



Pectin methylesterases induce an abrupt increase of acidic pectin during strawberry fruit ripening

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Summary

The decrease of strawberry (*Fragaria × ananassa* Duch.) fruit firmness observed during ripening is partly attributed to pectolytic enzymes: polygalacturonases, pectate lyases and pectin methylesterases (PMEs). In this study, PME activity and pectin content and esterification degree were measured in cell walls from ripening fruits. Small green, large green, white, turning, red and over-ripe fruits from the Elsanta cultivar were analyzed. Using the 2F4 antibody directed against the calcium-induced egg box conformation of pectin, we show that calcium-bound acidic pectin was nearly absent from green and white fruits, but increased abruptly at the turning stage, while the total pectin content decreased only slightly as maturation proceeded. Isoelectrofocalisation performed on wall protein extracts revealed the expression of at least six different basic PME isoforms. Maximum PME activity was detected in green fruits and steadily decreased to reach a minimum in senescent fruits. The preliminary role of PMEs and subsequent pectin degradation by pectolytic enzymes is discussed.

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Abbreviations: DA, degree of acetylation; DM, degree of methylesterification; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; IEF, isoelectrofocalisation; PG, polygalacturonase; PAE, pectin acetylerase; *pI*, isoelectric point; PL, pectate lyase; PLL, poly-L-lysine; PME, pectin methylesterase; PMSF, phenylmethylsulphonyl fluoride; PVPP, polyvinylpyrrolidone; Tris, trishydroxymethylamino-methane.

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Introduction

During maturation, the strawberry (*Fragaria × ananassa* Duch.) fruit undergoes softening of its texture and, in some cultivars, the shelf life is strongly limited by the loss of firmness. Plant tissue softening is due to cell wall degradation by hydrolytic enzymes, including pectate lyases (PLs), polygalacturonases (PGs) and pectin methylesterases (PMEs) (Brummell and Harpster, 2001).

The development and ripening of the non-climacteric strawberry fruit is under the control of auxin even if a small level of ethylene is produced and if transcription of ethylene biosynthesis genes and ethylene receptors genes is increased during fruit development (Trainotti et al., 2005). The transcription of new mRNA during maturation of strawberry fruit and its control by auxin have been demonstrated by Manning (1994, 1998) and others, e.g. Civello et al. (1996) and Medina-Escobar et al. (1997, 1998). Among these newly synthesized mRNAs, three PLs (Medina-Escobar et al., 1997; Benítez-Burraco et al., 2003) have been identified, while PGs seem to be absent or weakly expressed during maturation (Nogata et al., 1993; Redondo-Nevado et al., 2001). The silencing of PL slowed down strawberry softening during maturation (Jiménez-Bermúdez et al., 2002; Draye and Van Cutsem, unpublished results), suggesting that PLs could be a determinant factor of strawberry fruit softening.

PMEs are ubiquitous plant and microbial enzymes, and in *Arabidopsis* alone, 79 genes encode pectin esterases, indicating a massive investment of the plant in modifying pectin (The *Arabidopsis* Genome Initiative, 2000). In strawberry, different PME genes have been cloned and characterized (Castillejo et al., 2004). PME enzymes (EC 3.1.1.11.) likely play an important role in strawberry fruit softening by preparing the pectic substrate for the PLs. Indeed, PMEs catalyze the de-esterification of methyl esters of pectin, transforming a neutral polymer in a weakly esterified pectin and then in acidic pectate. This pectin acidification is an obligatory step before further degradation by either PGs or PLs can take place.

In this work, we used a specific monoclonal antibody to study pectin de-esterification by PME during strawberry fruit development, and to investigate whether egg box pectin detected by this antibody could be correlated to any ripening process.

Materials and methods

Plant material

Field-grown strawberry (*Fragaria × ananassa* Duch. Cv Elsanta) fruits were harvested at six different developmental stages: small green, large green, white, turning, red (ripe) and over-ripe (senescent). Pink to red stages were assessed by high-pressure liquid chromatography analysis of anthocyanins extracted following Mori et al. (1993). Fresh samples were ground in liquid nitrogen and 50 mg of powder was extracted over-night at 4 °C under constant shaking with 10 volumes of buffer A (acetic acid/acetonitrile/water; 20:25:55) diluted until 35%

(v/v) in 0.1% trifluoroacetic acid (TFA). Samples were centrifuged twice for 15 min at 15000g at 4 °C, and 20 µL of supernatants were injected on a Nucleosil 100-5 C₁₈ column. The elution was performed with buffer C composed of 25% buffer A and 75% buffer B (water/acetic acid/methanol; 80:15:15) at 40 °C, and the samples were read at 520 nm.

