Investigation of the Action Patterns of Pectinmethyl Esterase Isoforms through Kinetic Analyses and NMR Spectroscopy

IMPLICATIONS IN CELL WALL EXPANSION*

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Well characterized pectin samples were incubated with cell wall-bound and -solubilized pure isoforms of pectinmethyl esterase from mung bean hypocotyls (Vigna radiata). Both enzyme activity and average product structure were determined at intervals along the deesterification pathway at pH 5.6 and 7.6. The latter analyses were performed by 13C NMR spectroscopy, and the degree of esterification was probed by both 13C NMR and potentiometric measurements. A dichotomy was observed in the behavior of the α and γ isoforms when compared with that of the β isoenzyme. Ideal blockwise deesterification mechanisms reproduced the experimental average structures (methylster distribution) throughout the course of the reaction. In the case of the α and γ isoforms, a single chain mechanism associated with a free carboxyl group at the second nearest neighbor position could be postulated at pH 5.6, whereas some multiple attack character was required to reproduce the data at pH 7.6. Several mechanisms that differed from the preceding ones were compatible with the data for the β isoform at the two pH values. Both the nature of the polysaccharides produced in these reactions and the role of pectinmethyl esterase in the cell wall-stiffening process along the growth gradient are discussed.

Pectins that represent around 30% of the primary plant cell walls play a key role in plant physiology as well as in plant pathology. The general structure of pectic polymers consists of homogalacturonan linear chains (smooth regions) interspersed with highly branched galacturonic chains (hairy regions). Some of the galacturonic residues linked by α-1,4 glycosidic bonds are methyl-esterified at the carboxyl group. The degree of methyl-esterification (DE) varies greatly depending on the plant or- gan and the degree of differentiation of the cells. Young, plastic cell walls are generally characterized by a high content of highly methylated pectins that decreases in parallel with the loss of extensibility of the walls, whereas the amount of acidic residues increases (1–3). Moreover, the balance between high and low methylated pectins varies inside the wall of a single cell (4) generating microdomains. Not only the number but also the distribution of free, unesterified galacturionate carboxyl groups within the galacturonan regions will control the gellforming capacity of the pectin and thereby the porosity and the extensibility of the apoplast (5, 6). It is commonly accepted that the polygalacturonic backbone is polymerized in the cis-Golgi cisternae, methyl-esterified in the medial-Golgi, substituted with side chains in the trans-Golgi, and exported to the cell walls as a highly methylated polysaccharide (7). At a later stage, it is deesterified in muro by cell wall pectinmethyl esterases (PMEs). Many proteins exhibiting PME activity have been purified, and their biochemical features such as molecular weight, optimal pH, pI, and substrate specificity have been established (8). In some cases, the corresponding genes have also been cloned and sequenced (9–11), but so far three-dimensional models based on either crystallographic data or NMR analyses are lacking.

A limited number of investigations of the action pattern of PME have been reported (12–16). Partially deesterified pectin samples have been submitted to hydrolysis by endogalacturonases that are known to stop their attack in the vicinity of a methylated galacturonate unit, and the fragments released were subsequently characterized (13). In another approach, the activity coefficients of the PMEs (15, 16). The three mechanisms, which are generally evoked in the case of the plant PMEs, have also pointed to the importance of a free carboxyl group in the vicinity of the active site (13, 14, 17). A major drawback to the foregoing methods is that they provide only indirect information on the distribution of the carboxyl units after PME action and do not lead to a precise description of the reaction mechanism.

In quantitative studies of the action pattern of tomato PME, Grasdalen et al. have established the methoxyl distribution at various stages (DE) of the reaction with 1H NMR spectroscopy (15, 16). The three mechanisms, which are generally evoked in theoretical transformations of polysaccharides (18), were considered (see Scheme I): (a) multiple chain mechanism, where the enzyme-substrate complex dissociates after each reaction, resulting in conversion (deesterification) of just one residue for each attack; (b) single chain mechanism, where an enzymatic attack is followed by a contiguous conversion of all residues in

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†The abbreviations used are: DE, degree of methylsterification; PME, pectinmethyl esterases; DPn, degree of polymerization; MCM, multiple chain mechanism; SCM, single chain mechanism; MAM, multiple attack mechanism.
an E-block starting with the E-residue in the attacked UE-diad (where U and E are unesterified and esterified residues, respectively); and (c) multiple attack mechanism, where the enzyme deesterifies a limited average number of residues for every active enzyme-substrate encounter. Their results were best interpreted with the multiple attack mechanism, assuming that the enzyme attacked alternating UE sequences and deesterified linearly preferentially toward the reducing end.

