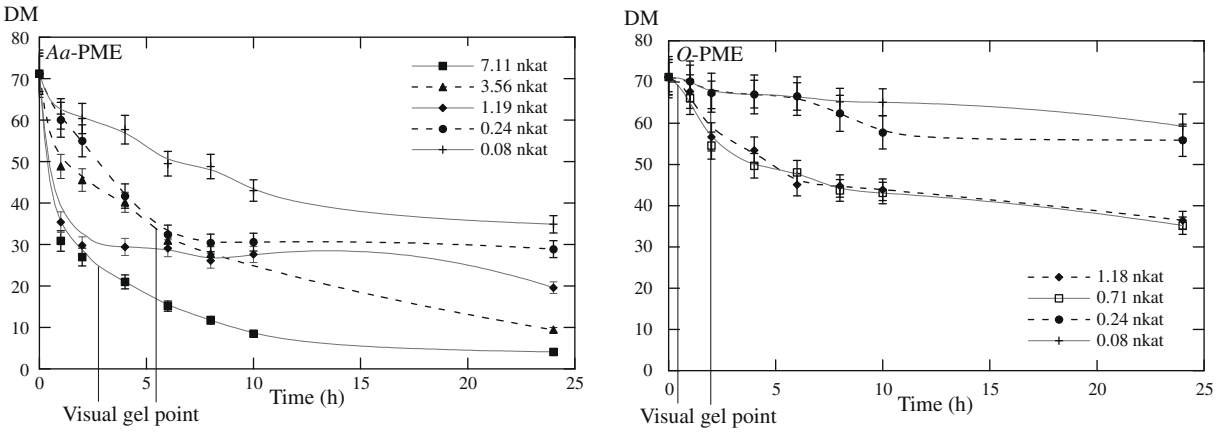

Fig. 1. DM kinetics of P71 with different activities of each enzyme, *Aa*–PME and *O*–PME.

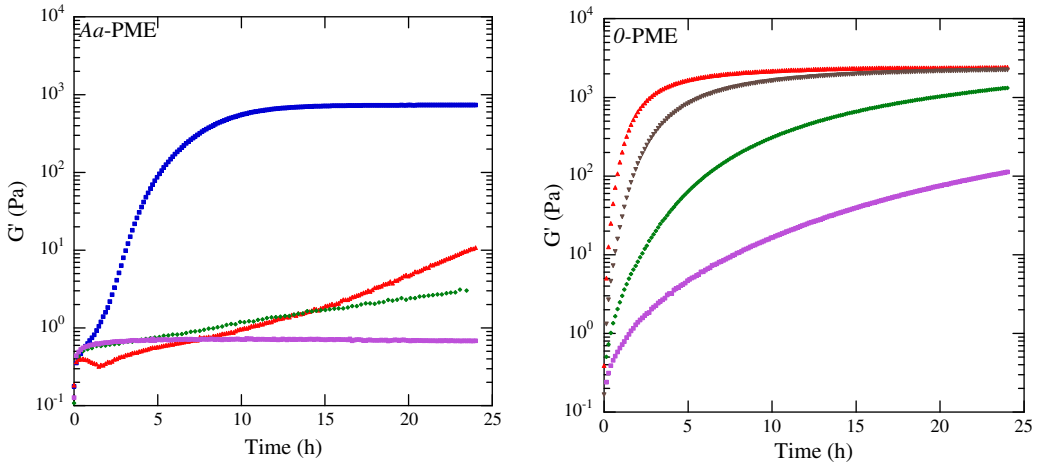

Fig. 2. Gel evolution as a function of time during *in situ* de-esterification of P71 reacting with different activities of each enzyme. Full markers, G′; empty markers, G″; square, 7.11 nkat; triangle upwards, 1.19 nkat; triangle downwards, 0.71 nkat; diamond, 0.24 nkat; circle, 0.08 nkat.

low compared to gel strength obtained with pre-deesterified pectin of similar DM in sodium chloride for example (Donato, Garnier, Novales, Durand, & Doublier, 2005; Ström et al., 2007).

For the *O*–PME de-esterified pectins, the gel evolution was rapid, in contradiction with the slow and moderate de-esterification reported in Fig. 1. Equilibrium was reached after 24 h for the two highest activities used, and the G′ value was very high around 2000 Pa to be related to final DM around 35 in both cases (Fig. 1). For the two lowest activities of *O*–PME, G′ did not reach any plateau, evidencing a continuous organization of the system. In these two last cases, G′ after 24 h were around 1500 and 100 Pa, for 0.24 and 0.08 nkat, respectively, the final DM values being above 50 (56 and 59, respectively, Fig. 1).

