Pectin Modification in Cell Walls of Ripening Tomatoes Occurs in Distinct Domains[1]

Nancy M. Steele[2], Maureen C. McCann*, and Keith Roberts

Department of Cell Biology, John Innes Centre, Norwich Research Park, Colney,
Norwich NR4 7UH, United Kingdom

The class of cell wall polysaccharides that undergoes the most extensive modification during tomato (*Lycopersicon esculentum*) fruit ripening is pectin. De-esterification of the polygalacturonic acid backbone by pectin methylesterase facilitates the depolymerization of pectins by polygalacturonase II (PGII). To investigate the spatial aspects of the de-esterification of cell wall pectins and the subsequent deposition of PGII, we have used antibodies to relatively methylesterified and nonesterified pectic epitopes and to the PGII protein on thin sections of pericarp tissue at different developmental stages. De-esterification of pectins and deposition of PGII protein occur in block-like domains within the cell wall. The boundaries of these domains are distinct and persistent, implying strict, spatial regulation of enzymic activities. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins strongly associated with cell walls of pericarp tissue at each stage of fruit development show ripening-related changes in this protein population. Western blots of these gels with anti-PGII antiserum demonstrate that PGII expression is ripening-related. The PGII co-extracts with specific pectic fractions extracted with imidazole or with Na_2CO_3 at $0°C$ from the walls of red-ripe pericarp tissue, indicating that the strong association between PGII and the cell wall involves binding to particular pectic polysaccharides.

During tomato (*Lycopersicon esculentum*) fruit ripening pectins in the cell walls of pericarp cells undergo extensive depolymerization. Pectic polysaccharides fall into three classes: HGA, RGI, and RGII (McCann and Roberts, 1991; Carpita and Gibeaut, 1993). The HGA molecules consist of a 1,4-linked α-D-galacturonic acid backbone and carry few side chains. RGI has been described as a "block" polymer (Jarvis, 1984), consisting of regions of "hairy," highly branched stretches of backbone comprising alternating galacturonic acid and rhamnosyl residues interspersed with long stretches of "smooth," HGA-like backbone carrying few side chains. The RGII molecules have a 1,4-linked α-D-galacturonic acid backbone with side chains that are extremely complex in sugar and are present in low

amounts within the cell wall (O'Neill et al., 1992). Borate diester cross-links may exist between dimers of RGII (Ishii and Matsunaga, 1996; Kobayashi et al., 1996).

In parenchyma tissue of some fruits and vegetables, including tomato, the pectins of the middle lamella are thought to be HGAs with a low degree of methylesterification (Selvendran, 1984). Calcium ions play a major role in binding these unesterified pectins together by forming Ca^{2+} cross-bridges between the negatively charged carboxylic acid groups of the galacturonosyl residues. The pectins of the primary cell wall are thought to be more highly branched, with longer side chains. The carboxylic acid groups on the molecules' backbone are extensively methylesterified, reducing the negative charge associated with them and thus the potential for Ca^{2+} cross-bridging. Ester linkages are thought to be involved in cross-linking this pectic gel (Selvendran, 1984).

Modifications to cell wall components must be performed by proteins that are active within the cell wall itself. The degree of pectic esterification drops from 90% in immature green fruit to 30% by the red-ripe stage of ripening. Most of this de-esterification occurs during the 4 d that are required for the fruit to ripen from breaker to pink (Koch and Nevins, 1989). The de-esterification event is presumed to be primarily the consequence of the activity of PME, which removes the methylester groups from the C6 position on the galacturonosyl residues of pectic polysaccharide backbones. The de-esterified HGA backbone is then susceptible to the activity of PG.

Three isozymes of PG (PGI, PGIIA, and PGIIB) have been isolated and shown to result from modifications to a single gene product (Dellapenna et al., 1990). PGIIA and PGIIB are differentially glycosylated. The enzyme designated PGI, with a molecular mass of approximately 100 kD (Tucker et al., 1980), comprises a 46-kD PGII polypeptide tightly complexed with a β-subunit or cofactor (Pogson and Brady, 1993). Results of the genetic manipulation of tomato (Dellapenna et al., 1990; Smith et al., 1990; Watson et al., 1994) and tobacco (Osteryoung et al., 1990) plants have led to the putative assignment of distinct hydrolytic activities to PGII and the β-subunit. The β-subunit has been proposed to function as an anchoring component for PGII and to be synthesized early in fruit development (Knegt et

[2] Present address: Division of Biological Sciences, The University of Edinburgh, Daniel Rutherford Building, The King's Buildings, Mayfield Road, Edinburgh EH9 3JH, UK.

* Corresponding author; e-mail maureen.mccann@bbsrc.ac.uk; fax 44-1603-501771.

Abbreviations: CWM, cell wall material; HGA, homogalacturonan; PG, polygalacturonase; PME, pectin methylesterase; RG, rhamnogalacturonan.

al., 1988). However, the extent of in vivo association to form PGI is controversial, with some evidence suggesting that the association may be an artifact of co-extraction of the two subunits (Moore and Bennett, 1994). Transgenic tomatoes with expression of the β-subunit down-regulated show increased pectin depolymerization, with the implication that the β-subunit may restrict the activity of PGII either by directly binding to it in vivo or by binding to its substrate (Watson et al., 1994).

Modifications to the pectic polysaccharides within the cell walls of the ripening pericarp tissue occur over a period of days. Both PME and PG are expressed by the cells of the ripening pericarp tissue at the breaker stage of development. However, full de-esterification of the pectins requires 4 d of PME action (Koch and Nevins, 1989), and pectic depolymerization continues until the tomato fruit becomes overripe (Huber and Lee, 1988), although most of the pectic depolymerization occurs at the turning stage (Dellapenna et al., 1990; Watson et al., 1994). In vitro studies show that the two enzymes are capable of substrate modification in much shorter periods of time, in minutes rather than days (Seymour et al., 1987b; Koch and Nevins, 1989). This indicates that pectin modification within the wall during ripening is a tightly regulated process.