The degree of polymerization (DPs) of the pectin samples used in this work was fairly low (n ~ 15 residues), and end group effects were necessarily important that might partially explain why the enzymatic deesterification deviated from an idealized block-producing mechanism. However, the major pitfall of these studies resides in the enzyme preparation as the tomato PME was not purified and contained several isoforms. Indeed, in most cases cell walls contain several PMEs differing in their biochemical properties and encoded by different genes (11). Moreover, the behavior of PME in solution sometimes differs from that exhibited by cell wall-bound enzymes (8), and it is known as the degree of multiple-attack.

**Scheme I.** E, U, and X are esterified, unesterified, and either esterified or unesterified residues, respectively; the esterified residue (underlines) to the left of \( \downarrow \) is the point of attack.

**MATERIALS AND METHODS**

### Pectin Samples—
The pectin sample (apple pectin classic AU 201 (DE 74.4%)) was graciously supplied by Herbstreith & Fox KG (Neuenburg, Germany). The thermal depolymerization protocol and the characteristics of the resulting polymers have been described previously (18). As regards the samples used in the present work, \(^{13}C\) NMR end group analysis indicated a DP of 100, whereas the one estimated (20) from the intrinsic viscosity ([η] = 0.5) was roughly 50. The totally esterified pectin was a gift from Dr. Baron.

### Enzyme Isolation and Assay—
Cell walls were isolated from the upper 2.5 cm of hypocotyl tissues of 3-day-old seedlings of mung bean (Vigna radiata) L. Wilczek according to a procedure previously described (1). PMEs bound to the cell wall fragments were solubilized with wall fragments with 1 M NaCl, and the different isoforms were recovered according to Bordenave and Goldberg (21, 22). Assay for PME activity estimation (total volume 6 ml) contained 30 mg of thermally depolymerized polymers. PME activity was measured titrimetrically by following the release of protons in the presence of 150 mM NaCl. The protons were titrated with 10 mM NaOH under nitrogen, the pH being maintained at 5.6 or 7.6 with a automatic titrator (TTT 80, Radiometer). The reaction rate, as \( \text{m} \mu \text{Eq}^{-1} \text{gPectin}^{-1} \text{h}^{-1} \), was measured at 25 °C.

### Deesterification Simulations—
Starting samples containing all possible distributions of the methoxyl groups for a 10-residue fragment of given DE (i.e. 210, 120, 45, 10 fragments for DE values of 60, 70, 80, and 90%, respectively) were first generated as a linear two-dimensional array. A polydisperse population (between 100 to 4000 chains) of polymers of given DP (\( n = 25, 50, 100, \) or 200) and DE (73%) with a random sequence were then built from these 10-residue fragments using a random number generator. The DE and the triad populations (EEE, EEU, UEE, UEU, EUE, UEU, EUU, and UUU, where the underscore designates the residue that is either being considered in the simulation algorithm or detected in NMR spectra) and the homopolymer E-block and U-block populations (for block-lengths of 1–100 residues) of the resulting two-dimensional arrays were evaluated and compared with the corresponding data of the pectins used for assaying enzyme activity. The enzymatic reactions were simulated as follows (see Scheme I). Random generation of the point of attack (the point of attack \( \text{E} \rightarrow \text{X} \) or \( \text{PA} \) is defined by the integers, which correspond to the chain and residue positions in the two-dimensional array), evaluation of constraint criteria, and sequential (single chain mechanism) or single residue (multiple chain) deesterification toward a given end of the macromolecule until a deesterified unit or the end of the chain was encountered (or a given number of residues, \( k \), in the case of the multiple attack mechanism). For suitable decrements in the DE (5%), the fractional triad populations (EEE, EEU, UEE, UEU, EUE, UEU, EUU, and UUU) and the homopolymer E-block and U-block populations of the resulting sample of polydisperse polymers were evaluated. The average E- and U-block lengths, \( N_E \) and \( N_U \), which included all the E or U residues except the isolated ones (EEU or EUE), were as follows: \( N_k = ((\text{DE} \cdot \text{UEU} \cdot \text{EUE}) \cdot (1 - \text{DE}) \cdot \text{EUE} / \text{UUE} \cdot \text{UEU}) \). The following R-factor has been used to evaluate the quality of the fits with the experimental data: \( \text{R} = \text{m}(\text{EEU}_{\text{expt}} - \text{EEU}_{\text{calc}}) + (\text{EEU} + \text{UUE}_{\text{expt}} - \text{UUE} + \text{UEU}_{\text{calc}}) + (\text{UEU} - \text{UEU}_{\text{calc}}) \), where the summation extends over all the DE values for which data were collected. Replacing the sums by the corresponding squared experimental average deviations afforded the experimental R-factor. The standard deviation in the triad populations, estimated from simulations initiated with different seeds for the random number generator was <1%.