In order to investigate the organization of the systems, time sweep tests were followed by frequency sweep tests. Figs. 3 and 4 show the mechanical spectra recorded after 24 h de-esterification *in situ* with *Aa*–PME and *O*–PME, respectively. Systems obtained by *Aa*–PME action exhibited gel behaviour (G′ > G″ and plateau in G′ at low frequency) but the shape of the curves depended strongly on the activity used. At the highest activity used (7.11 nkat), a strong gel was observed with no dependence of G′ with the frequency and a value of G′ around 800 Pa. The gels became very tenuous as the activity decreased, leading to a weak structure for 0.08 nkat (very strong dependence of G′ with the frequency, excepted at low frequency, and G′ at 0.01 rad/s around 0.5 Pa). With *Aa*–PME treatment it was possible to obtain strong gels only with the highest activity and the gelification was a consequence of the large decrease of pectin DM. With all the activities used, gels obtained by *O*–PME demethoxylation showed behaviour of strong gels, although at 0.08 and 0.24 nkat pectins had DM above 50 and the final DMs were around 36 by using 0.71 or 1.18 nkat. These results suggest that the structure of the gels obtained with *O*–PME is determined by the presence of blocks of de-esterified GalA residues appearing during demethoxylation process.

Consequently, they suggest that DM value is not the unique key parameter to investigate the *in situ* de-esterification of the pectin. The different patterns of action of the two PMEs, combined with the different rates of DM decrease, seem to be very important. In the case of *O*–PME, de-esterification proceeds slowly with introduction of blocks of free carboxylic acids, leading to a very strong texturation of the system, while in the case of *Aa*–PME, DM decreased very rapidly, with very low final DM, but the structuration by randomly distributed carboxylated groups was more difficult, suggesting that the structuration proceeded at the very beginning of the demethoxylation, hampering the reorganization of the system.

With pre-deesterified commercial pectins having a DM close to 30, system gelation in presence of the same amount of calcium is easily obtained and equilibrium is reached far before 24 h (Ström et al., 2007). To investigate the possibility of our system to gel at longer time, HM pectin and *Aa*–PME at 0.24 nkat activity were incubated for 24 days (Fig. 5). DM decreased fast to 30 in the very beginning of enzymatic treatment (first day) and a further constant decrease of DM was observed to reach a final value of DM below 10 at the end of the experiment. G′ was very low at the beginning of the kinetics, and increased only after 6 days of incubation, whereas visual gelation was observed after 90 h. A plateau around 540 Pa was reached after 23 days of incubation. These data show that *Aa*–PME can produce strong gels in the experimental conditions but only when DM was lower than 30, which is a low value for LM pectin gelation. The slow evolution of G′ concomitantly with the large decrease of DM confirms again the random mode of action of *Aa*–PME and obtaining of strong gels at the end of incubation time is rather the result of a large DM decrease.

3.3. DM and DB comparison

Confronting data of DM decrease after treatment with both PMEs and rheological properties of the gels obtained as a result of PME action suggested that the distribution pattern of free carboxyl groups may be the key parameter to explain the gel ability. To investigate the pattern of de-esterification, enzymatic fingerprinting of the pectins was performed using endoPG II. Since the preferred substrate of endoPG II is polygalacturonic acid or pectins having long blocks of free GalA, the more blocky structure the pectin has, the more degradation products are obtained.

The amounts of GalA released after endoPG II treatment allow to assign a quantitative measurement of the blocky structure (Daas, Meyer-Hansen, Schols, De Ruiter, & Voragen, 1999). The so-called degree of blockiness (DB) is calculated from the amount of non methyl-esterified monomer (1^0), dimer (2^0), trimer (3^0) released by the enzyme and is expressed as a percentage of the amount of non methyl-esterified GalA present in the pectin. Thus, the DB will be higher for a block wise distribution than for a random distribution. In the presence of PME, the amount of non methyl-esterified GalA is increasing along the incubation, meaning that the reference used for DB calculation is changing. For this reason, it is more appropriate to use the absolute DB (DBabs) for which the amount of free oligomers is expressed as a percentage of total GalA.