Wall protein extraction

Proteins were extracted from fruits at the six main stages. Unless otherwise stated, all operations were carried out at 4 °C. Fresh samples were crushed in liquid nitrogen and dissolved by vortexing in four volumes of buffer A (76 mM K₂HPO₄; 27 mM KH₂PO₄; 25 mM ethylene diamine tetraacetic acid (EDTA); 1 mM cysteine; 50 g/l polyvinylpolypyrrolidone (PVPP); 1 mM phenylmethylsulphonyl fluoride (PMSF); pH 7.3). The soluble proteins were extracted for 30 min under constant shaking. The supernatants of a 30-min centrifugation at 3000g were discarded and the pellets resuspended by vortexing in four volumes of wall protein extraction buffer B (76 mM K₂HPO₄; 27 mM KH₂PO₄; 25 mM EDTA; 2 M NaCl; 1 mM cysteine; 50 g/L PVPP; 1 mM PMSF; pH 7.3). After 60 min under constant shaking and 30 min centrifugation at 3000g, the supernatants were centrifuged further for 30 min at 14000g. The final supernatants were dialysed over-night against distilled water, concentrated with Millipore ultra-free column and stored at -80 °C.

PME activity testing

PME activities of wall protein samples from the six main ripening stages were assayed. Two micrograms of protein samples were incubated at room temperature in 100 µL of 1% citrus pectin (degree of esterification 90%; Sigma p-9561) containing 0.002% methyl red in trishydroxymethylaminomethane (Tris)/HCl 20 mM, EDTA 5 mM, NaCl 160 mM at pH 7.6. The absorbance spectra were recorded every 5 min for 2 h between 350 and 700 nm (Shimadzu UV-1601PC). Acidic solutions had an absorption peak at 550 nm and alkaline solutions at 410 nm. To quantify PME-induced acidification, the absorption spectra were integrated from 350 nm to the isobestic point at 465 nm (Area A) and from the isobestic point to 550 nm (Area B) for each data point. The Area B to Area A ratios were plotted as a function of time after subtracting control values obtained from samples without protein and ratio values at time zero.

Isoelectrofocalisation (IEF) and zymograms

PME from strawberry fruits were separated by IEF according to their isoelectric points (pI). The six main stages of development were compared. IEF were performed on an LKB 2117 Multiphor II electrophoresis unit using Ampholine PAG plate pH 3.5–9.5 (Amersham 80-1124-80). In all tests, 5 µg of proteins were applied directly on the gel near the cathode. Migration

parameters were: 40 V/cm, 2 mA/cm and 0.6 W/cm. Gels were run for 120 min at 11 °C without pre-focusing.

Following IEF, PME activity was assayed at 25 °C. The IEF gel was first equilibrated for 20 min in Tris/HCl 20 mM, EDTA 5 mM, pH 8.5 buffer and then incubated for 60 min in 1% citrus pectin (degree of esterification 90%; Sigma p-9561) dissolved in Tris/HCl 20 mM, EDTA 5 mM and NaCl 160 mM at pH 7.6. The gel was rinsed in distilled water to remove surface pectin, incubated in 0.05% ruthenium red for 30 min, and de-stained in distilled water until red bands corresponding to PME isoenzymes appeared on a clear background. For *pI* determination, a gel lane was cut in 1 cm² pieces from anode to cathode, the pieces were incubated over-night in 1 ml distilled water, and the pH measured with a microelectrode. The linearity of the pH gradient allowed estimation of the *pI* of the isoforms all along the gel.

Inhibition enzyme-linked immunosorbent assay (ELISA) test

ELISA tests were performed in polystyrene 96-well plates (Nunc-Immuno module Maxisorp F-8). Plates were treated for 60 min with 50 µL/well of 0.05 mg/mL poly-L-lysine (PLL) hydrobromid solution (Sigma P-1399) at room temperature. PLL in excess was washed with 250 µL of T/Ca/Na buffer (Tris-HCl 20 mM, pH 8.2, CaCl₂ 0.5 mM, NaCl 150 mM). About 50 µL of PGA (Sigma P-3889) 1 mg/mL solution was added to each well and incubated over-night at 4 °C. PGA in excess was washed with 250 µL of T/Ca/Na buffer. Non-specific adsorption was blocked by incubating a 250 µL/well of 2% milk powder solution prepared in T/Ca/Na buffer for 120 min at 37 °C. Inhibiting polysaccharides were prepared by chemical de-esterification: 25 mg of crushed fruit were solubilized in 1.25 mL NaOH 50 mM (pH 12.5) and incubated for 30 min at 4 °C under shaking. After incubation, 11.25 mL 1% milk powder in T/Ca/Na were added to de-esterify samples to reduce pH. Native samples were prepared similarly but NaOH was replaced by distilled water. About 12.5 mL of 2F4 antibody culture supernatant diluted 32 × in 1% milk powder in T/Ca/Na was added to both native and de-esterified samples. The samples were incubated for 30 min at room temperature under shaking before centrifuging for 10 min at 7500g at 4 °C. The 50 µL aliquots of the supernatants were dispensed to the ELISA microwells with three replicates. Plates were incubated for 60 min at 37 °C. After eight washes with washing buffer (1% milk powder in T/Ca/Na and 0.1% tween-20), 50 µL of horse radish peroxidase-sheep anti-mouse-labeled immunoglobulin (1:5000 in 1% milk powder in T/Ca/Na) was added to the wells for 60 min at 37 °C. After eight more washes with the washing buffer, 100 µL of enhanced K-Blue TMB substrate (Neogen 308177) was added to each well and the plate was incubated in the dark for 20 min at room temperature. The absorbance was read at 450 nm. The quantification of acidic pectin present in the samples, whether native or chemically de-esterified, was carried out with reference to a calibration curve made of PGA concentrations between 0.002 and 20 µg/mL. Three replicates per