### Potentiometry—
The degree of esterification was potentiometrically estimated as previously reported (1). Briefly, samples were dialyzed and weighed, and a portion was set aside for colorimetric estimation of the galacturonic acid content according to Blumenkranz and Asboe-Hansen (23).

### NMR Spectra—
In the case of a random distribution of methoxyl groups, thermally depolymerized pectins with a wide range of DE (35–75%) can be analyzed at room temperature, as the major fraction of the polymers adopts the random-coil conformation and is detected by NMR spectroscopy (19). However, because of a change in the distribution of the methoxyl groups upon deesterification with PME and/or the salt concentration, the pectins incubated with PME were much more prone to aggregation than the starting material. To optimize the signal-to-noise ratio in the \(^{13}C\) spectra, the samples were desalted on an ion-exchange resin (DOWEX, H\(^+\) form) and then dialyzed at 4 °C to obtain a less acidic pH (4 to 6 depending on the DE). Finally, EDITA (0.5 eq) was added to remove divalent cations. Totally decoupled \(^{13}C\) spectra were recorded at 80 °C on aqueous solutions of the pectins (~2% w/v) at 100.6 MHz.
120H, the weight and the uronic acid content were again determined to assure that all of the starting pectin was recovered from the ion-exchange resin. The sample was then neutralized under nitrogen flux with sodium hydroxide. After saponification (4 h, 4 °C) the new negative charges were estimated as above. The degree of esterification was calculated as the number of new charges over the total amount of galacturonic acid.

RESULTS

**Relationships between PME Activity and DE**—To visualize the effects of the degree of methylesterification of the substrate on the reaction rate of the deesterification process, plots of activity versus DE have been given in Fig. 1 for the long term incubations, which were performed either at pH 5.6 (○) or pH 7.6 (●). In all cases the initial reaction rate (i.e. the rate at DE 70–75%) at pH 5.6 has been normalized to 100. A, crude cell wall fragments containing all PME isoforms; B, PMEs solubilized from the cell walls; C, cell wall fragments treated successively with 0.2, 0.4, and 0.5 M NaCl, which contain only isoform PMEα; D, E, and F, purified isoforms PMEα, PMEγ, and PMEβ, respectively.

A dichotomy appears in the behavior of the three purified enzymes. Two of them, PMEα and PMEγ, reacted in a similar way to decreasing DE. At pH 7.6, plots of the reaction rate versus DE presented a plateau for high DE values (74–45%) followed by an abrupt regular drop to zero at lower DE values (24 and 32% for α and γ, respectively, Fig. 1, D and E). At pH 5.6, the decrease in activity started at DE values of about 65–70%, and it was sharper for PMEα than for PMEγ. At high DE, contrary to cell wall fragments or isoform mixtures, the maximal activity was higher at pH 5.6 than at pH 7.6, a situation that persisted over a larger range of DE values in the case of PMEγ. These data explain why PMEα and PMEγ have been reported to display different pH sensitivities when incubated with citrus pectin whose DE is around 65%; with such substrates PMEγ exhibits an acidic optimal pH, whereas PMEα activity is barely modified between pH 5.6 and 7.6 (21). The behavior of PMEβ differs markedly from that of the other two isoforms (Fig. 1F); at pH 7.6, for high DE values a plateau in activity is not detected, and for all DE values, activities are higher than at pH 5.6. This tendency was observed both when the enzyme was bound (Fig. 1C) and when it was solubilized (Fig. 1F), the only difference being an attenuation in the effect of the pH after solubilization of the enzyme. Finally, when a totally esterified pectin sample was incubated with PMEβ, enzymic activity was negligible.