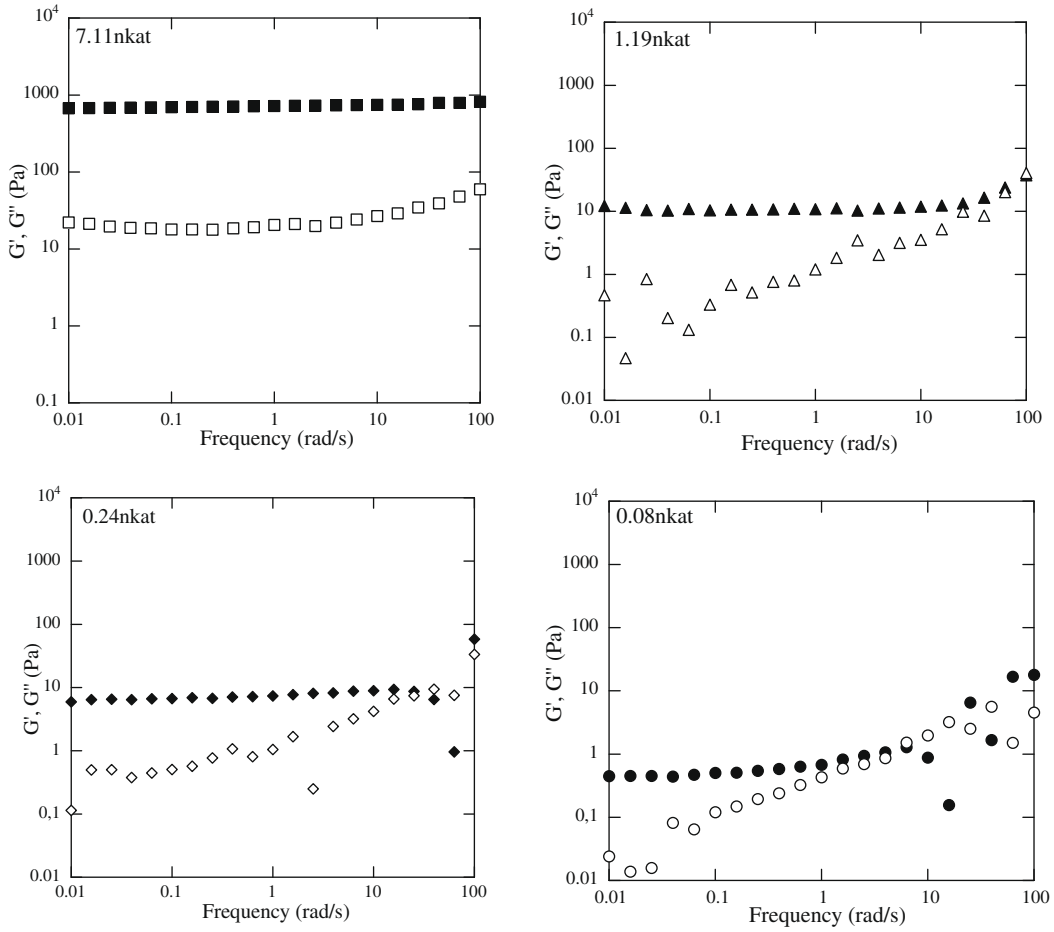

Fig. 3. Mechanical spectra of gels obtained by *in situ* de-esterification of P71 with *Aa*–PME. The amount of activity is indicated in each graph. Full markers, G′; empty markers, G″.

Initial DBabs of P71 was determined to be 3% and the effects of *Aa*–PME and *O*–PME were compared at 0.24 and 1.19 nkat (Table 3). The smallest decrease of DM of pectin treated with *O*–PME resulted in doubling DBabs after 120 min of treatment with 0.24 nkat *O*–PME. After 24 h DBabs was 28.6% when DM was above 55. On the contrary 24 h treatment with the same activity of *Aa*–PME induced a large decrease of DM (down to 29) with a moderate increase of DBabs (18.6%). To easily compare the effect of the two enzymes, 2 h incubation with 0.24 nkat *Aa*–PME induced a DM of 55 and a DBabs of 5.4, whereas *O*–PME induced the same range of DM after 24 h incubation, for a DBabs of 28.6, i.e. a ratio around 5 between the two values. Thus when the same DM was reached with the two enzymes, there were five times more oligomers belonging to the de-esterified domains after *O*–PME treatment than after *Aa*–PME treatment.

The importance of presence of block structures in pectins is highlighted when DM, DBabs and G′ are plotted together as a function of time (Fig. 6). G′ increased as DM decreased and DBabs increased for the two enzymes. The increase of G′ is linked to the increase of DBabs induced by *O*–PME de-esterification, and the steeper rise of DBabs is observed, the sharper is the gel evolution. For the *Aa*–PME a moderate increase of DBabs is observed although the sharp decrease of DM. This resulted in a slowly evolving gel system, which did not reach equilibrium with time. Gel formation using *Aa*–PME occurred when the DM decreased around 20, and in this case, the presence of more blocky structures was a consequence of the low DM. Determination of the visual gelation point of the investigated systems showed that *O*–PME induced gel formation from the beginning of the incubation – after 120 and 20 min of treatment, for 0.24 and 1.19 nkat, respectively. Gelation strongly depended on DBabs increase from 3 (initial value) to 6.1 and 5.2 for 0.24 and 1.19 nkat, respectively, and occurred even for DM values of 67 and 70.