sample were performed, and each experiment was repeated at least five times.

Results

Modulation of PME activity during strawberry development

To investigate the role of PMEs in strawberry fruit softening, we tested both PME activity during fruit development and pectin modifications, particularly de-esterification of methylesterified homopolygalacturonic acid that allows pectin to adopt the “egg box” conformation.

PME activity during development

Since PMEs are ionically linked to plant cell walls, their extraction necessitates buffers with high salt concentrations, despite the fact that some reports describe “soluble” PME isoforms weakly linked to the cell wall (Bordenave and Goldberg, 1994; Micheli et al., 2000). We examined PME localization during strawberry development by assaying wall and soluble proteins in a cup plate activity test (Downie et al., 1998; Ren and Kermodé, 2000) and found that PMEs were exclusively localized to the cell walls (data not shown).

Protons released in the incubation medium by PME action on methylesterified pectin were assayed colorimetrically with methyl red, a pH indicator that is yellow in alkaline conditions and turns red between pH 4.2 and 6.2. Since protein synthesis in strawberry fruit is constant all along fruit development and continues until the last stages of fruit development (Civello et al., 1996), we used 2 µg of wall proteins from each developmental stage as well as one unit of a commercial PME from orange peel (Sigma, P-5400) as positive control. Distilled water and wall proteins denatured for 10 min at 95 °C were the negative controls. After 2 h incubation at 25 °C, a gradient of color had developed from red for the small green fruits and the Sigma PME control, to yellow for the senescent fruits, the distilled water and the boiled extracts (data not shown). PME activity was thus strong in the early stages of maturation and decreased to no activity in old fruits.

Figure 1 shows the kinetics of color change of the pH indicator. For all samples, the ratio of integrated absorbance values increased very steeply for early maturational stages and very slightly for the late stages. In most cases, the curves reached a plateau that was lower as maturation progressed. The sigmoidal response of the late samples was due

to the initial neutralization of the small amount of buffer used to bring all samples at the optimal pH of activity of PME's at the beginning of the experiments. The initial linear part of the curves was used to compare PME activity between samples. PME activity was clearly highest in green fruits and steadily decreased until senescence.

PME isoforms during strawberry fruit development

One of the main modes of regulation of the activity of most plant enzyme systems is the expression of particular isoforms that are regulated spatially and/or temporally. To assess the presence of such isozymes, we performed a zymogram analysis of PME's on wall protein extracts from fruits at different maturational stages.

Figure 2 presents the results of a typical IEF separation of 5 μ g wall protein samples from fruits at each of the main developmental stages. Six PME isoforms, differing by their pI's, were detected at the basic end of the gel, regardless of the developmental stage. The six isoforms had pI's of 9.5, 9.3, 9.0, 8.9, 8.5 and 8.2. To verify whether the PME isoforms detected were fruit-specific, IEF was performed with wall extracts from young leaves (Figure 2), stolons and other vegetative organs, and identical results were obtained (data not shown).

Low methylesterified pectin content during fruit development

To assess the role of pectin in strawberry softening, the content in low methylesterified