To investigate modifications to the pectic polymers that occur in situ within the cell wall during tomato fruit ripening, antibodies against methylesterified and nonesterified pectic polymers and PGII were used on thin sections of tomato pericarp tissue at different developmental stages of ripening. The association between the PGII protein and the cell wall was further investigated by the use of SDS-PAGE and western analysis of proteins strongly associated with, and co-extracting with, the pectic polysaccharides of the ripening pericarp cell wall.

MATERIALS AND METHODS

Biological Material

Tomatoes (*Lycopersicon esculentum* cv Ailsa Craig) at the immature green, mature green, breaker, turning, orange, red, and red-ripe stages were obtained from Unilever Research (Colworth, UK), a kind gift from J. Barroclough and Dr. M. Gidley.

Sample Fixation and Embedding

One- to 2-mm-thick blocks of tomato pericarp tissue were fixed in 2.5% (w/v) glutaraldehyde in 0.05 M sodium cacodylate adjusted to pH 7.2. Care was taken to ensure that the piece spanned the entire width of the tissue, including the epidermis and the endocarp.

The tissue pieces were then low-temperature-embedded for immunogold labeling, as described previously (Wells, 1985; Wells et al., 1994). Thin sections (0.1 μm) were cut on an ultramicrotome (Reichert-Jung Optische Werke AG, Vienna, Austria) and picked up on carbon-coated, plastic-filmed gold grids (Agar Scientific, Ltd., Stansted, UK).

Immunogold Labeling

Thin sections on filmed gold grids were incubated on drops of either 10% (w/v) sheep serum (for labeling with JIM 5 and JIM 7 monoclonal antibodies [Knox et al., 1990]) or 2% (w/v) powdered milk, 3% (w/v) BSA, and 1% (w/v) Tween 20 (for the anti-PGII antibody) in PBS to block for 10 min. The grid was then placed on a drop of primary antibody diluted in a blocking solution and left at 4°C overnight. After six washes of 10 min each in PBS, the grids were placed on drops of goat anti-rat IgG coupled to 10-nm-diameter colloidal gold particles at a dilution of 1:1000 in a blocking solution and incubated at 4°C for 1 h. After thorough washing in distilled water, the sections were stained with 5% uranyl acetate for 10 min, washed thoroughly in distilled water, and then stained with saturated lead citrate for 30 s. The sections were washed thoroughly and viewed in a transmission electron microscope (1200 EX, Jeol). Representative micrographs were selected from many micrographs of at least four sections from each of three fruits at each developmental stage.

Protein Extraction

Pericarp tissue was homogenized in a sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% [v/v] glycerol, 5% [v/v] β-mercaptoethanol, 2% [w/v] SDS, and 0.01% [w/v] bromphenol blue) and boiled for 5 min to extract total protein. Proteins from the detergent-cleaned walls were extracted by boiling in the sample buffer for 5 min. The supernatant was collected by centrifugation at 15,000g for 10 min and the proteins were acetone-precipitated. The pellet was then resuspended in the sample buffer.

Antibody Production

Preimmune screens of the LOU-C rats were performed using the methods described by Harlow and Lane (1988). Western blots of proteins extracted from pericarp tissue and cleaned cell walls of both immature green and red-ripe tomatoes were blocked with 3% (w/v) BSA, 2% (w/v) powdered milk, and 1% (w/v) Tween 20 in PBS and probed with preimmune sera. If these sera failed to recognize any of the protein bands that were present, the rat was selected for inoculation with the antigen. Rats were immunized with 75 μg of PGII in 125 μL of PBS mixed with 125 μL of complete Freund's adjuvant. After 3 weeks a boost inoculation was given (75 μg of PGII), but this and subsequent inoculations were in incomplete Freund's adjuvant. A third and final inoculation was given 3 weeks after the second. The PGII protein was a kind gift from Dr. Greg Tucker (University of Nottingham, UK).

Preparation of Tomato Pericarp CWM

CWM was prepared and polymer fractions extracted, dialyzed, and freeze-dried as described by McCann et al. (1990).

Detergent Extraction of CWM

CWM was glass-on-glass-homogenized on ice in 1 mL of buffer (40 mM glycylglycine [pH 7.5], 1% [v/v] Triton

X-100, and 500 mM KCl) per 100 mg fresh weight of cell walls. The homogenate was centrifuged at 1000g for 10 min at 4°C, and the pellet was washed three times in 40 mM glycylglycine (pH 7.5) and collected by centrifugation at 4°C (Atwell and ap Rees, 1977).

Pectin-Associated Proteins

Freeze-dried pectic fractions were boiled in the sample buffer at 3 mg mL^{-1}, and the supernatant was collected by centrifugation at 15,000g for 10 min. The proteins were then acetone-precipitated and resuspended in the sample buffer.

One-Dimensional Polyacrylamide Gels

Proteins were separated by electrophoresis through a denaturing SDS-polyacrylamide gel of 14 or 10% (w/v) (Laemmli, 1970), using a dual mini-slab kit (AE-6400, Atto Corp., Tokyo, Japan). Samples were solubilized in the sample buffer at 100°C for 5 min and loaded into the resolving gel via slots in the stacking gel of 2.5% polyacrylamide (w/v). The polyacrylamide gel was run for approximately 2 h at 170 V. The following molecular mass markers were used: myosin (200 kD), phosphorylase b (97.4 kD), BSA (69 kD), ovalbumin (46 kD), carbonic anhydrase (30 kD), trypsin inhibitor (21.5 kD), and lysozyme (14.3 kD).

Western Analysis

SDS-PAGE-separated proteins were electroblotted (Towbin et al., 1979) onto nitrocellulose membrane (Hybond C Super, Amersham) before probing with primary antibody, using secondary antibodies conjugated with alkaline phosphatase (Sigma) to detect binding.