Since *Aa*–PME produced strong gel only at the highest activity (7.11 nkat), it was of interest to follow changes of DBabs in these conditions (Table 4). Results demonstrate that along with the fast decrease of DM, DBabs increased steep rise, and at the visual gel point (165 min), DBabs was already 33. After 24 h the DBabs was almost doubled but this was also accompanied by an almost complete de-esterification.

Taken all together, these results show that both DM and DBabs are involved in the gelation process but none of them is directly related with gel strength.

3.4. Influence of the gelled state on the enzyme behaviour

In order to investigate if the gel structure has an influence on the enzymatic de-esterification, P71 was de-esterified in the same conditions as above but without calcium, and then the pre-deesterified pectin was recovered and allowed to gel by mixing it with calcium. *Aa*–PME decreased pectin DM to the same value with or without Ca (DM = 29, Tables 3 and 5) while DBabs was significantly lower without calcium (18.6 in presence of calcium and 13.6 without calcium). In the opposite, with *O*–PME, the residual DM was 55.8 and DBabs was 28.6 in the presence of Ca (Table 3) whereas DM was only 42.6 and DBabs was 22.2 in the absence of Ca (Table 5). It was observed that Ca largely enhanced *O*–PME action, when the enzyme works in soluble homogeneous medium (Table 2). This

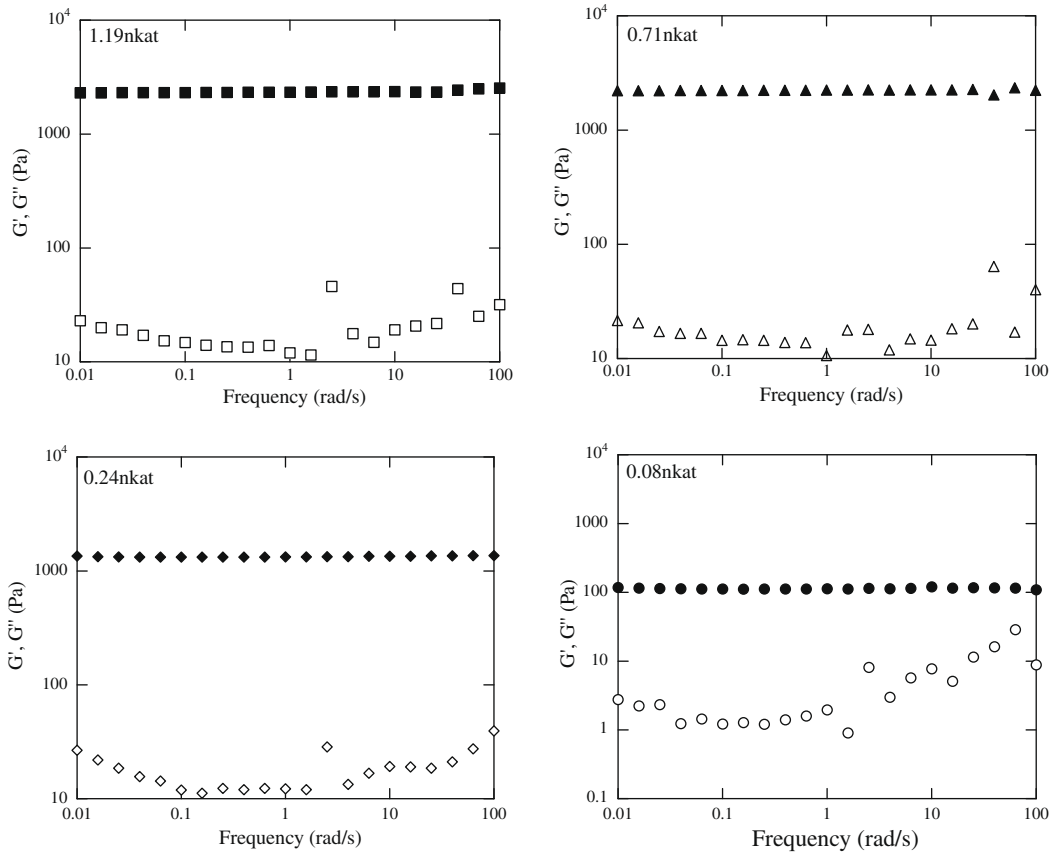

Fig. 4. Mechanical spectra of gels obtained by *in situ* de-esterification of P71 with *O*–PME. The amount of activity is indicated in each graph. Full markers, G′; empty markers, G″.