**Triad Populations and Block Lengths of Pectin Incubated with PME Estimated from 13C NMR Spectra**—It has been demonstrated that the populations of triad structures such as UEU, EEE, UUU etc., and the average length of the E-blocks of
pectins can be estimated from the corresponding ¹H (15, 16) or ¹³C (24) NMR spectra (Fig. 2). The 100.6 MHz ¹³C NMR triad population data for different incubation periods with the various enzyme preparations have been collected in Table I along with the corresponding DE values. These latter data were in good agreement with the DE independently established by potentiometry, indicating that the random-coil species detected with the latter technique was representative of the polymer as a whole. The discrepancies in the minimum DE values obtained either during the kinetic activity measurements (Fig. 1) or in the course of NMR analyses (Table I) stems from a regain in enzyme activity upon raising the pH from 3 to 4–6 during the preparation of the NMR samples (see “Materials and Methods”). For example, upon lowering the pH of a solution containing PMEβ to 3 and storing at 5 °C for 2 weeks, the enzyme recovers 5 and 16% of its normal activity when the pH is raised to 5.6 and 7.6, respectively.

From inspection of the triad populations, it can be seen that the results obtained by incubation with either PMEα or PMEβ, at a given pH were analogous (see DE 23% at pH 5.6 for PMEα and PMEβ). As regards the esterified residues (XEX) at pH 5.6, the EEE and UEE populations were the major and minor ones, respectively, at DE values as low as 23%. In contrast, at pH 7.6, the EEE and EEU + UEE populations were similar, and the UEU population became significant below DE values of 50%. Subsequently, all the data for PMEα and PMEβ were simultaneously fitted to the same theoretical plots of triad populations versus DE to increase the number of experimental points. In the case of incuba

The single chain and multiple attack mechanisms (SCM and MAM) were next systematically evaluated with 0–3 constraints. In the case of the latter mechanism, multiple attack leading to the deesterification of 2–5 (degree of multiple attack, k in the scheme) residues/active encounter was considered. Simulations with more than one constrained or specified residue led to monotonously decreasing reaction profiles for the EEE and EEU + UEE triad populations, which did not reproduce the experimental data and gave very poor agreement for UEU. However, in the case of simulations with one constraint, reasonable fits to the experimental data were observed for some of the SCM and MAM simulations. Generally speaking, the UEU versus DE curve varied significantly when the neighboring constrained U residue was located between 1 and 3 residues away from the point of attack, but when the constrained residue was placed at an even greater distance to the point of attack, subsequent variations in the UEU versus DE profiles became negligible.

In the case of the incubations with the α and γ isoforms at pH 5.6, the best fit was obtained for all the esterified triad populations with the SCM reaction as long as the constrained U residue was separated from the point of attack by one unit ([XUXE]↓), and plots of the corresponding theoretical triad populations (solid lines () and () for DP values of 200 and 25, respectively) have been given in Fig. 3A (experimental data at pH 5.6 []). An R² value of 0.0123 was calculated for the fit between the experimental and theoretical data, and it was analogous to the experimental precision (R²).
Table I

Triad populations (EEE, EEU+UEE, UEU and UUU) as a function of DE estimated from 100.6 MHz 13C NMR spectra of pectin samples incubated with PME from Vigna Radiata