RESULTS

Pectic De-Esterification

The JIM 5 antibody recognizes a relatively unesterified pectic epitope, whereas the JIM 7 antibody recognizes a relatively methylesterified pectic epitope (Knox et al., 1990). Weak labeling with the JIM 5 antibody in green pericarp tissue showed that the unesterified pectic epitope was confined to the middle-lamellar region of the wall (Fig. 1a). However, there was strong labeling with the JIM 7 antibody throughout the primary cell wall of the green fruit (Fig. 1b). The labeling pattern of the JIM 5 antibody within the cell walls of red-ripe pericarp showed that there had been a change in the localization of the unesterified pectic epitope. In contrast to being localized exclusively to the middle-lamellar region, the epitope was now throughout the primary cell wall (Fig. 1c). Labeling of the red-ripe pericarp cell walls with JIM 7 showed that there were still
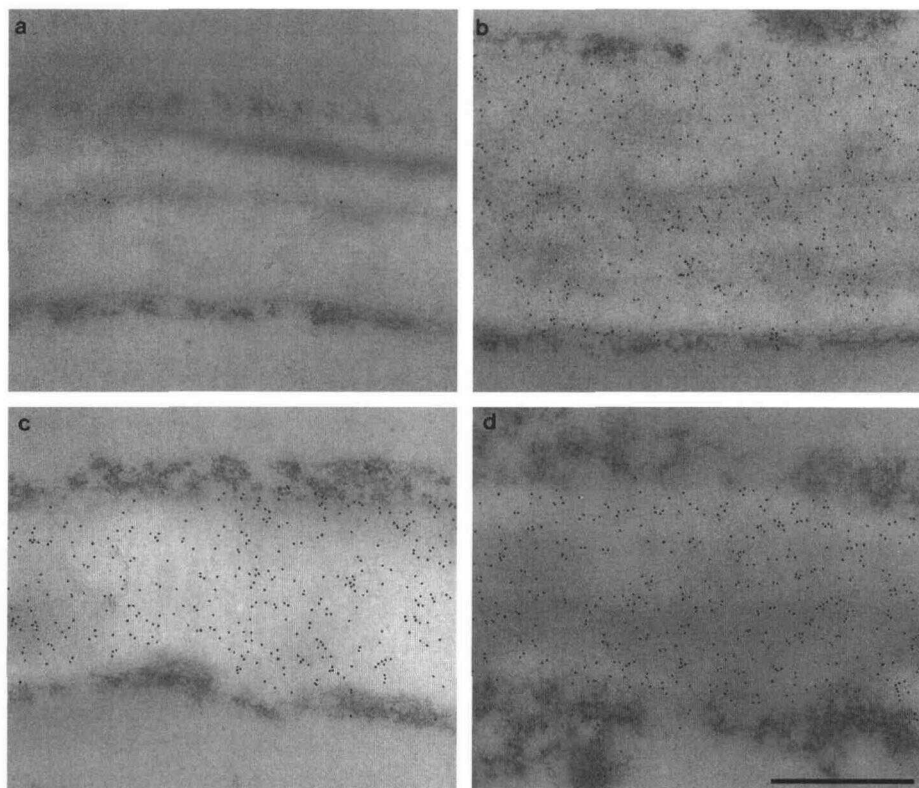

Figure 1. a, The unesterified pectin recognized by JIM 5 is localized to the middle-lamellar region of the immature green pericarp cell wall. b, The methylesterified pectin recognized by JIM 7 is distributed throughout the primary cell wall in the immature green pericarp tissue. c, In contrast, the unesterified pectin in red-ripe pericarp tissue is present throughout the primary cell wall. d, Methylesterified pectin is present throughout the cell wall of red-ripe fruit. Scale bar = 500 nm.

large amounts of methylesterified pectic epitopes throughout the cell wall (Fig. 1d). However, within the lengths of the cell walls that were labeled with JIM 7, there were occasionally small areas in which the middle-lamellar region had a reduced density of JIM 7 label (Fig. 2a). In some extreme cases there was almost complete removal of the methylesterified epitope from the primary cell wall in these areas, leaving label localized only in the wall immediately adjacent to the plasma membrane (Fig. 2b). The cell walls appear wider in Figure 2 than in Figure 1, since glancing sections have been selected to illustrate the point.

Pectin de-esterification, from the middle lamella throughout the entire primary cell wall, occurred in a block-wise fashion. Individual cell walls were de-esterified independently from their neighbors, and the process appeared to occur in discrete domains along the length of each wall. These domains eventually joined together, until JIM 5 labeled throughout the wall at the red-ripe stage. This de-esterification was first apparent in the pericarp walls of breaker fruit. At this stage, some regions of the wall showed middle-lamellar localization of the relatively un-esterified pectic epitope, whereas other regions showed labeling throughout the wall (Fig. 3). By the orange stage, the majority of the cell wall was labeled with JIM 5. Figure 4 shows that the pectin within the wall of one cell can remain relatively methylesterified, whereas the wall of the neighboring cell can undergo de-esterification, which is a cell-autonomous event.

PGII Deposition

Once de-esterified, the pectic polysaccharides are susceptible to the activity of PGII, which hydrolyzes the polygalacturonic acid backbone of the polysaccharide (Seymour et al., 1987a). A polyclonal antiserum against PGII was raised to localize this enzyme during ripening.
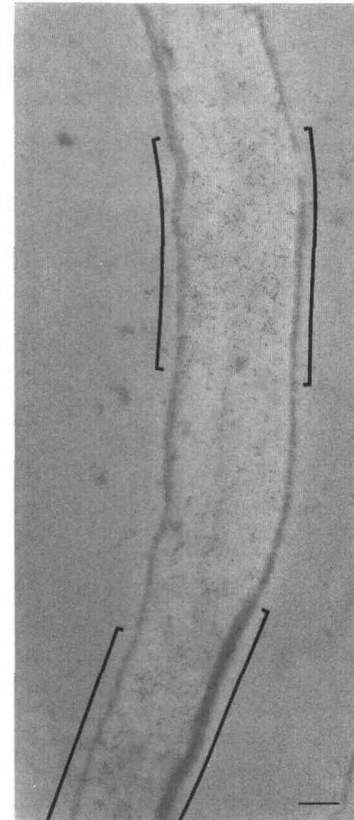