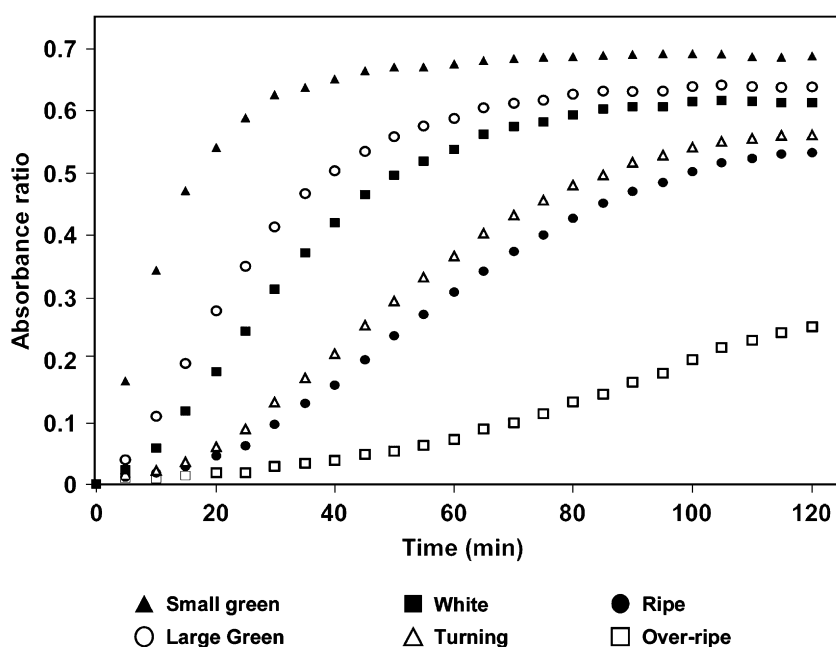


Figure 1. PME activity at the main developmental stages of strawberry ripening. The kinetics of enzyme activity was assayed colorimetrically on citrus pectin (degree of methylesterification 90%) using methyl red as pH indicator. The PME action was strongest in small green fruits and continuously decreased during fruit development.

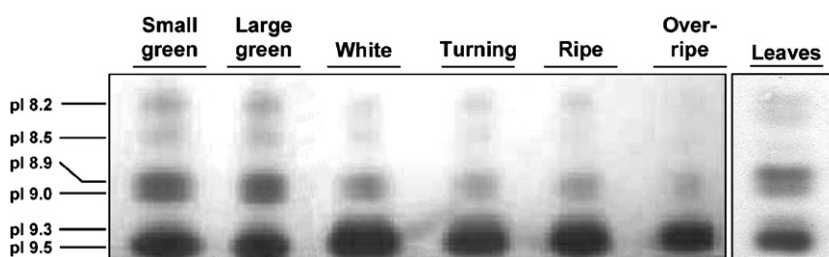


Figure 2. Isoelectrofocalisation and zymogram analysis of PME isoenzymes isolated from strawberry fruit cell walls (small green to over-ripe stages) and young leaves. Six basic isoenzymes were detected between pI's 8.2 and 9.5 in all samples. Equal amounts of proteins were deposited on each lane, indicating a decreasing PME activity along fruit development.

pectin of the fruit was followed during development with a monoclonal antibody. The 2F4 monoclonal antibody very specifically recognizes low methylesterified (up to 30%) homopolygalacturonic sequences dimerized through Ca^{2+} bridges according to a structure called “egg boxes” (Liners et al., 1989; Liners et al., 1992). We used an indirect ELISA test of the inhibitor type. The test consists of incubating the antibody and the antigen to be tested (crushed fruits in this case) together in solution. After pelleting the antibody–antigen complexes formed, the amounts of remaining free antibodies present in the supernatant were quantified on standard pectin immobilized in the micro-wells of a test plate. This technique has several advantages: (1) the antigen to be tested is not adsorbed on any surface, and its accessibility for the antibody is thus optimal; (2) the degree of methylesterification of the sample pectin does not interfere with binding to the microwell since the antigen–antibody reaction occurs in solution; (3) It allows a very reliable quantitative estimate of the acidic pectin present in the samples by reference to a calibration curve.

Native and de-esterified samples were tested. The amounts of acidic pectin present were estimated from water-crushed fruits. The total amount of pectin (i.e. the acidic pectin plus the methylesterified pectin) was obtained from alkaline-de-esterified (NaOH 50mM, pH 12.5) water-ground fruits.

Figure 3A shows the amounts of acidic pectin present in fruits. The results at different maturational stages are not comparable with each other because the cell weights were not constant along development (Cheng and Breen, 1991). In other words, mature fruits contain much more water than small green fruits. However, for an equal amount of ground tissue, the acidic pectin contents of the three first stages of maturation were about $140\ \mu\text{g}$ per gram of fruit. The acidic pectin content increased at the turning stage ($473\ \mu\text{g}$ per gram tissue) and culminated in senescent fruits ($841\ \mu\text{g}$ per gram). The total amount of homopolygalacturonic acid (both acidic and methylesterified) was determined by the same procedure but on chemically de-esterified ground tissue. Figure 3B shows that the total amount of pectin present decreased from a relatively constant value of about $4\ \text{mg/g}$ sample at early stages to $2.6\text{--}2.8\ \text{mg/g}$ in ripe and over-ripe fruits, respectively. Such a decrease is also observed in the Californian strawberry variety Yolo between the unripe and ripe stages (Koh and Melton, 2002). The ratio of acidic to total (acidic plus methylesterified) pectin was calculated at each ripening stage (Figure 3C). The proportion of

