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Immobilized and Free Apoplastic Pectinmethylesterases in Mung Bean Hypocotyl

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The nature and the action pattern of apoplastic pectinmethylesterase (PME) isoforms were investigated in mung bean [*Vigna radiata* (L.) Wilzeck] hypocotyls. Successive extractions of neutral and alkaline PME isoforms present in hypocotyl native cell walls (referred to as PE_1, PE_2, PE_3, PE_4, with increasingly basic isoelectric points) revealed that solubilization of PE_1, PE_2, and PE_4 did not induce any significant decrease in the cell-wall-bound PME activity. The in vitro de-esterification occurring when isolated cell walls were incubated with pectin resulted, then, from the activity of PE_3. In addition, pH control of PME activity was shown to be much stronger for enzymes bound to cell walls, in their native state or reintroduced after solubilization, than for enzymes in solution. Mature cell walls showed much more activity than young cell walls, and were relatively enriched in two acidic PME isoforms missing in young cell walls. One acidic PME was also detected in the extracellular fluid. The acidic and neutral isoforms that could be easily transferred from their binding sites to their substrate might be those involved in the demethylation process developing along the mung bean hypocotyl.

It has often been suggested that PMEs, due to their sequestration in the cell wall and their ability to generate protons, should be playing a major role in cell wall turnover. On the one hand, pectins have been shown to be esterified in the Golgi (Kauss and Swanson, 1969) and to be exported to the apoplasm still highly methylated (Liners and Van Cutsem, 1992; Zhang and Staehelin, 1992). Cell wall PMEs should then generate in muro acidic domains along the galacturonan chain and, consequently, modulate the cation-exchange capacity of the walls. On the other hand, PME activity has been reported to control apoplastic pH and, in turn, cell expansion processes (Nari et al., 1986; Ricard and Noat, 1986; Moustacas et al., 1991). During the past few years, the regulation of plant cell wall PME by the wall electrostatic potential has been thoroughly investigated in Ricard's laboratory (Moustacas et al., 1991; Nari et al., 1991; Charnay et al., 1992). This activity was shown to be strictly regulated by the ionic composition of its microenvironment. Additionally, numerous isoforms have been isolated from plant cell walls (Gaffe et al., 1992; Bordenave and Goldberg, 1993; Lim and Chung, 1993), but their respective functions and action patterns have not been determined.

The mung bean [*Vigna radiata* (L.) Wilzeck] hypocotyl represents a good material in which to investigate the possible involvement of PME activity in the modifications of pectins that occur during the maturation of the cells. Indeed, along this axis young, plastic cell walls are characterized by a significant content of highly methylated pectins and a low content of acidic polygalacturonans, in contrast to older, stiff cell walls, which contain mainly acidic galacturonans and fewer esterified galacturonic acids (Goldberg et al., 1986). Moreover, several PME isoforms have been isolated from this material (Bordenave and Goldberg, 1993). Therefore, mung bean hypocotyl cell walls were further investigated to determine the role of each PME isoform. With this object, we first investigated the strength of the binding of the isoforms to the cell walls and the behavior of both solubilized or immobilized enzymes. The possible presence in the apoplastic fluid of free-moving PME was also investigated. The aim of these experiments was to determine whether ionic interactions between PMEs and the cell wall might influence PME mobilities and enzyme activities. Finally, the changes in the isoform pattern along the hypocotyl were also studied and are discussed in relation to the modifications occurring in the pectic fraction.

MATERIALS AND METHODS

Plant Material

Cell walls were isolated from the upper 2.5 cm of hypocotyl tissues of 3-d-old seedlings of mung bean [*Vigna radiata* (L.) Wilzeck], according to a previously described procedure (Goldberg et al., 1986). In addition, cell walls were also isolated from successive segments sectioned along the hypocotyl, referred to as segments A (hooks 5 mm long), B and C (10 mm long each), and D (20 mm long).