Figure 3. Labeling with the JIM 5 antibody shows that the ripening-related de-esterification of pectins is first apparent in the pericarp cell walls of breaker fruit. It occurs in a block-wise fashion, with individual walls de-esterified independently from their neighbors, and in discrete domains along their length. The brackets indicate the discrete domains of the de-esterified pectic epitope, whereas the unmarked regions of the wall maintain middle-lamellar localization of the unesterified pectin. Scale bar = 500 nm.
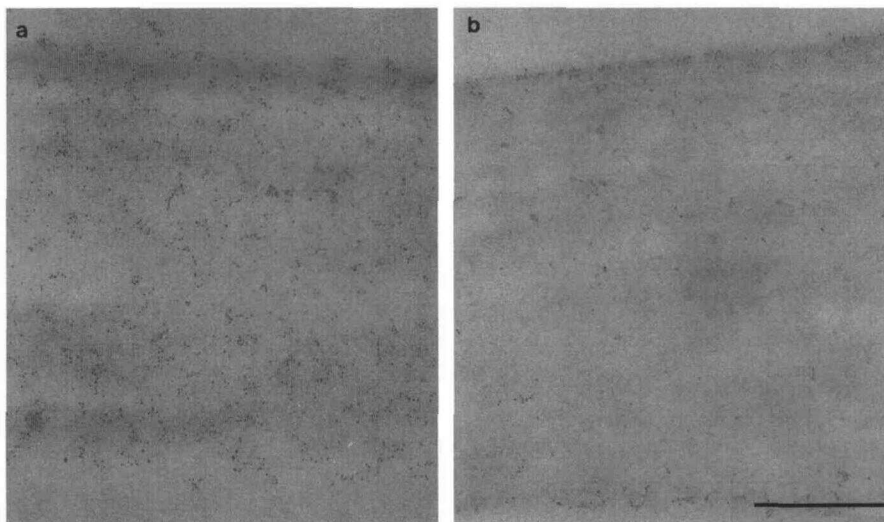

Figure 2. a, Within the regions of pericarp walls of red-ripe tomatoes that label with the JIM 7 antibody, small areas were occasionally seen in which the middle lamella showed a reduced labeling density. b, In some cases, this reduced labeling density was extreme, with JIM 7 localized to the regions of the wall immediately adjacent to the plasma membrane. Scale bar = 500 nm.
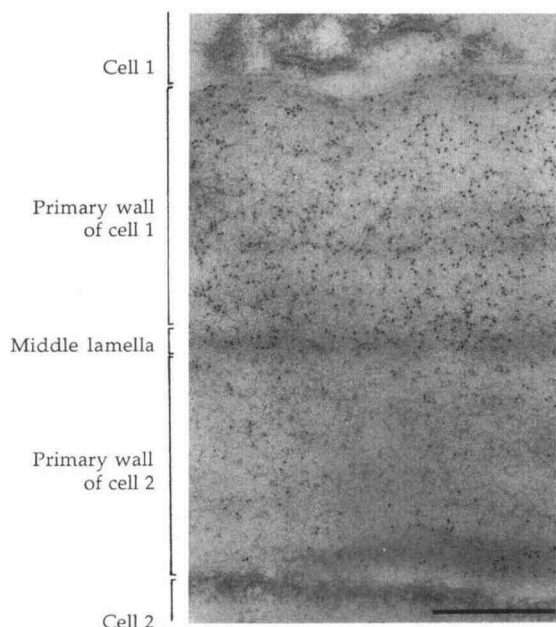
Figure 4. In pericarp cell walls of orange tomatoes, labeling with the JIM 5 antibody shows that the pectin within the wall of one cell has undergone de-esterification, whereas domains of the neighboring cell wall remain methylesterified. Scale bar = 500 nm.

The rat preimmune serum showed no cross-reactivity with proteins that were extracted from green or red-ripe tomato pericarp tissue (Fig. 5a) on western analysis. After immunization with the purified PGII protein, the rat antiserum cross-reacted with a protein of about 46 kD, which was extracted from pericarp tissue and detergent-cleaned walls of ripe fruit, that was absent from the green fruit (Fig. 5b), as well as the PGII preparation. The antiserum was then used to label thin sections of London Resin White-embedded pericarp tissue of tomato at the immature green, mature green, breaker, turning, orange, red, and red-ripe developmental stages.

Cell walls of green pericarp tissues showed very weak labeling (Fig. 6a), but not above the background. The walls of the red-ripe pericarp were strongly labeled with the anti-PGII antiserum (Fig. 6d). There had been a large increase in the quantity of the protein present within the cell walls of the fruit pericarp tissue, and this labeling was cell wall-specific.

The deposition of the PGII protein within the walls occurred in a block-wise fashion, in a pattern similar to that of wall de-esterification. Each domain of the primary cell wall labeled with the anti-PG antisera independently of the neighboring wall and of adjacent regions along its own length. As ripening progressed, the domains joined together and the whole wall became labeled. This effect was first seen in the walls of breaker fruit, in which distinct regions of the anti-PGII reactivity could be seen in the individual cell walls (Fig. 6b). By the orange stage, the majority of the walls labeled with the anti-PGII antibody and the block-wise deposition was still apparent (Fig. 6c). Once the fruit reached the red-ripe stage, all of the regions

within the primary cell wall was labeled with the antibody, showing uniform labeling along their lengths.

Labeling of Wall-Associated Protein

Walls were prepared by grinding pericarp tissue from each developmental stage in liquid nitrogen, and then thawing and homogenizing in a buffered solution of Triton X-100 to remove all of the remaining cytoplasmic contaminants and residual membranes (Atwell and ap Rees, 1977). Proteins from these clean walls were then extracted by boiling them in the sample buffer, and collected in the supernatant after centrifugation. Proteins that were extracted from these walls are likely to have been quite strongly associated with the wall polymers, since they remained after the homogenization.