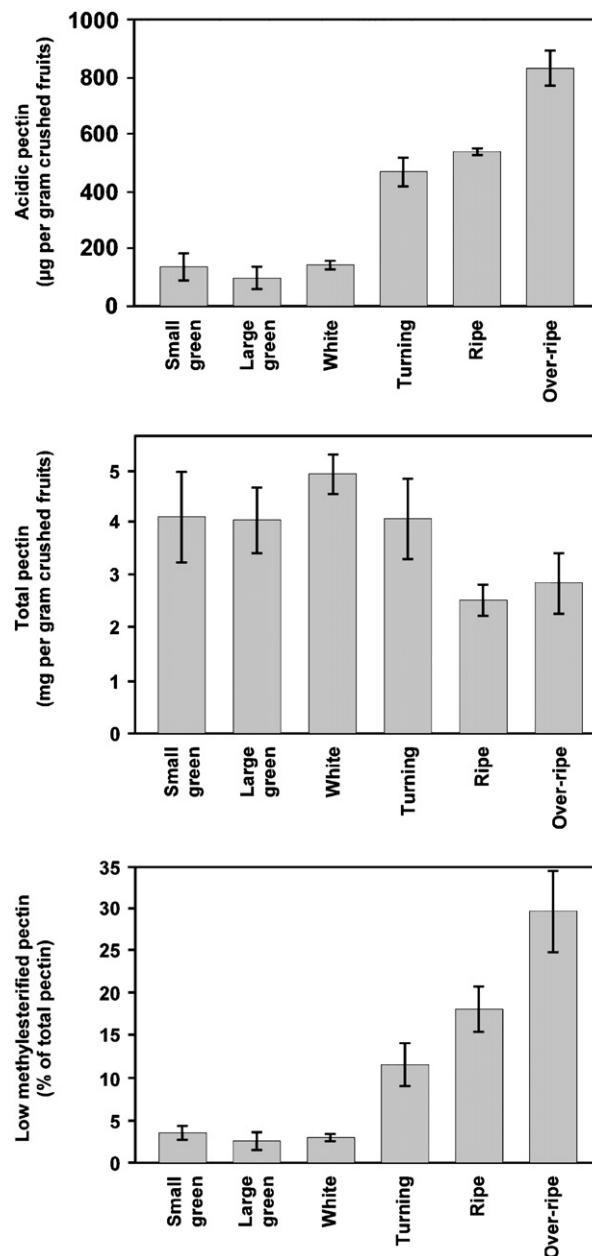


Figure 3. (A) Acidic pectin detected in strawberry fruits at different stages of maturation using the 2F4 monoclonal antibody specific of egg boxes, a calcium-induced conformation of acidic pectin. The absorbance values obtained from crude samples in an inhibiting ELISA test were expressed as acidic pectin contents ($\mu\text{g/g}$ crushed sample) using a calibration curve made from Sigma PGA. A dramatic increase in content of acidic pectin started at the turning stage. (B) Total pectin (acidic+methylesterified) recognized by the 2F4 antibody after chemical de-esterification of the samples (mg pectin/g crushed sample). The total amount of homopolygalacturonic acid content decreased by the end of ripening. (C) Proportion of acidic pectin present in fruits. The ratios calculated from values in (A) and (B) show that the proportion of acidic pectin was negligible in green and white fruits and strongly increased from the turning to the over-ripe stages of ripening.

weakly methylesterified (acidic) pectin was low and stable at around 3% for the small green, large green and white fruits. It sharply increased to 12% in turning, 18% in ripe and 30% in over-ripe fruits.

Acetylation strongly lowers the affinity of acidic pectins for bivalent cations (Renard and Jarvis, 1999). A 12.5% acetylation is sufficient for preventing acidic pectins from gelling (Kravtchenko et al., 1992), but lower levels are generally found in plants (with the exception of sugar beet and apple) that have pectins with DA up to 35%, depending on the species, the tissue and the extraction method (Voragen et al., 1995; Bonnin et al., 2003; Mohamed et al., 2003). Since preliminary tests to detect acetylase activity in strawberry were unsuccessful (Cécile Thonar, personal communication), pectin modifications detected by the 2F4 antibody resulted from the action of PME and not PAs.

Discussion

Beside pigments and organoleptic components accumulation, wall softening is an essential feature of ripening that results from middle lamella and/or primary wall polysaccharide modifications. These wall modifications are due primarily to wall enzymes including PLs, PGs and PMEs. Since pectin solubilization increases during strawberry ripening, one of the main softening enzymes involved could be PLs, of which under-expression in transgenic plants yields fruits with reduced softening (Jiménez-Bermúdez et al., 2002; Draye and Van Cutsem, unpublished results). However, PL and PG activity depend on pectin methylesterification (Scavetta et al., 1999; Herron et al., 2000; Wakabayashi et al., 2003). Methylesterification protects pectin from pectinolytic enzymes because PL and PG substrate consists of low methylesterified pectin. PME action is thus a pre-requisite before degradation by PG and PL can take place.