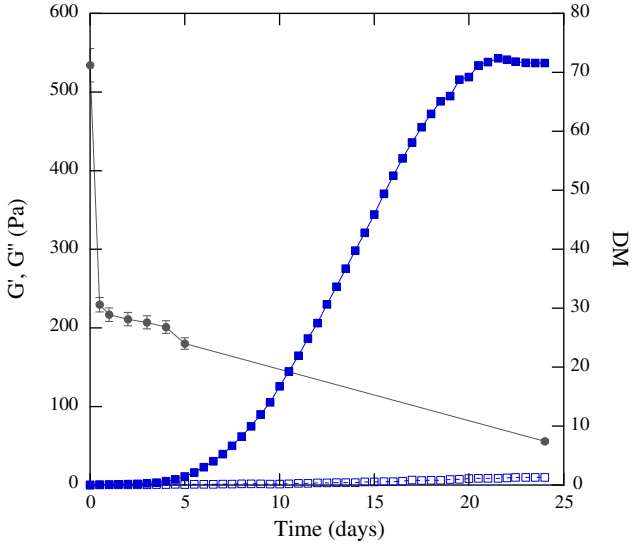

Fig. 5. Incubation of P71 with *Aa*–PME (0.24 nkat) in the presence of Ca for a 24 days incubation time. Full circles, DM; full squares, G′; empty squares, G″.

can suggest that the enzyme is more mobile in solution than in the gel probably because of less constraint, leading to a more rapid turn over.

Visco-elastic properties of Ca–pectin gels obtained by these two different ways were compared (Fig. 7). As already observed, the system using *Aa*–PME *in situ* did not reach equilibrium while gel obtained with *O*–PME *in situ* almost reached the equilibrium after

Table 3
DM and DBabs of P71 de-esterified by different amounts of the two PMEs in the presence of Ca. The initial values of DM and DBabs were 71 and 3.0, respectively. The standard error is 5% for the DM and 8% for the DBabs.

	Time of incubation	DM (%)	DBabs (%)
Aa–PME activity (nkat)			
0.24	120 min	55.0	5.4
0.24	24 h	28.9	18.6
1.19	20 min	59.3	4.4
1.19	24 h	19.6	25.3
O–PME activity (nkat)			
0.24	120 min	67.4	6.1
0.24	24 h	55.8	28.6
1.19	20 min	70.1	5.2
1.19	24 h	36.5	44.6

24 h. On the contrary, gel prepared with pectin pre-deesterified with both PMEs reached the equilibrium very fast. For the pectin pre-deesterified by *O*–PME (DM 42.6, DBabs 22.2), gelation occurred faster and equilibrium was reached after about 1 h, without further changes of the gel features. However, final G′ value was 10 times lower than when the pectin was de-esterified *in situ*, although the DM was also lower, but in agreement with the evolution of the DBabs value (22 for pre-deesterified *vs* 29 after *in situ* de-esterification). For the pectin previously de-esterified by *Aa*–PME (DM 29, DBabs 13.6), gelation occurred also faster but gels evolved slowly during 24 h. In this case, final G′ value was 10 times higher than when the pectin was de-esterified *in situ*, which is in contradiction with the DBabs values (13.6 for pre-deesterified *vs* 18.6 for *in situ* de-esterified pectin), the DM being the same for the two modes of de-esterification. However, it has to be noticed