Analysis

PME activity was measured titrimetrically by following the increase in free carboxyl groups. The carboxyl groups released by PME from 0.25% *Citrus* pectin (Sigma) in the presence of different concentrations of NaCl were titrated with 10 mM NaOH under nitrogen, the pH being maintained at chosen values ranging from 5.2 to 8.0 with an automatic titrator (TTT 80, Radiometer, Copenhagen, Denmark).

Protein concentrations were determined according to the micromethod of Bradford (1976) using a Bio-Rad kit and lysozyme as standard.

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Abbreviations: pI, isoelectric point; PME, pectinmethylesterase.

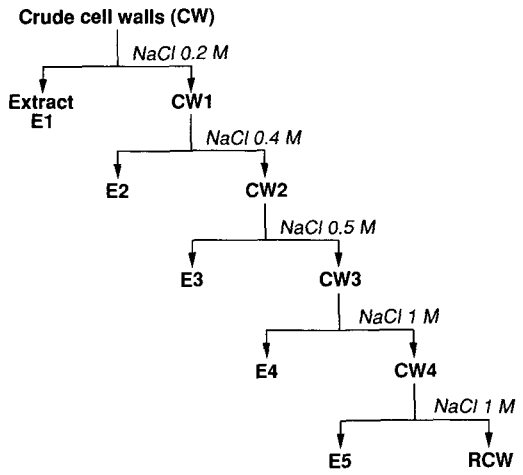


Figure 1. Flow chart for sequential extractions of PME from isolated cell walls.

Electrophoretic Procedures

IEF was performed on ultrathin (0.5 mm) polyacrylamide slab gels (10% acrylamide, 0.26% bisacrylamide) containing 10% Pharmacia ampholines (pH range 3–10) (Bertheau et al., 1984). The IEF was run on an LKB Multiphor 2117 (Pharmacia, Uppsala, Sweden) using an LKB 2103 power supply of 30 W for 1 h. One molar NaOH and 1 M SO_4H_2 were used, respectively, as catholyte and anolyte. After focusing, gel sections were collected to determine the pH gradient. The PME active bands were revealed with the agar-pectin sandwich technique (Bertheau et al., 1984) using ruthenium red purchased from Aldrich. IEF gels were scanned with an Image Analyzer (Vilber Lourmat, Marne-la-Vallée, France) using Bioprofil (Vilber Lourmat) software.

Extraction of PME from Isolated Cell Walls

PMEs were eluted sequentially by successive NaCl treatments of cell walls (Fig. 1). All steps were conducted at 4°C. At each step, the cell walls were incubated for 30 min in NaCl solution, the concentration of which ranged from 0.2 to 1 M, then filtered through a cheesecloth and further submitted to the next NaCl treatment.

Immobilization of Extracted PME

Mini-chromatography columns were packed with 5 mL of a suspension of completely eluted cell walls presenting no detectable PME activity. After rinsing the column with distilled water, 1 to 2 mL of dialyzed enzymatic extracts were passed through the column, which was then rinsed again with 10 mL of distilled water. The cell walls were then resuspended in water and assayed for their PME activity as described above. Enzymatic extracts were also immobilized on CM Sepharose CL 6B and the activity loaded on the gel was estimated.

Extraction of Intercellular Fluid

Hypocotyl segments were immersed in ice-cold distilled water and infiltrated in vacuo for three periods of 1 min each. The segments were then placed vertically in a 50-mL polypropylene centrifuge tube provided with perforations (around 2 mm in diameter) at the bottom. The tube was placed in a 225-mL plastic centrifuge bottle so that the tube was held by its rim and remained suspended in the bottle. The apparatus was centrifuged at 1000*g* for 10 min at 4°C. After centrifugation, the fluid that collected at the bottom of the bottle was concentrated by ultrafiltration on an Amicon (Beverly, MA) PM10 membrane and then submitted to IEF. PME activities were detected with the agar-pectin sandwich technique.