Homogalacturonan is largely synthesized in the Golgi system and ends up in secretion vesicles (Moore et al., 1991; Liners and Van Cutsem, 1992; Sherrier and VandenBosh, 1994). Homogalacturonan methyltransferases are present in the Golgi lumen, and newly produced pectin is highly methylesterified (81% methylesterification in sunflower, Mohamed et al., 2003). *In muro* pectin can be largely acidic, as in raspberry, where by the end of maturation, fruit pectin esterification has dropped down to 7% (Stewart et al., 2001). Since, to our knowledge, no weakly esterified pectin has ever been detected in any Golgi compartment, acidic pectin found in cell walls must result from

wall PME action. To follow wall-bound PME activity during fruit development, we used a colorimetric test and we looked for the presence of PME isoforms by zymogram analysis of samples from the main maturational stages.

The PME activity detected by zymogram during fruit development revealed the presence of at least six main basic isoforms (Figure 3). The six isozymes were not, however, specific of fruits since we obtained identical gel profiles on several other organs of the strawberry. Such ubiquitous PMEs are described, for example, in trembling aspen, in which one neutral isoform is expressed in all organs during dormancy as well as during growth (Micheli et al., 2000). No acidic PME could be detected in our tests at any developmental stage.

From the colorimetric results, it appeared that there were two main stages of pectin network modification during strawberry fruit development: an early one between the small green and white stages with high PME activity (Figures 1 and 2) and little acidic (egg box) pectin (Figure 3), and a late one between the turning and over-ripe stages characterized by a rapidly decreasing level of PME activity and a steeply increasing amount of egg box pectin.

At first sight, it could seem paradoxical that the highest PME activity occurred during these early stages for which the de-esterified pectin content measured remained low and did not increase before the turning stage, when PME activity had already decreased.

A first explanation is that the early phase of PME activity coincides with cell division and elongation processes that occur at the beginning of fruit growth. The massive neo-synthesis of highly methylesterified pectin during the green and white stages occurs because cell number and volume dramatically increase at that moment. This would counterweight the activity of PMEs, preventing detection of significant amounts of acidic pectin by ELISA tests.

On the other hand, the 2F4 antibody specifically binds acidic pectin chains dimerized through calcium ions ("egg box" dimers) when the degree of methylesterification is lower than 30% and provided uninterrupted blocks of at least nine consecutive galacturonic residues (Liners et al., 1992). Pectin de-esterification by PMEs can be either sequential along pectin chains (block-wise de-esterification), which favors egg box formation, or random over the pectin network (like chemical de-esterification), which delays formation of "egg boxes" (Grasdalen et al., 1996; Limberg et al., 2000; Micheli, 2001; Willats et al., 2001). We do not know whether strawberry PMEs process wall pectin

sequentially or randomly but, the weak detection of acidic pectin in early stages could simply be due to the kinetics of enzyme activity: even sustained enzymatic de-esterification during these early stages could not be detected by the 2F4 antibodies as long as the mean methylesterification degree of pectin had not fallen below 30%. It is not until the late maturation phase that PME could de-esterify the remaining methylesterified galacturonic residues below that 30% DM, allowing the pectin to adopt the egg box conformation.

Other factors could also interfere with PME activity. First, progressive wall acidification due to PME action can gradually slow down the enzyme's hydrolytic activity (Moustacas et al., 1991). Even if present and functional on zymograms run at pH 7.6, PMEs would not necessarily be active at lower pH *in muro*. This slow down would be more pronounced at early stages when PME activity is at a maximum. Second, PME-inhibiting proteins could be present at any stage of maturation. These inhibitors, although still not characterized in strawberry, are well known in kiwi, potato, banana or *Arabidopsis* (McMillan and Pérombelon, 1995; Wolf et al., 2003). These molecules can be of either pectin- or protein-type. Protein-type inhibitors show a strong identity with the pro-sequence of immature PMEs and are active on numerous PMEs from different plants. Since we assayed enzymes in non-denaturing conditions, PME-inhibitor complexes could have been maintained in some samples.

Increasing acidic pectin content during fruit development has been reported for different fruits. Wakabayashi et al. (2003) have shown that PME or weak alkali de-esterified polyuronides treated with either avocado or tomato PGs caused extensive molecular mass downshifts, paralleling those observed during avocado fruit ripening. Here we specifically show that egg box pectin resulting from de-esterification of contiguous methylesterified pectin residues started increasing abruptly at the turning stage, which coincides with the stage at which the fruit also starts softening. The temporal expression of the three PL genes described in strawberry (Benítez-Burraco et al., 2003) nicely coincides with the kinetics of egg box pectin production during fruit maturation. De-esterification of neutral into egg box pectin and PL production appear to be simultaneous processes during strawberry maturation.

The PME activity pattern and acidic pectin contents we measured are perfectly compatible with a general process of strawberry fruit maturation that would consist of strong early activity of PME enzymes followed later by other pectolytic

enzymes. In strawberry, these latter enzymes would most likely be PLs whose specific expression in the fruit (Medina-Escobar et al., 1997) begins at the start of maturation in variety Chandler (Benítez-Burraco et al., 2003) but also in Elsanta (Draye and Van Cutsem, unpublished data). Accordingly, PL down-regulation in transgenic plants results in firmer fruits (Jiménez-Bermúdez et al., 2002; Draye and Van Cutsem, unpublished data).