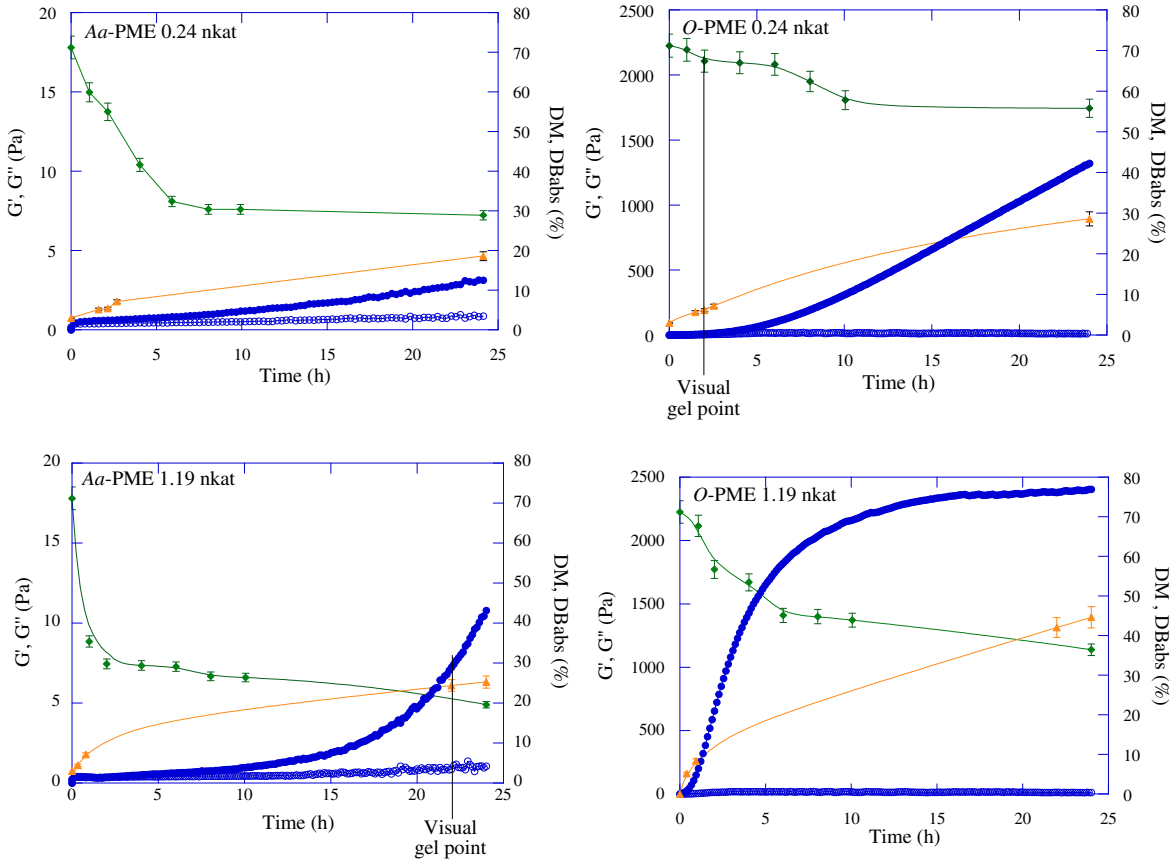

Fig. 6. DM (lozenges), DBabs (triangles) and gel evolution (full circles: G′, empty circles: G″) of systems containing P71 and *Aa*–PME (left hand) and *O*–PME (right hand) at 0.24 nkat (up) and 1.19 nkat (down).

Table 4
DM and DBabs of P71 de-esterified by 7.11 nkat *Aa*–PME in the presence of Ca. The standard error is 5% for the DM and 8% for the DBabs.

Time of incubation	DM (%)	DBabs (%)
0 min	71.0	3.0
135 min	26.0	29.6
165 min	23.0	32.3
195 min	22.0	31.4
24 h	4.1	61.4

Table 5
DM and DBabs of P71 de-esterified by different amounts of the two PMEs in the absence of Ca. The initial values of DM and DBabs were 71 and 3.0, respectively. The standard error is 5% for the DM and 8% for the DBabs.

	Time of incubation	DM (%)	DBabs (%)
Aa–PME activity (nkat)			
0.24	120 min	52.8	4.0
0.24	24 h	28.8	13.6
1.19	20 min	58.3	5.2
1.19	24 h	19.8	16.2
O–PME activity (nkat)			
0.24	120 min	57.7	4.8
0.24	24 h	42.6	22.2
1.19	20 min	65.1	5.6
1.19	24 h	28.0	45.6

that in the case of *in situ* de-esterification, both DM and DBabs were changing all along the reaction time, which allowed the structuration of the medium to be much more progressive than when the pectin was pre-deesterified. Nevertheless, it can be seen in Table 5 that after 2 h reaction with both enzymes at 0.24 nkat, DM and DBabs values were in the same range (DM 52.8 for *Aa*–PME and 57.7 for *O*–PME, DBabs 4 for *Aa*–PME and 4.8 for *O*–PME), although a higher G′ modulus was obtained for *O*–PME pectin gel.

These very different behaviours demonstrate the effect of the medium on the enzyme action and also show the importance of the dynamics of gel formation on the visco-elastic properties of pectin gels.

4. Discussion

De-esterification is an important method for modification of the physico-chemical properties of pectin. The use of enzymatic treatment allows to alter pectin DM by obtaining a more ordered distribution of de-esterified GalA. In solution, plant PMEs follow a processive mode of action (Catoire et al., 1998) whereas fungal PMEs do not (Grasdalen et al., 1996; van Alebeek et al., 2003). However, it was shown that even in solution, the processive mode of action of apple PME can be modulated by pH (Denès et al., 2000).