The silver-stained gel (Fig. 7a) showed many changes in the repertoire of proteins extracted from the detergent-cleaned walls of pericarp tissue at different stages of fruit ripening. A sister gel was western-blotted overnight and probed with the anti-PGII antiserum. The western blot showed the appearance of the PGII protein in the population of extracted wall proteins from the breaker stage of ripening (Fig. 7b). The band intensity increased progressively from a faint, but distinct, band in the breaker wall extract to a broad, more-diffuse band in the red-ripe extract.

Labeling of Protein Co-Extracting with Pectins

Pectic polysaccharides were obtained by sequentially extracting tomato pericarp walls of green and ripe fruit with two consecutive treatments each of 2 M imidazole and 50 mM Na_2CO_3 to extract Ca^{2+}-chelated pectins and ester-linked pectins, respectively. This yielded the first and second imidazole-extractable fractions and the first and second Na_2CO_3-extractable fractions, which were all
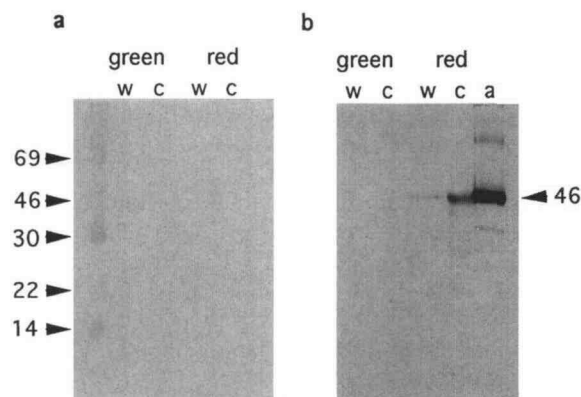

Figure 5. a, Western blot of a polyacrylamide gel of cell wall (w) and of whole-tissue (c) protein extracts of immature green and red-ripe pericarp tissue probed with rat preimmune serum shows no cross-reactivity. b, Western blot of a sister gel to that used in the preimmune screen, with an additional lane containing a sample of the PGII preparation used to inoculate the rat (a). The antiserum recognizes a 46-kD band present in the cell wall and whole-tissue extracts of ripe pericarp, which is absent in the extracts from green fruit, in addition to bands in the PGII preparation.
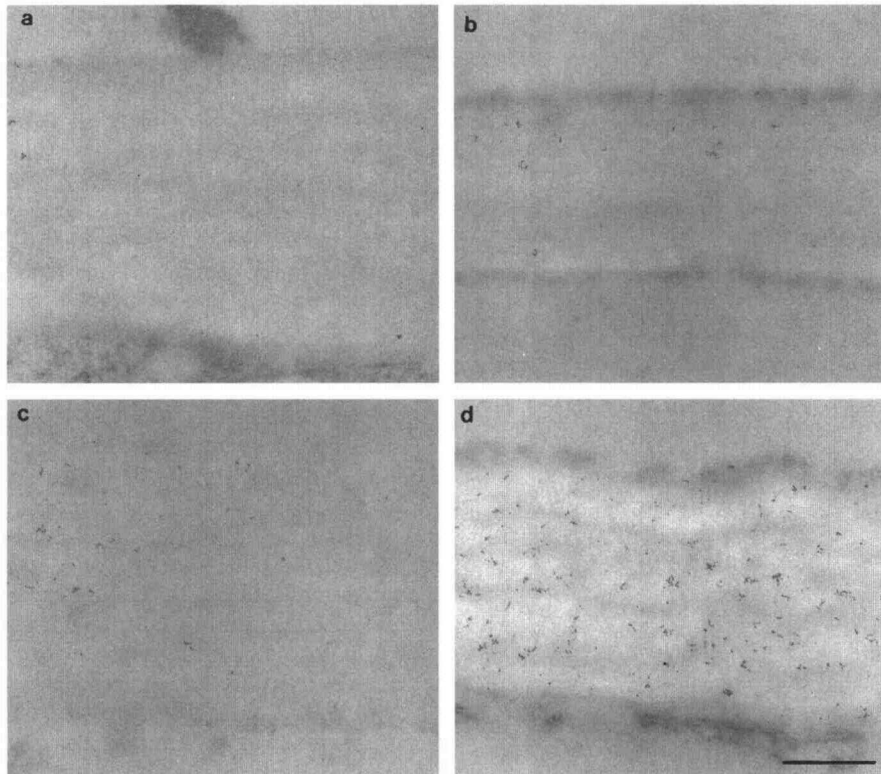
Figure 6. Thin (100-nm) sections of low-temperature, London Resin White-embedded tomato pericarp tissue at different developmental stages have been labeled with an anti-PGII antiserum. Epitope localization was visualized using a 10-nm colloidal-gold-conjugated secondary antibody. a, Immature green pericarp tissue labels very weakly and the signal is not above the background. b, Deposition of the PGII protein at the breaker stage of pericarp ripening has occurred into one cell wall, leaving the neighboring cell wall unlabeled by the antiserum. c, By the orange stage of tomato ripening, block-wise deposition of the protein has generated domains of cell wall that label with the PGII antiserum, whereas adjacent regions are unlabeled. d, Cell walls of red-ripe pericarp tissue are strongly labeled with the antiserum. Scale bar = 500 nm.

neutralized, filtered, dialyzed against distilled water at 4°C, and freeze-dried.

A sample of 3 mg of each fraction was added to 1 mL of the sample buffer and boiled at 100°C for 5 min. The supernatant was collected after centrifugation and acetone-precipitated, and the protein pellet was resuspended in the sample buffer. The silver-stained gel (Fig. 8a) shows the relative proportion of protein that was co-extracted from the cell wall with each pectic fraction. The amount of protein associated with the first imidazole-extractable fraction of both green and red-ripe fruit is significantly higher than the amount associated with later fractions. The remaining pectic fractions of the green fruit had little or no detectable protein associated with them, whereas several bands were present in the second imidazole- and first Na_2CO_3-extractable fractions from the red-ripe fruit. Two ripening-related bands of particularly strong intensities, one at approximately 46 kD and the other at approximately 35 kD, were associated with the imidazole-extractable fractions.