| Enzyme | DE | pH = 5.6 | | pH = 7.6 |
|--------|----|----------|----|----------|
|         |    | EEE | EEU | UEU | UUU |    | EEE | EEU | UEU | UUU |
| α      | 13.5 | 6  | 4  | 3.5 | 80 | 8 | 8 | 8 | 8 | 8 | 81.5 |
| 23     | 11  | 7.5 | 4.5 | 59.5 | 21.5 | 5 | 6.5 | 10 | - | - | - |
| 44.5   | 21.5 | 12.5 | 9.5 | 33.5 | 48 | 18.5 | 17.5 | 12 | - | - | - |
| 50.5   | 27 | 16 | 7.5 | 27.5 | 54 | 24 | 20 | 10 | 27.5 |
| γ      | 23 | 10.5 | 8 | 4.5 | 61.5 | 12 | 4 | 2.5 | 5.5 | 81.5 |
| 46.5   | 23 | 15.5 | 8 | 36 | 38 | 14.5 | 10.5 | 13 | 32 |
| 50     | 26.5 | 17 | 6.5 | - | 40 | 15 | 14 | 11 | 37 |
| β      | 19 | 4.5 | 6 | 8 | 59.5 | 33 | 15 | 12 | 8 | 49 |
| 27     | 6.5 | 12.5 | 8 | 66.5 | 37.5 | 15 | 16 | 6.5 | 45 |
| 37.5   | 15.5 | 15 | 7 | 43 | 39 | 16.5 | 15 | 8.5 | 37 |
| 42.5   | 16 | 19 | 7.5 | - | 40 | 16.5 | 15 | 8.5 | 37 |
| 49     | 20.5 | 21.5 | 7 | 33 | 45 | 20.5 | 19 | 5.5 | 29.5 |
| α,β,γ  | 40 | 21 | 11.5 | 7.5 | 40.5 | 34 | 11.5 | 14.5 | 8 | 47 |
| Cell wall fragments | 35.5 | 13.5 | 14.5 | 7.5 | 40 | 40 | 16.5 | 16.5 | 7 | 38 |
| 43     | 20.5 | 13 | 9.5 | 38 | 42 | 16.5 | 18 | 7.5 | 40.5 |
| 51     | 6 | 15 | 9 | 53.5 | 42 | 16 | 19 | 7 | 42.5 |
| Eluted cell wall fragments | 51 | 165. | | 22 | 12.5 |

Table II

Estimations of the agreement between theoretical and experimental triad populations (R-factors x 10^-3) for simulations of various mechanisms (see Scheme I) of PME-mediated deesterification of pectins

Partial R-factors for individual triad populations (EEE, EEU+UEE, UEU) have also been given. Data in italics indicate the mechanisms fitting the three populations. Rf = \sum((EEE\text{expt} - EEE\text{calc})^2) + (EEU\text{expt} - EEU\text{calc})^2 + (UEU\text{expt} - UEU\text{calc})^2, where the summation extends over all the DE values in which data were collected. The experimental R-factor (bold) was obtained by replacing the sums in this expression by the corresponding squared experimental average deviations.

| Enzyme | pH | Exp | SCM XUXE | X | XUE | | XUXE | X | XUE | | SCM XUXE | X | XUE |
|--------|----|-----|----------|| | |----------|| | | |----------|| | |
| αγ,β,γ | 2.00 | 1.25 | 2.35 | 3.92 | 15 | 1.42 |
| (0.88) | (0.57) | (1.00) | (2.10) | (9.70) | (0.09) |
| (0.60) | (0.16) | (0.22) | (1.56) | (2.39) | (0.33) |
| (0.52) | (0.52) | (1.13) | (0.26) | (2.91) | (1.10) |
| αγ,β,γ | 2.08 | 1.11 | 0.51 | 3.52 | 6.53 | 7.16 |
| (0.76) | (0.35) | (0.15) | (0.13) | (3.94) | (1.98) |
| (0.68) | (0.10) | (0.20) | (1.51) | (2.20) | (0.33) |
| (0.64) | (0.66) | (0.18) | (1.88) | (0.39) | (4.85) |
| β,γ,δ | 1.44 | 1.30 | 2.07 | 0.30 | 5.15 | 3.21 |
| (0.56) | (0.19) | (0.09) | (0.12) | (3.16) | (1.62) |
| (0.99) | (1.40) | (0.11) | (0.17) | (0.24) | |
| (0.44) | (0.12) | (0.58) | (0.07) | (3.06) | (1.35) |
| β,γ,δ | 1.68 | 1.12 | 2.68 | 1.17 | 11.2 | 1.83 |
| (0.72) | (0.08) | (0.34) | (0.72) | (7.56) | (0.72) |
| (0.72) | (0.68) | (1.72) | (0.38) | (0.56) | (0.08) |
| (0.24) | (0.38) | (0.34) | (0.07) | (3.06) | (1.03) |