RESULTS

Sequential Extractions of Cell Wall PMEs

To investigate which isoforms were actually the most active in situ, we attempted to sequentially solubilize these proteins and to determine the activity of the progressively extracted cell walls. For that purpose, isolated cell walls were treated successively with saline solutions of increasing concentration as illustrated in Figure 1. After each treatment, PME activity remaining in the cell walls and PME activity extracted with NaCl were estimated in the presence of either 150 mM NaCl or 75 mM $MgCl_2$, concentrations known to be especially effective for stimulating the de-esterification of *Citrus* pectin by mung bean isolated cell walls (Goldberg et al., 1992b). Yields of the extractions are reported in Figure 2. The highest activity was recovered in extract E_2 (obtained with 0.4 M NaCl) regardless of the nature of the cation (Na^+ or Mg^{2+}) added in the assays. In spite of solubilization of PME by the first saline treatments (0.2 and 0.4 M NaCl), the treated cell walls still exhibited almost the same activity as did the native, unextracted cell walls (around 450 neq min^{-1} mg^{-1} cell walls

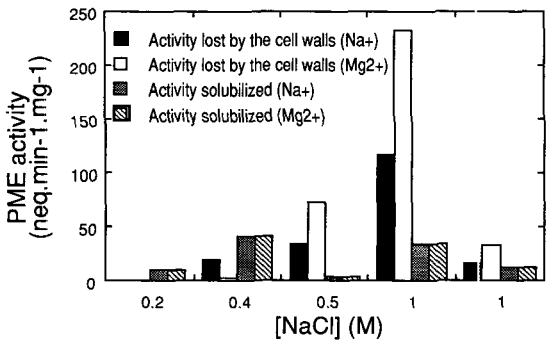


Figure 2. Yields of successive NaCl treatments. Activities were estimated in the cell walls and in the saline extracts in the presence of either 150 mM NaCl or 75 mM $MgCl_2$ and expressed as neq H^+ min^{-1} mg^{-1} cell walls. Cell walls were successively treated with 0.2, 0.4, 0.5, and 1 M (twice) NaCl solutions. Activity lost by the cell walls through one treatment corresponds to the difference between PME activity bound to the cell walls before and after NaCl treatment. Activity solubilized corresponds to the PME activity recovered in the different extracts, E_1, E_2, E_3, and E_4.

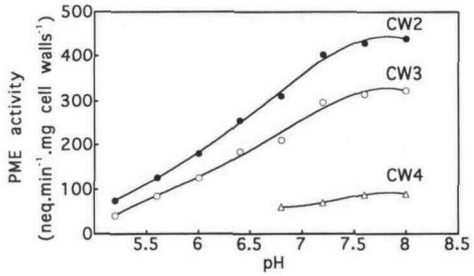


Figure 3. pH plots of PME still bound to cell walls after NaCl treatments. Reaction rates were measured in a pH-stat at several pH values ranging from 5.2 to 8.0 in the presence of 150 mM NaCl and under nitrogen flux. Activities are expressed as neq H^+ min^{-1} mg^{-1} cell walls. Curves labeled CW_2, CW_3, and CW_4 are for PME activity remaining bound to cell walls after 0.4 M (CW_2), 0.5 M (CW_3), and 1 M (CW_4) NaCl treatments.

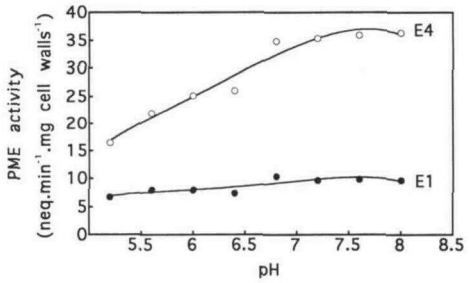


Figure 5. pH plots of PME solubilized with 0.2 M (E_1) and 1 M (E_4) NaCl. Reaction rates were measured in a pH-stat at several pH values ranging from 5.2 to 8.0, in the presence of 150 mM NaCl and under nitrogen flux. Activities are expressed as neq H^+ min^{-1} μg^{-1} protein.