In conclusion, the PME activities detected on zymograms and in *in vitro* assays showed a continuous decrease from the earliest small green to the late over-ripe stage. This activity led to an abrupt increase in acidic pectin right from the turning stage and up to the over-ripe stage. This steadily increasing content in acidic pectin that can adopt the egg box conformation was parallel to the well-known onset of fruit softening at the turning stage. The role of strawberry fruit PME would therefore be to de-esterify pectin to a point where it can adopt the egg box conformation recognized by the 2F4 antibody and is a suitable substrate for pectolytic enzymes such as PLs.

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References

- Benítez-Burraco A, Blanco-Portales R, Redondo-Nevado J, Bellido ML, Moyano E, Caballero J-L, et al. Cloning and characterization of two ripening-related strawberry (*Fragaria × ananassa* cv. Chandler) pectate lyase genes. *J Exp Bot* 2003;54:633–45.
- Bonnin E, Le Goff A, van Alebeek G-JWM, Voragen AGJ, Thibault J-F. Mode of action of *Fusarium moniliforme* endopolygalacturonase towards acetylated pectin. *Carbohydr Polym* 2003;52:381–8.
- Bordenave M, Goldberg R. Immobilized and free apoplastic pectinmethylesterases in mung bean hypocotyl. *Plant Physiol* 1994;106:1151–6.
- Brummell DA, Harpster MH. Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. *Plant Mol Biol* 2001;47:311–40.
- Castillejo C, de la Fuente JI, Iannetta P, Botella MA, Valpuesta V. Pectin esterase gene family in strawberry fruit: study of FaPE1, a ripening-specific isoform. *J Exp Bot* 2004;55:909–18.
- Cheng GW, Breen PJ. Activity of phenylalanine ammonia-lyase (PAL) and concentrations of anthocyanins and phenolics in developing strawberry fruit. *J Am Soc Hortic Sci* 1991;116:865–9.