* n indicates the number of residues X inserted.

praisal of Table II suggests that the SCM mechanism involving attack of UE diads (XUXE) also gives a good fit to the experimental data (Rf 1.42). However, when one inspects the Rf values for the individual triad populations (Table II, values in italics), it can be seen that this mechanism is far from reproducing the experimental populations for UEU (0.0113 versus 0.0052 for Rf and Rf\text{expt}, respectively). As these latter populations are low, their contribution to the Rf values is often masked by that of the much larger EEE and the EEU + UEU triad populations. Comparison of the UEU population versus DE for long (DP 200 (Fig. 3A, solid lines (□)) and short (DP 25 (Fig. 3A, solid lines (□)) chains shows two effects; for the shorter chains this population is smaller at low DE, and the reaction stops at higher DE than for the longer chains. At pH 7.6, the best results for incubations with the α and γ isozymes were obtained for MAM involving the deesterification of 2-5 residues with the free carboxyl unit in the same position (XUXE) (Fig. 3A, experimental and theoretical data, ■ and dotted lines, respectively).

In the case of the β isozyme, many of the SCM and MAM simulations (without constraints, XUXE, EUE, and XUEX) gave the same reasonable fits to the experimental data. SCM and MAM mechanisms without constraints or with only an esterified residue in the vicinity of the point of attack could be rejected, as the β isozyme was unable to deesterify totally esterified pectin. The smallest Rf value was obtained when the free carboxyl group was between 3 to 10 residues away from the point of attack (XUXE), but other mechanisms reproduced all the individual triad populations within the experimental precision. The SCM (XUXE and MAM (XUXE, k = 2) mechanisms, which gave the best fit to the experimental NMR data for PME, and PME were notable exceptions. Here, in spite of the low Rf, certain individual triad populations gave very poor fits. A plot of the SCM triad profiles...
for the most favorable mechanism for the β isofrom (solid lines; XUXE □) has been given in Fig. 3B along with the corresponding experimental data at pH 5.6 and 7.6 □ and □, respectively.

Finally, the plots of the average length of the E- and U-blocks \((N_E\) and \(N_U\)) versus the DE have been given in Fig. 4A (●, α and γ isoforms; ○, β isoform). Similar profiles were obtained for all the satisfactory mechanisms of a given isoform irrespective of the pH. As can be seen in Fig. 4A, in the case of the α and γ isoforms, the average E-block length

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**Fig. 3.** Plots of the best fits to experimental triad populations (left EEE, middle EEU+UEE and right UEU) at pH 5.6 (○) and pH 7.6 (□) for simulations of ideal blockwise deesterification mechanisms. A, α and γ isoforms (above): XUXE \(\times\) X and SCM for DP 200 (solid line and ○), MAM with \(k = 2\) (dotted line and ×), and SCM for DP 25 (solid line and +). B, β isoform (below): XUXE \(\times\) X and SCM (solid line and +). Error bars for the experimental data have also been indicated.

**Fig. 4.** A, plots of the evolution of the average E-block \((N_E, \text{ left})\) and U-block \((N_U, \text{ right})\) lengths versus DE for the simulations of ideal blockwise deesterification mechanisms in Fig. 3: ●, α and γ isoforms; ○, β isoform. B, plots of the evolution of the UEE and UEEE (left) and EUE and EUEU (right) populations with DE for the simulations of ideal blockwise deesterification mechanisms in Fig. 3: ● and □, α and γ isoforms; β, ○ and □.
remains constant (4.8) as the DE decreases from 74% to about 15% and then increases when the DE drops to below 10%. In contrast, for the β isomorf, the average E-block length decreases slowly with the DE% and then increases sharply when the DE drops to below 10%. The experimental data confirm this behavior for incubations with the α and γ isomors on the one hand and the β isomorf on the other. In parallel, differences in the theoretical populations of short E-blocks (i.e. UEU and UEEU) for the preferred mechanisms for the α and γ isomors (on the left, ○ and ●) and the β isomorf (on the left, ○ and □) are also observed (Fig. 4B). The former mechanisms (α/γ) lead to much stronger populations for UEU than UEEU, whereas the latter one (β) produces more similar populations for these short blocks. Inspection of the average U-block lengths and the populations for the short U-blocks as a function of DE in Figs. 4, A and B showed that fairly analogous distributions were produced by all the isomors.