at pH 7.6, with 150 mM NaCl). These data might suggest that some isoforms are very poorly active in situ, since their solubilization did not reduce cell wall PME activity. A significant decrease of the cell wall activity was noticed only after the 0.5 M NaCl treatment, and the largest decrease was measured after the first 1 M NaCl treatment. Besides, it was also noted that, as expected from previous results (Goldberg et al., 1992b), all wall-bound activities, contrary to solubilized ones, were higher when estimated with 75 mM $MgCl_2$ than with 150 mM NaCl. After being treated with 0.5 M NaCl, the first treatment that decreased the cell wall activity, the cell walls (CW_3) exhibited the same sensitivity to pH as the native walls, CW_0 (Fig. 3). Isoform composition of the successive extracts was checked by IEF. As shown in Figure 4, we observed that the treatment with 0.2 M NaCl solubilized only one isoform (apparent pI around 7.5), which was similar to that called PE_2 in a previous report (Bordenave and Goldberg, 1993). The other, faint neutral isoform (PE_1) occasionally detected in crude cell wall extracts likely corresponded to a degraded form of PE_2. As expected from previous experiments with purified isoforms (Bordenave and Goldberg, 1993), the first eluate (0.2 M NaCl), which contained only PE_2, was weakly sensitive to pH (Fig. 5). In contrast, extract E_4, obtained with 1 M NaCl and containing only an alkaline isoform similar to that previously called PE_3, exhibited a pH optimum around 7.6 (Figs. 4 and 5). All these data suggest that the de-esterification that occurs when isolated cell walls are incubated with *Citrus* pectin is due mainly to PE_3, the other isoforms being unable to work on exogenous pectin when they are embedded in the cell walls. Enzymatic activity of isolated cell walls would then result from the activity of the most tightly bound isoform.

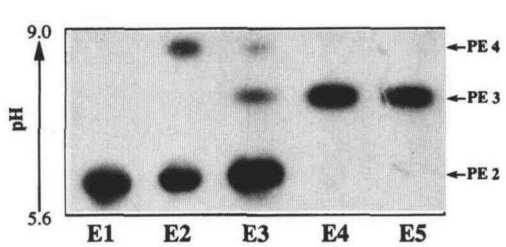


Figure 4. IEF of the successive saline extracts E_1, E_2, E_3, E_4, and E_5. PME activities were detected by the agar-pectin sandwich technique (Bertheau et al., 1984).

PME Present in Intercellular Fluid

The possible occurrence of free PME isoforms that are mobile in the intercellular fluid was then investigated. Interstitial fluids were collected from the upper half of mung bean hypocotyls, concentrated, and submitted to IEF. As illustrated in Figure 6, lane IF, only one acidic protein (apparent pI around 5) exhibiting PME activity was detected in this fluid. In the apoplasm, free and bound PME isoforms are, then, very different, which shows the remarkable diversity and complexity of PME activities in this compartment.

Development of Isoform Pattern along the Hypocotyl

Cell walls were isolated from four successive segments (A, B, C, and D) sectioned along 3-d-old hypocotyls, from the top to the bottom of this axis, as represented in Figure 6. Enzymes were then extracted from the cell walls with 1 M NaCl. Aliquots of the successive eluates were diluted so that they exhibited equivalent PME activity and then submitted to IEF; after detection of PME activities, IEF gels were scanned lane by lane with a spectrodensitometer. Activity of each isoform could thus be estimated and then expressed as neq H^+ min^{-1} mg^{-1} cell walls. Figure 7 shows that much more PME activity was solubilized from mature, stiff cell walls (segments D) than from young, plastic ones, regardless of the nature of the isoform. Moreover, along the hypocotyl, the relative proportions of the different isoforms changed, PE_4 and PE_3 becoming the main isoforms solubilized from mature cell walls. Additionally, after IEF of undiluted eluates (Fig. 6, lanes A, B, C, and D), two slightly active spots corresponding to PMEs with acidic pI values (around 4 and 5) were detected, particularly in the extract isolated from the lower parts of the hypocotyls. Similar acidic isoforms were also detected, after concentration, in the extract E_1 (data not shown) obtained by incubating crude cell walls in 0.2 M NaCl. Moreover, one of these acidic proteins, here called PE_5, is similar to the one detected in the intercellular fluid (Fig. 6, lane IF). These data

reveal that inside the apoplasm, the acidic PMEs are either free in the intercellular fluid or weakly bound to the cell walls.