In the present work, pectin gelation was examined as related to distribution of free galacturonic acid released by mild enzymatic pectin demethoxylation in the presence of calcium, i.e. in the same time as the gelation process. Our investigations showed that the typical behaviour of PME in solution is also observed when the enzymes are acting in a gelled structure. *Aa*–PME action on pectins in the presence of calcium was accompanied with a steep decrease of DM and a moderate increase of block structures of free GalA residues. This resulted in no or weak gel formation and stronger gels could be obtained only if the DM decreases below 20. *O*–PME

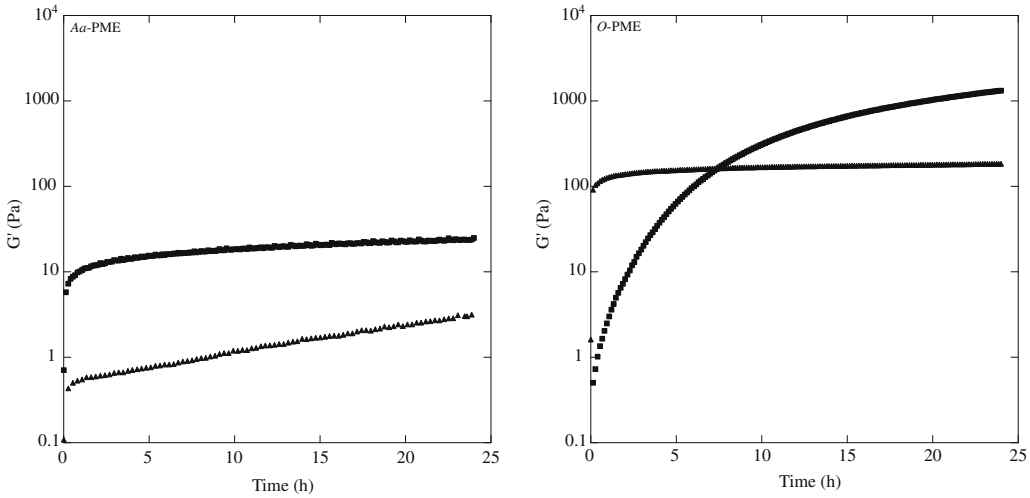

Fig. 7. Comparison of evolution of gels obtained from P71 by *Aa*–PME treatment (left hand) and *O*–PME treatment (right hand) *in situ* (squares) and with pre-deesterified P71 (triangles).

treatment led to a slight decrease of DM and gelation of pectins was visible at DM near 70, this value being usually considered as incompatible with calcium-induced gelation. Strong gels can be obtained relatively fast, even by using low amount of enzyme. To explain these observations, the distribution of methyl groups was quantified by calculating the DBabs and we observed that *O*–PME causes a much larger DBabs increase than *Aa*–PME. This is in agreement with Ström et al. (2007) who suggested that G′ of calcium–pectin gels correlates better with pectin DBabs than with DM, whatever the de-esterification mean. They also showed a DM threshold around 70 for Ca-induced gelation of pectins having a block wise distribution of free GalA. Nevertheless, they proceeded to de-esterification in solution, and rheological measurements were further performed in the presence of calcium. In the present study, the two processes of de-esterification and calcium binding were simultaneous, and the Fig. 7 shows that DM and DBabs values can affect in different ways the visco-elastic properties of pectin gels. Not only these parameters can be seen as governing the gelation properties, the kinetics of formation being totally different in the case of pre- or *in situ* de-esterification. The dynamics of gel formation appear to be the key parameter to be taken into account.

Therefore, the enzymes have different behaviours in solution and in gel. This can be linked either to the presence of calcium or to the physicochemical constraints due to network formation. Indeed by plotting together DM and DBabs (Fig. 8), we demonstrate that calcium enhances processivity of *O*–PME as well as *Aa*–PME, *i.e*. the ability of the enzyme to repetitively continue its catalytic function without dissociating from its substrate. On the other hand, it can be seen with *Aa*–PME that the decreases of DM in the presence or in the absence of calcium are merged whereas the DBabs are not. On the contrary with *O*–PME, both DM decrease and DBabs increase are significantly different in the presence and in the absence of calcium. Presence of di- or trivalent cations has been shown to influence the activity of microbial (Laurent, Kotoujansky, & Bertheau, 2000) as well as plant PME (Schmohl, Pilling, Fisahn, & Horst, 2000). Calcium could affect the enzyme or the substrate, by modifying their conformation or by altering the enzyme–substrate interactions. In the case of *Aa*–PME demethoxylation, numerous short zones of demethoxylation were formed, leading to junction zones of low energy, contributing weakly to the rheological properties of the gel. On the other hand with *O*–PME, long zones of demethoxylated GalA were formed, leading to efficient binding of calcium. Moreover, we have shown by measuring the activity on two different pectins that both enzymes were sensitive