A gel of proteins co-extracting with the sequentially extracted pectic molecules from green and red-ripe pericarp cell walls was western-blotted overnight and probed with the anti-PGII antiserum. The PGII epitope co-extracted with pectic polymers from red-ripe fruit cell walls, being present within both imidazole-extractable fractions and the first Na_2CO_3-extractable fraction (Fig. 8b). There was no labeling of proteins extracted from the pectic fractions of unripe pericarp cell walls.

DISCUSSION

De-Esterification during Ripening

PME activity is first detected in the pericarp tissue at the breaker stage, with pectic de-esterification occurring during the 4 d that are required for the fruit to ripen to pink (Koch and Nevins, 1989). Immunolabeling with the monoclonal antibody JIM 5, which recognizes a relatively unesterified pectic epitope, has shown that unesterified pectin is localized in the middle lamella in green pericarp cell walls of cherry tomatoes, but is present throughout the primary cell wall in red-ripe fruit (Roy et al., 1992). Using this antibody to label thin sections of London Resin White-embedded pericarp tissue at all stages of ripening of tomatoes between green and red-ripe shows that de-esterification of pectic polymers is first apparent at the breaker stage. The de-esterification occurs in block-like units or domains, in which the unesterified pectic epitope is present throughout the primary cell wall rather than
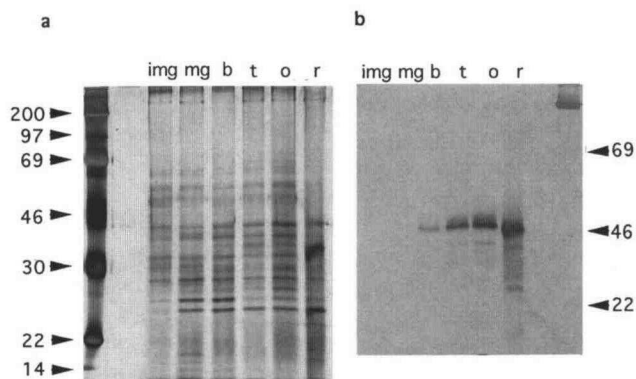
Figure 7. a, Cell walls of pericarp tissue at all stages of ripening were detergent-cleaned by homogenization in buffered Triton X-100 to remove cytoplasmic contaminants and residual membranes. The silver-stained gel shows the population of wall-associated proteins extracted from these walls by the sample buffer at the immature green (img), mature green (mg), breaker (b), turning (t), orange (o), and red-ripe (r) stages of ripening. Arrowheads indicate position of marker proteins. b, Western blot of a sister gel of proteins extracted in the sample buffer from the cell walls of the pericarp tissue at progressive stages in the ripening process probed with the anti-PGII antiserum. The blot shows the PGII epitope is first apparent in the population of proteins extracted from cell walls of pericarp tissue at the breaker stage of ripening. The intensity of the resulting band on the western blot increases as ripening progresses.

being confined to the middle-lamellar region. Each domain is independent of the adjacent regions of the wall and of the cell wall of the neighboring cell. As fruit ripening progresses to the orange stage, only small regions of the primary cell wall are devoid of the unesterified pectic epitope. The boundaries to these block-like domains are very distinct, with little gradation between areas of high and low JIM 5-labeling densities.

Domains of pectin de-esterification may be established as a result of one or more of three possible mechanisms. Localized deposition of PME, followed by rapid immobilization into the cell wall matrix, would prevent diffusion of the enzyme to new sites of activity and preserve the distinct boundaries of each domain. Alternatively, the enzyme may be soluble, and the activity regulated by immobilized inhibitors or activators localized in the block-like domains or provided by the microenvironment of the substrate. Modification of local pH or Ca^{2+} ion concentration may alter the local charge and conformation of the pectins in each domain, and so affect the enzyme's access and binding to the substrate, although tight restriction of movement of H^+ and Ca^{2+} ions within an aqueous environment such as the cell wall seems unlikely. Conditions within the cell walls of green fruit may inhibit PME activity, allowing secretion of the enzyme into the cell wall without significant pectin de-esterification. The third mechanism involves modification of the substrate itself, with unmasking of the methylesterified groups on the pectic polysaccharide to allow PME action in individual domains along the length of each wall. This masking must be of a nature that does not interfere with antibody recognition, however, since both green and red-ripe fruit pericarp cell walls are highly

reactive to the JIM 7 monoclonal antibody that recognizes methylesterified pectic polymers. One such steric effect would be the presence of ester cross-linking between pectin molecules, preventing hydrolysis by both PME and PGII, while not inhibiting recognition of epitopes by antibodies.

Roy et al. (1992) observed a disappearance of the JIM 7-reactive epitope from the middle-lamellar region during ripening. In contrast, only an occasional reduction in the density of labeling of the methylesterified pectic epitope has been observed in these studies. The walls of the ripe pericarp usually showed a similar JIM 7 labeling density to those of the green tissue. Koch and Nevins (1989) reported that 30% of the pectic methylesterification remains in the red-ripe fruit cell walls. These methylester groups may be inaccessible to the PME, or may simply be on regions of the pectin that do not fulfill the conformational requirements of the enzyme. The density of label seen in the cell walls of green fruit may represent maximum labeling within the restrictions caused by steric hindrance of the large antibody-gold complexes. Although the degree of methylesterification of the pectins decreases during ripening, there may be sufficient epitopes still present to allow near-maximal labeling densities with JIM 7.
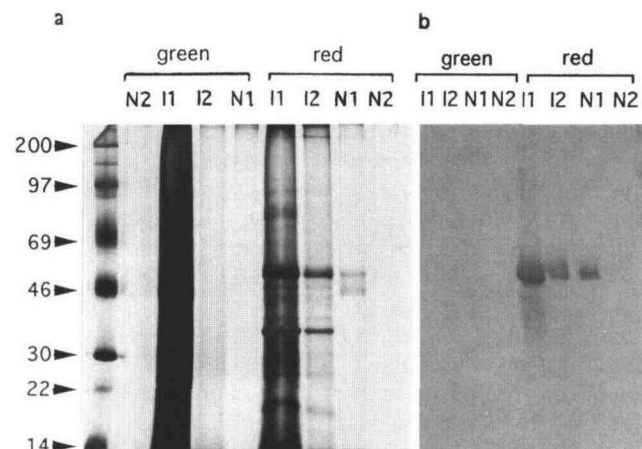