Immobilization of Solubilized Cell Wall PME

The presence in the apoplasm of free and immobilized PME isoforms raises the question of their possible relationships. Weakly bound enzymes might be solubilized, then transferred via the apoplastic fluid and eventually refixed. To test such a hypothesis, isolated cell walls were incubated with 1 M NaCl and the solubilized proteins were further immobilized on cell walls previously treated with 1 M NaCl and thus free of PMEs. Some characteristics of immobilized, solubilized, and refixed PME activities are reported in Figure 8. At low saline concentration (45 mM NaCl), immobilized and free enzymes exhibited different pH plots (Fig. 8A): the ratio of activities (reaction rate at pH 7.6:reaction rate at pH 5.6) was in all cases lower for enzymes in solution than for bound enzymes. High saline concentration (225 mM NaCl) increased drastically the activity of isolated cell walls but did not affect their pH optimum. Activity of solubilized enzymes was only weakly stimulated by the increase in NaCl concentration and remained approximately constant from pH 5.2 to pH 8.

In contrast, when the enzymes were reimmobilized on eluted cell walls, the pH sensitivity seemed to become dependent on the saline concentration: at low ionic strength, activity of reimmobilized enzymes was about 5-fold lower at acidic pH than at around pH 7, which mimicked on a reduced scale the behavior of native cell walls, but at high saline concentration the inhibition induced by acidic pH decreased markedly. Similar results were obtained with PME reimmobilized on CM Sepharose (data not shown). As a matter of fact, this behavior resulted from a progressive resolubilization of the enzymes, the free enzymes being little sensitive to pH. In contrast, PME activity of samples solubilized from native cell walls with 225 mM NaCl was negligible when compared to that of enzymes that remained bound to the cell walls.

These data pointed out significant differences between solubilized and immobilized enzymes and, besides, underscored the fact that a saline concentration sufficient for solubilizing most of the refixed PME was much less effective on enzymes bound to native cell walls. Moreover, the difference between reaction rates estimated at pH 7.6 and pH 5.6

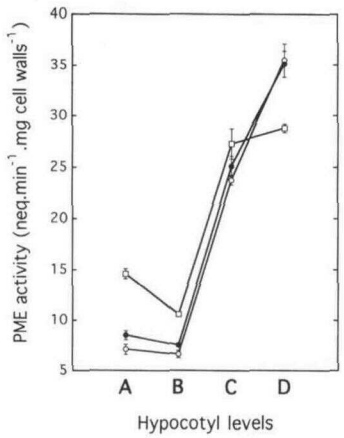


Figure 7. Development of the PME isoform pattern along the mung bean hypocotyl. PMEs were extracted from cell walls isolated from successive parts of the hypocotyl and submitted to IEF. The gels were scanned with a spectrodensitometer. Three groups of PME activities were estimated: PE_1 and PE_2 together (□), PE_3 (●), and PE_4 (○). Activities are expressed as neq H^+ min^{-1} mg^{-1} cell walls.

strongly decreased after solubilization and was partly restored when the enzymes were reimmobilized; however, after refixation, the initial maximal activity at pH 7.6 was not recovered.