**DISCUSSION**

The data reported above shed new light on the reactions in the apoplasm, which modify the structural features of the pectic polymers. Previous investigations on pectin from mung bean hypocotyl (1) have shown that the DE decreases along the growth axis from 58.4 to 44.5%, whereas the PME activity solubilized from the cell walls doubles. This suggested that these enzymes were involved in the accumulation of galacturonic in mature stiff cell walls. It is well known that the pH of the apoplasm is acidic (pH 5.5–6.0), and under these conditions, the V_{max} of the different PME isomors is markedly different, because studies with citrus pectin (DE 65%) have led to activities of 0.4, 0.08, and 0.8 μeq min^{-1} μg^{-1} for PME_{α}, PME_{γ}, and PME_{β}, respectively (25). These data show that PME_{α} and PME_{γ} activities are undoubtedly those that contribute the most to the decrease in DE in vivo. Fig. 1 indicates that, at acidic pH, the activity of these two isomors drops abruptly for DE values below 60%. The balance between methyl and free carboxyl groups can then be considered as a key factor in the control of PME-mediated deesterification. This feedback control will prevent the generation of totally deesterified polymers but allows the formation of long U-blocks along the polymer chain. The presence of these blocks in mature cell walls favors the formation of junction zones via calcium ions and will then contribute to the cell wall stiffening that develops along the hypocotyl.

An explanation for the dichotomy in the action patterns of the PME isomors from V. radiata has been obtained by simulation of the triad populations established from 13C NMR. In the case of the pure α and γ isomors, a single chain mechanism requiring a free carboxyl group at the second nearest neighbor position (XUXE ⊑ X) gave the best fit at pH 5.6, whereas at pH 7.6 the reaction displayed multiple attack character involving the deesterification of only between 2 and 5 residues. Thus, the pH strongly effects the efficiency of the deesterification process. A change in reaction mechanism with a variation in pH has been reported for porcine pancreatic α-amylase (26). In contrast, successful simulation of the action pattern of the β included a specific interaction (E or U) at least three residues away from the active site (e.g. XUXXE ⊑ X). It should be emphasized that the methoxyl distribution in the polysaccharide produced by PME-mediated deesterification depends on the conditions (constrained residues) governing the reaction and not on the type of process (MCM, SCM, or MAM). It would appear that the distance between crucial complementary polar groups and the catalytic site varies slightly in going from the α/γ isomors to the β isomorf, but three-dimensional structures for all the isomors will be required to fully interpret these results. At present, only the complete amino acid sequence of PME has been determined (25).

Interestingly, the lowest DE values attained experimentally for the pure isomors (for example, 13.5–23% at pH 5.6) are significantly higher than those predicted by the simulations (<5.5 to 2.5% for chains with DP = 50–100), which implies that the reaction stops because of factors that are not accounted for in the simulation algorithm (i.e. reasons other than a lack of substrate with a suitable methoxyl distribution). One possible explanation is that the free carboxyl group on the pectin near the active site may become protonated, thus weakening the polar interaction. However, van der Waals interactions between some of the methoxyl groups and hydrophobic amino acids may make an important contribution to the binding energy and partially explain why, when the methoxyl group population or DE decreases below 45–55%, the enzymatic activity drops very abruptly. In the case of protein-protein interactions, the stabilization from a hydrophobic contact has been estimated to be about half of that produced by complementary polar groups (27), whereas in the case of protein-carbohydrate interactions (28), the favorable effects of electrostatic and van der Waals interactions probably contributes to pectin-PME binding.

The methoxyl group distribution in pectins incubated with the α and γ or β PME isomors from V. radiata is significantly different, particularly with respect to the short E-blocks. The block-producing character of the deesterification process leads to highly aggregated polymers, and in the future, such enzymes may find industrial applications as they should be able to confer tailor-made rheological properties to commercial pectins.

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