Figure 8. Pectic polymers were obtained by sequential extraction of pericarp cell walls of tomatoes at the immature green stage of development. This yielded the first imidazole-extractable fraction (I1), the second imidazole-extractable fraction (I2), the first Na_2CO_3-extractable fraction (N1), and the second Na_2CO_3-extractable fraction (N2). This extraction sequence was also carried out on red-ripe pericarp cell walls, yielding the I1, I2, N1, and N2 fractions. a, A sample of 3 mg of each fraction was extracted in 1 mL of the sample buffer for 5 min, the proteins were collected by centrifugation, and an equal volume of sample buffer from each extraction was run on a 10% polyacrylamide gel. The resulting silver-stained gel shows the relative proportion of protein that co-extracted from the cell wall with each pectic fraction. Note that the green N2 fraction is loaded out of sequence in the lane next to the markers. There are bands co-extracting with pectic polymers from ripe pericarp cell walls, which are absent from the pectic fractions from green pericarp cell walls. b, Western blot of a sister gel probed with the anti-PGII antiserum. The banding pattern shows the PGII epitope to be present in the population of proteins co-extracting with the first and second imidazole-extractable and first Na_2CO_3-extractable fractions from red-ripe pericarp cell walls. No labeling was seen of proteins co-extracting with the pectic polymers of green fruit.

The contribution of as yet uncharacterized pectic esterases in the ripening-related de-esterification of pectic polymers must also be considered. Phenolics and other sugar hydroxyl groups may be esterified to uronic acids, and their removal would also reveal possible JIM 5 epitopes. Pectin synthesis continues throughout ripening (Mitcham et al., 1989). The pericarp cells undergo extensive growth during the transition of the fruit from immature to mature green and there may be modifications or even changes in pectic composition during these and subsequent stages. These observations may help to explain the massive increase in JIM 5 labeling, without a concomitant decrease in the labeling density of JIM 7. However, the strict regulation of activity proposed for PME must also be exerted over any other esterase to maintain the observed de-esterification of cell wall pectins in block-like domains.

PGII Deposition during Ripening

The deposition of PGII protein also occurs in a block-wise fashion, occurring first in breaker tissue, with most of the walls labeling with the anti-PGII antiserum by the orange stage of tomato ripening. Once the red-ripe stage is reached, all of the walls of the pericarp label with the anti-PGII antiserum. Like the distinct boundaries seen in the domains of wall undergoing de-esterification, the boundaries to the blocks of wall containing the PGII protein are quite persistent.

The de-esterification of pectic epitopes and deposition of the PGII protein occur synchronously, since both are first apparent in the pericarp tissue at the breaker stage of fruit ripening. Since pectins are de-esterified in block-like domains, coordination of the deposition of PME and PGII within each cell wall is very important. The β-subunit is deposited into the cell wall as the tomato grows from immature to mature green. If PGI formation is indeed an in vivo event, then rapid immobilization of the newly deposited PGII protein through binding to the β-subunit would prevent diffusion to new sites of activity within the wall matrix. The β-subunit may also restrict the activity of PGII until later stages of ripening (Watson et al., 1994).

An indication that the PGII protein becomes tightly, but noncovalently, associated with the cell wall matrix is that PGII is first extractable from walls of breaker fruit, with a progressive increase in the quantity of protein present as walls from riper fruit are extracted. A consequence of the restricted mobility of the PGII protein within the wall is that progressive degradation of the polyuronides will require continual synthesis and deposition of new PGII, rather than being a consequence of the repetitive action of individual protein molecules. Monitoring of PG mRNA and protein accumulation has shown that continual synthesis of new enzyme occurs throughout the ripening process (Biggs and Handa, 1989). In in vitro incubations the amount of pectin released from an enzymically inactivated wall is proportionally related to the amount of PGII that is added, and no PGII is recovered in a cell-free homogenate (Huber and Lee, 1988). The size characteristics of the pectic products released are also very similar, regardless of the

actual amount of PGII added, although the quantity of product increases with the amount of PGII. This implies that once bound to the cell wall, PGII is not released to diffuse to new sites of activity, and also that the extent to which hydrolysis of the pectic backbone proceeds is relatively independent of the quantity of enzyme present.

Whereas the PGII protein was absent from the populations of proteins co-extracting with pectins from green pericarp cell walls, it was present within the populations extracted from the first and second imidazole-extractable pectic fractions, and from the first Na_2CO_3-extractable fraction from red-ripe pericarp cell walls. This indicates that the PGII protein had formed strong, but noncovalent, associations with pectic polysaccharides, perhaps mediated through binding to the β-subunit (Watson et al., 1994).

Sugars released from the wall during in vivo degradation of pectin contain a high proportion of acidic sugars, from which Seymour et al. (1987b) inferred that they originate from the unbranched middle-lamellar pectins. Those released from in vitro incubations contained more neutral sugars, indicating a more highly branched, primary cell wall pectic substrate. Seymour et al. (1987b) concluded that the conditions of the wall in vivo limited the mobility of PGII, confining it to the middle-lamellar region. However, the immunolocalization of the PGII peptide presented here shows that the protein is present throughout the primary cell wall.