DISCUSSION

The data reported above reveal that, in mung bean hypocotyl cell walls, PME isoforms are differently bound to their natural matrix. Some of them (the acidic and the neutral ones) can be easily solubilized, whereas others, like PE_3, are very tightly bound, their solubilization requiring an ionic strength equal to 1000 mM. Although this isoform was shown to be probably the only one acting on exogenous pectin when isolated cell walls were incubated with *Citrus* pectin, it is difficult to imagine how, in muro, this protein can reach its substrate and move along a methylated polygalacturonic acid chain. Indeed, the high ionic strength necessary for solubilizing PE_3 would plasmolyze and in turn damage the surrounding cells. Nevertheless, it cannot be excluded that in some precise points inside the glycoproteic network, tightly bound PME molecules are submitted to high saline concen-

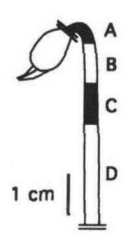
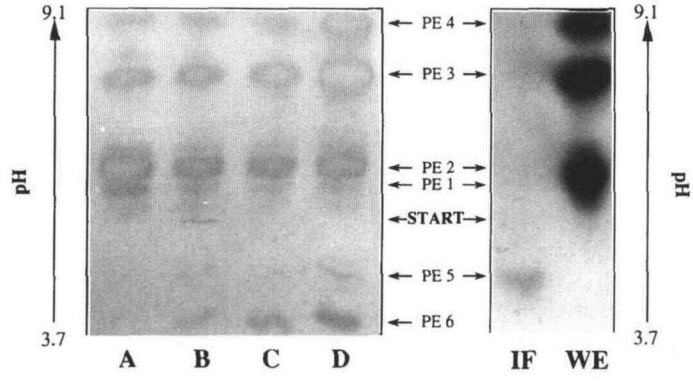


Figure 6. IEF of PMEs solubilized from cell walls isolated from the successive parts of the hypocotyls (A, B, C, and D) and of PME present in the intercellular fluid (IF). WE, Crude saline cell wall eluate from the upper 2.5 cm of mung bean hypocotyls.



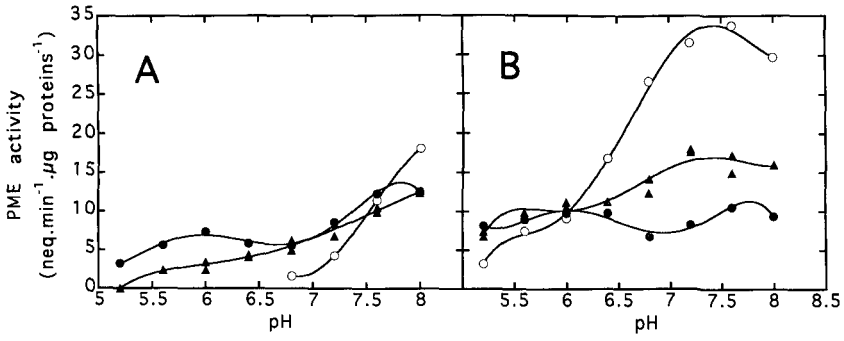


Figure 8. pH plots of solubilized and immobilized cell wall PMEs estimated in 45 (A) or 225 (B) mM NaCl incubation assays. ●, PME activity of solubilized enzymes; ○, PME activity of isolated cell walls in their native state; ▲ PME activity immobilized on cell walls previously treated with 1 M NaCl. Reaction rates were measured in a pH-stat at several pH values ranging from 5.2 to 8.0 under nitrogen flux. Activities are expressed as neq H^+ min^{-1} μg^{-1} protein.

trations and can thus be transferred to their substrate; however, in any case, this can only apply to a few of them. In contrast, free and weakly bound PME (mainly acidic or neutral isoforms) might be involved in the de-esterification process developing inside the cell walls along the hypocotyl.