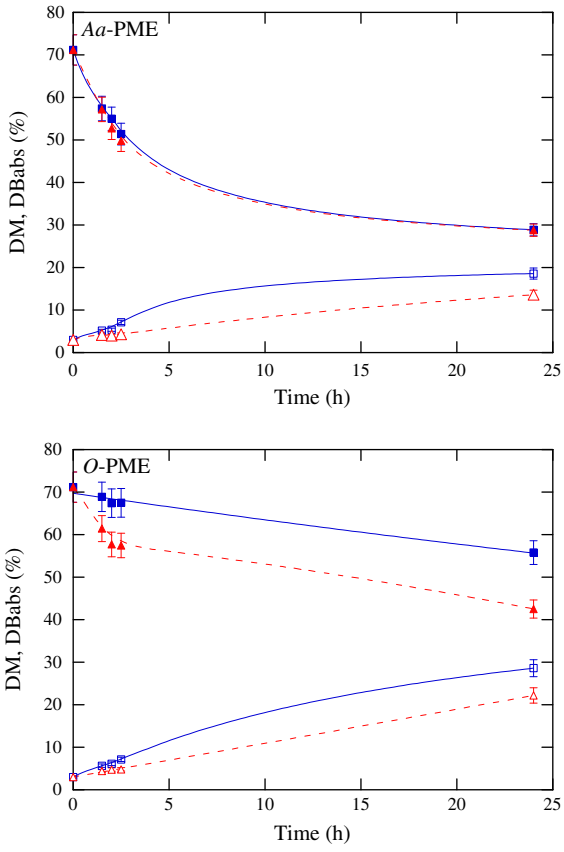

Fig. 8. Comparison of DM (full markers) and DBabs (empty markers) obtained through *in situ* demethylation of P71 with *Aa*–PME or *O*–PME in presence (squares) and absence (triangles) of Ca.

to the DM of their substrate but in opposite sense (Table 2), *O*–PME being more efficient when the substrate is less methyl-esterified. Similarly, both lemon and apple PMEs have their optimal activity for DM 36 (Macdonald & Evans, 1996; Macdonald, Evans, & Spencer, 1993) and their activity decreases when the DM increases. A different behaviour was shown for the three PME isoforms purified from mung bean hypocotyl for which the activity drops down when the DM decreases below 60 (Catoire et al., 1998).

The second hypothesis to explain the discrepancy between PME action in solution and in gel is the construction of the network and the physical constraints that it would induce.

Having in mind the nature of weak and strong gels obtained with *Aa*–PME and *O*–PME and shown by mechanical spectra (Figs. 3 and 4) it can be deduced that strong gel structure inhibits to a certain extent PME activity, as DM decrease is lower than the one observed in solution, even in the presence of calcium. This might be due to a lower diffusion of the enzyme in the gel, even if the pore size in pectin gel is difficult to investigate as the structure of pectin/calcium gel is in dynamic equilibrium with mobile calcium ions leading to a permanent reorganization, as shown by the evolving conservative modulus even after 24 h. Löfgren, Guillotin, Evenbratt, Schols, and Hermansson (2005) followed the kinetics of gel formation using HM pectin in the presence of sucrose. They showed by transmission electron microscopy that in these conditions the gel microstructure was not affected by the distribution of methyl ester in the absence of calcium. But it can be hypothesized that when calcium is added, its distribution will be influenced by the distribution of free carboxyl groups. Since plant PMEs are responsible for de-esterification of pectins *in vivo* (Jarvis & McCann, 2000) it can be speculated that Ca concentration and location are important factors regulating the PME activity and mode of action in the cell wall.

5. Conclusions

For the first time, a PME was incorporated in a pectin/calcium system in the course of gelation. Our results show that the addition of PME and calcium ions to HM pectins may lead to gelation even with DM around 70. The presence of PME during the gel formation allows to delay the equilibrium and thus to propose new stimulable gelation systems. Moreover, the enzyme behaviour observed in this work gives new insights to explain the role of calcium *in muro*.

Our results also show that *O*–PME was more efficient in solution than in a hydrated Ca–pectin solid matrix, and that both enzymes were more processive in Ca–pectin gel than in solution. A more processive de-esterification leads to a faster gel formation and higher gel strength. However, it was not possible to discriminate if these differences were due to the presence of calcium or to the physical state of the medium. The pectin network formation should be considered as a dynamic phenomenon, where calcium creates some specific junction zones with a continuous turn over.

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