- Civello PM, Chaves AR, Añón MC. Change in protein composition during strawberry (*Fragaria × ananassa* Duch.) fruit ripening. *J Food Biochem* 1996;20:135–53 and in mealy fruit. *Plant Physiology* 100, 1203–1210.
- Downie B, Dirk LMA, Hadfield KA, Wilkins TA, Bennett AB, Bradford KJ. A gel diffusion assay for quantification of pectin methylesterase activity. *Anal Biochem* 1998; 264:149–57.
- Grasdalen H, Andersen AK, Larsen B. NMR spectroscopy studies of the action pattern of tomato pectinesterase: generation of block structure in pectin by a multiple-attack mechanism. *Carbohydr Res* 1996;289: 105–14.
- Herron SR, Benen JAE, Scavetta RD, Visser J, Jurnak F. Structure and function of pectic enzymes: virulence factors of plant pathogens. *Proc Natl Acad Sci USA* 2000;97:8762–9.
- Jiménez-Bermúdez S, Redondo-Nevado J, Muñoz-Blanco J, Caballero JL, López-Aranda JM, Valpuesta V, et al. Manipulation of strawberry fruit softening by anti-sense expression of a pectate lyase gene. *Plant Physiol* 2002;128:751–9 The World Avocado Congress III, 277–284.
- Koh TH, Melton LD. Ripening-related changes in cell wall polysaccharides of strawberry cortical and pith tissues. *Postharvest Biol Technol* 2002;26:23–33.
- Kravtchenko TP, Voragen AGJ, Pilnik W. Analytical comparison of three industrial pectin preparation. *Carbohydr Polym* 1992;18:17–25.
- Limberg G, Körner R, Buchholt HC, Christensen TMIE, Roepstorff P, Mikkelsen JD. Analysis of different de-esterification mechanisms for pectin by enzymatic fingerprinting using endopectin lyase and endopolygalacturonase II from *A. Niger*. *Carbohydr Res* 2000;327: 293–307.
- Liners F, Van Cutsem P. Distribution of pectic polysaccharides throughout walls of suspension-cultured carrot cells: an immunocytochemical study. *Protoplasma* 1992;170:10–21.
- Liners F, Thibault JF, Van Cutsem P. Influence of the degree of polymerization of oligogalacturonates and of esterification pattern of pectin on their recognition by monoclonal antibodies. *Plant Physiol* 1992;99: 1099–104.
- Liners F, Letesson J-J, Didembourg C, Van Cutsem P. Monoclonal antibodies against pectin. Recognition of a conformation induced by calcium. *Plant Physiol* 1989;91:1419–24.
- Manning K. Changes in gene expression during strawberry fruit ripening and their regulation by auxin. *Planta* 1994;194:62–8.
- Manning K. Isolation of a set of ripening-related genes from strawberry: their identification and possible relationship to fruit quality traits. *Planta* 1998;205: 622–31.
- McMillan GP, Pérombelon MCM. Purification and characterization of a high *pI* pectin methyl esterase isoenzyme and its inhibitor from tubers of *Solanum tuberosum* subsp. *tuberosum* cv. Katahdin. *Physiol Mol Plant Pathol* 1995;46:413–27.
- Medina-Escobar N, Cárdenas J, Moyano E, Caballero JL, Muñoz-Blanco J. Cloning, molecular characterization and expression pattern of a strawberry ripening-specific cDNA with sequence homology to pectate lyase from higher plants. *Plant Mol Biol* 1997;34: 867–77.
- Medina-Escobar N, Cárdenas J, Muñoz-Blanco J, Caballero JL. Cloning and molecular characterization of a strawberry fruit ripening-related cDNA corresponding to a mRNA for a low-molecular-weight heat-shock protein. *Plant Mol Biol* 1998;36:34–42.
- Micheli F. Pectin methylesterases: cell wall enzymes with important roles in plant physiology. *Trends Plant Sci* 2001;6:414–9.
- Micheli F, Sundberg B, Goldberg R, Richard L. Radial distribution pattern of pectin methylesterases across the cambial region of hybrid aspen at activity and dormancy. *Plant Physiol* 2000;124:191–9.
- Mohamed SA, Christensen TMIE, Mikkelsen JD. New polygalacturonases from *Trichoderma reesei*: characterization and their specificities to partially methylated and acetylated pectins. *Carbohydr Res* 2003; 338:515–24.
- Moore PJ, Swords KMM, Lynch MA, Staehelin LA. Spatial organization of the assembly pathways of glycoproteins and complex polysaccharides in the Golgi apparatus of plants. *J Cell Biol* 1991;112: 589–602.
- Mori T, Sakurai M, Shigeta J-I, Yoshida K, Kondo T. Formation of anthocyanins from cells cultured from different parts of strawberry plants. *J Food Sci* 1993; 58:788–92.
- Moustacas A-M, Nari J, Borel M, Noat G, Ricard J. Pectin methylesterase, metal ions and plant cell-wall extension. *Biochem J* 1991;279:351–4.
- Nogata C, Ohta H, Voragen AGJ. Polygalacturonase in strawberry fruit. *Phytochemistry* 1993;34:617–20.
- Redondo-Nevado J, Moyano E, Medina-Escobar N, Caballero JL, Muñoz-Blanco J. A fruit-specific and developmentally regulated endopolygalacturonase gene from strawberry (*Fragaria × ananassa* cv. Chandler). *J Exp Bot* 2001;52:1941–5.
- Ren C, Kermod AR. An increase in pectin methyl esterase activity accompanies dormancy breakage and germination of yellow cedar seeds. *Plant Physiol* 2000;124:231–42.
- Renard CMGC, Jarvis MC. Acetylation and methylation of homogalacturonans 1: optimisation of the reaction and characterisation of the products. *Carbohydr Polym* 1999;39:201–7.
- Scavetta RD, Herron SR, Hotchkiss AT, Kita N, Keen NT, Benen JAE, et al. Structure of a plant cell wall fragment complexed to pectate lyase C. *Plant Cell* 1999;11:1081–92.
- Sherrier DJ, VandenBosh KA. Secretion of cell wall polysaccharides in *Vicia* root hairs. *Plant J* 1994; 5:185–95.
- Stewart D, Iannetta PP, Davies HV. Ripening-related changes in raspberry cell wall composition and structure. *Phytochemistry* 2001;56:423–8.

- The Arabidopsis Genome Initiative. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* 2000;408:796–815.
- Trainotti L, Pavanello A, Casadoro G. Different ethylene receptors show an increased expression during the ripening of strawberries: does such an increment imply a role for ethylene in the ripening of these non-climacteric fruits? *J Exp Bot* 2005;56:2037–46.
- Voragen AGJ, Pilnik W, Thibault J-F, Axelos MVA, Renard CMGC. Pectins. In: Stephen AM, editor. *Food polysaccharides and their applications*. New York: Marcel Dekker; 1995. p. 287–339.
- Wakabayashi K, Hoson T, Huber DJ. Methyl de-esterification as a major factor regulation the extent of pectin depolymerization during fruit ripening: a comparison of the action of avocado (*Persea americana*) and tomato (*Lycopersicon esculentum*) polygalacturonases. *J Plant Physiol* 2003;160:667–73.
- Willats WGT, Orfila C, Limberg G, Buchlot HC, van Alebeek G-JWM, Voragen AGJ, et al. Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls. Implications for pectin methyl esterase action, matrix properties, and cell adhesion. *J Biol Chem* 2001;276:19404–13.
- Wolf S, Grsic-Rausch S, Rausch T, Greiner S. Identification of pollen-expressed pectin methylesterase inhibitors in *Arabidopsis*. *FEBS Lett* 2003;555:551–5.