Acidic PMEs are known in fungi (Baron et al., 1980; Markovic et al., 1983) but, until now, acidic isoforms have been reported only rarely in plant cell walls (Lin et al., 1989; Komae et al., 1990), all other PMEs being cationic proteins. Interestingly, the level of acidic isoforms increased in the lower parts of the hypocotyl known to be enriched in acidic galacturonan and impoverished in esterified pectins. The free-moving acidic PME might then be involved in the in muro de-esterification of methylated pectins. Indeed, according to Markovic and Kohn (1984), acidic PMEs cause a random cleavage of esterified carboxyl groups, which fits well with the presence in mung bean hypocotyl mature cell walls of a pectic fraction with a low degree of esterification and methylesters randomly distributed along the chain (Goldberg et al., 1994). Neutral isoforms, which can be easily removed from their support (0.2 M NaCl partly solubilized these isoforms, as represented in Fig. 3), should also be involved in the in muro de-esterification. However, since no neutral isoforms could be detected in the interstitial fluid, this might suggest that either only a few molecules are actually solubilized or that most of them become rapidly linked to weak negative charges present in the cell walls.

All of these observations suggest that only a small fraction of PME molecules might be involved at a given time in the de-esterification of methylated pectins, which would explain how highly methylated pectins can be present in the hypocotyl (Goldberg et al., 1986), although isolated cell walls exhibit very high PME activity toward exogenous pectin, activity sufficient on paper to de-esterify in a few minutes all the pectins present in the cell walls. Some years ago, Seymour et al. (1987), studying the differential effects of pectolytic enzymes on tomato polyuronides in vivo and in vitro, also concluded that there was a controlled restriction of pectolytic activity in vivo. The spatial regulation of pectin distribution reported by Knox et al. (1990) might be due either to distinct localization inside the cell walls of esterified pectins and PME molecules or to a strict control of these activities by their microenvironment. Using monoclonal antibodies, Vian and Roland (1991) observed that in mung bean hypocotyl sections, esterified pectins were spread throughout the thickness of the walls, whereas acidic pectins were localized principally in cell junctions and in the middle lamella. Preliminary observations of transverse sections of hypocotyls immunolabeled with polyclonal anti-PME immune serum (Goldberg et al., 1992a) indicated that the PME molecules were located mainly in cell junctions, which fits with the occurrence of acidic pectins in these areas. However, these locations also contained methylated rhamnogalacturonan, which suggests that the demethylation process might be limited by sequestration of the most active enzymes through very strong linkages. This sequestration is probably progressive, depending on the deposition rate of newly synthesized polysaccharides. According to this scheme, the first secreted PME molecules, present in the older parts of the cell walls, middle lamella, and cell junctions, would be the most active and would give rise to the acidic domains detected in these areas.

Moreover, when bound to the cell walls, some PME isoforms (PE_3) exhibited a very high activity that was drastically increased in the presence of ions, whereas others (PE_2 or PE_4) were unable to de-esterify pectin regardless of the cation present in the assay mixture. In contrast, all the solubilized isoforms were active on methylated pectin but the recovered activity was obviously lower. When embedded inside the glycoproteic network, the PME molecules might be submitted to constraints inducing important conformational modifications that can either increase or decrease their de-esterification rate, as already reported for PME (Borrego et al., 1989) and phosphatase activities (Ittah, 1992). The different activities of the bound PME isoforms can be due to the chemistry of their respective microenvironments. Information on the spatial distribution of each isoform and on the structure of its surroundings would provide a useful tool to ascertain the possible involvement of each PME in the turnover of the pectins inside the cell walls. Changes in gene expression during maturation will take part in the development of in muro PME activity by changing both the isoform pattern and the overall structure of the cell walls. Concerning the ionic control of immobilized and solubilized enzymes, our experimental data confirm that the PME activity of isolated cell walls is strongly modulated by the salt concentration in the reaction mixture, this control being nevertheless weaker when the enzymes are solubilized.

These data suggest that cations known to affect the PME activity indirectly by interacting with the negative charges of the pectin substrate (Moustacas et al., 1991; Charnay et al., 1993) might also affect directly the kinetic behavior of the bound enzymes. Since PMEs can amplify their enzyme ac-

tivities according to structural fluctuations in their environment, the ionic composition of the moving intercellular fluid might represent a key factor for the control of cell wall PMEs, ionic interactions between PMEs and the cell wall influencing both the PME mobility and enzyme activity.

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