Koch and Nevins (1989) reported that addition of PME to cell wall preparations in vitro caused complete de-esterification of wall pectins within 10 min. However, the de-esterification of the cell wall in vivo takes 4 d, despite the presence of significant quantities of PME activity in the tissue at the breaker stage. Our observations strongly suggest that this is due to compartmentation of the de-esterification event in localized domains of the wall at the breaker stage. The regulation of PME activity in vivo could be the result of mechanisms such as substrate modification, restricted access to the substrate by compartmentation of the enzymes, or the presense of inhibitors of enzyme activity, such as the inhibition of PGII activity by the diffusible products of pectin depolymerization, which was observed by Rushing and Huber (1990). For PG, however, our results show that the PGII protein has limited mobility within the wall, and that consideration of other models is unnecessary.

LITERATURE CITED

Atwell BJ, ap Rees T (1977) Distribution of protein synthesized by seedlings of *Oryza sativa* grown in anoxia. J Plant Physiol 123: 401–408

Biggs MS, Handa AK (1989) Temporal regulation of polygalacturonase gene-expression in fruits of normal, mutant, and heterozygous tomato genotypes. Plant Physiol 89: 117–125

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J 3: 1–30

Dellapenna D, Lashbrook CC, Toenjes K, Giovannoni JJ, Fischer RL, Bennett AB (1990) Polygalacturonase isozymes and pectin depolymerization in transgenic *rin* tomato fruit. Plant Physiol 94: 1882–1886

Harlow E, Lane D (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 139–242

Huber DJ, Lee JH (1988) Uronic-acid products release from enzymically active cell-wall from tomato fruit and its dependency on enzyme quantity and distribution. Plant Physiol 87: 592–597

Ishii T, Matsunaga T (1996) Isolation and characterization of a boron-rhamnogalacturonan II complex from cell walls of sugar beet. Carbohydr Res 284: 1–9

Jarvis MC (1984) Structure and properties of pectin gels in plant-cell walls. Plant Cell Environ 7: 153–164

Knegt E, Vermeer E, Bruinsma J (1988) Conversion of the polygalacturonase isoenzymes from ripening tomato fruits. Physiol Plant 72: 108–114

Knox JP, Linstead PJ, King J, Cooper C, Roberts K (1990) Pectin esterification is spatially regulated both within cell walls and between developing-tissues of root apices. Planta 4: 512–521

Kobayashi M, Matoh T, Azuma J-I (1996) Two chains of rhamnogalacturonan II are cross-linked by borate-diol ester bonds in higher plant cell walls. Plant Physiol 110: 1017–1020

Koch JL, Nevins DJ (1989) Tomato fruit cell-wall. I. Use of purified tomato polygalacturonase and pectin methyl-esterase to identify developmental changes in pectins. Plant Physiol 91: 816–822

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

McCann MC, Roberts K (1991) Architecture of the primary cell wall. *In* CW Lloyd, ed, The Cytoskeletal Basis of Plant Growth and Form. Academic Press, London, pp 109–129

McCann MC, Wells B, Roberts K (1990) Direct visualisation of cross-links in the primary cell wall. J Cell Sci 96: 323–334

Mitcham EJ, Gross KC, Ng TJ (1989) Tomato fruit cell-wall synthesis during development and senescence. *In vivo* radiolabeling of wall fractions using [^{14}C]sucrose. Plant Physiol 89: 477–481

Moore T, Bennett AB (1994) Tomato fruit polygalacturonase isozyme. I. Characterization of the β subunit and its state of assembly in vivo. Plant Physiol 106: 1461–1469

O'Neill MP, Albersheim P, Darvill A (1992) The pectic polysaccharides of primary cell walls. *In* PM Dey, ed, Methods in Plant Biochemistry, Vol 2. Academic Press, London, pp 415–441

Osteryoung KW, Toenjes K, Hall B, Winkler V, Bennett AB (1990) Analysis of tomato polygalacturonase expression in transgenic tobacco. Plant Cell 2: 1239–1248

Pogson BJ, Brady CJ (1993) Accumulation of the β subunit of polygalacturonase I in normal and mutant tomato fruit. Planta 191: 71–78

Roy S, Vian B, Roland JC (1992) Immunocytochemical study of the deesterification patterns during cell-wall autolysis in the ripening of cherry tomato. Plant Physiol Biochem 30: 139–146

Rushing JW, Huber DJ (1990) Mobility limitations of bound polygalacturonase in isolated cell-wall from tomato pericarp tissue. J Am Soc Hortic Sci 115: 97–101

Selvendran RR (1984) The plant cell wall as a source of dietary fiber: chemistry and structure. Am J Clin Nutr 39: 320–337

Seymour GB, Harding SE, Taylor AJ, Hobson GE, Tucker GA (1987a) Polyuronide solubilization during ripening of normal and mutant tomato fruit. Phytochemistry 26: 1871–1875

Seymour GB, Lasslett Y, Tucker GA (1987b) Differential effects of pectolytic enzymes on tomato polyuronides *in vivo* and *in vitro*. Phytochemistry 26: 3137–3139

Smith CJS, Watson CF, Morris PC, Bird CR, Seymour GB, Gray JE, Arnold C, Tucker GA, Schuch W, Harding S, and others (1990) Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes. Plant Mol Biol 14: 369–379

Towbin J, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350–4354

Tucker GA, Robertson NG, Grierson D (1980) Changes in polygalacturonase isoenzymes during the "ripening" of normal and mutant tomato fruit. Eur J Biochem 112: 119–124

Watson CF, Zheng L, Dellapenna D (1994) Reduction of tomato polygalacturonase β subunit expression affects pectin solubilization and degradation during fruit ripening. Plant Cell 6: 1623–1634

Wells B (1985) Low temperature box and tissue handling device for embedding biological tissue for immunostaining. Micron Microsc Acta 16: 49–53

Wells B, McCann MC, Shedletzky E, Delmer D, Roberts K (1994) Structural features of cell-walls from tomato cells adapted to grow on the herbicide 2,6-dichlorobenzonitrile. J Microsc 173: 155–164