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Uronic Acid Products Release from Enzymically Active Cell Wall from Tomato Fruit and Its Dependency on Enzyme Quantity and Distribution[1]

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ABSTRACT

Isolated cell wall from tomato (*Lycopersicon esculentum* Mill. cv Rutgers) fruit released polymeric (degree of polymerization [DP] > 8), oligomeric, and monomeric uronic acids in a reaction mediated by bound polygalacturonase (PG) (EC 3.2.1.15). Wall autolytic capacity increased with ripening, reflecting increased levels of bound PG; however, characteristic oligomeric and monomeric products were recovered from all wall isolates exhibiting net pectin release. The capacity of wall from fruit at early ripening (breaker, turning) to generate oligomeric and monomeric uronic acids was attributed to the nonuniform ripening pattern of the tomato fruit and, consequently, a locally dense distribution of enzyme in wall originating from those fruit portions at more temporally advanced stages of ripening. Artificial autolytically active wall, prepared by permitting solubilized PG to bind to enzymically inactive wall from mature-green fruit, released products which were similar in size characteristics to those recovered from active wall isolates. Extraction of wall-bound PG using high concentrations of NaCl (1.2 molar) did not attenuate subsequent autolytic activity but greatly suppressed the production of oligomeric and monomeric products. An examination of water-soluble uronic acids recovered from ripe pericarp tissue disclosed the presence of polymeric and monomeric uronic acids but only trace quantities of oligomers. The significance in autolytic reactions of enzyme quantity and distribution and their possible relevance to *in vivo* pectin degradation will be discussed.

The softening of many fruit types is known to involve the enzyme PG[2] (EC 3.2.1.15) (9). However, it is also recognized that the enzyme does not operate in an autonomous fashion. Factors known to affect the hydrolytic capacity of PG include pH (17, 20–22), calcium (6, 29, 41), and the structural features of the pectins (25, 26, 31). Other factors which have received little attention as potential regulative features include the quantity, distribution, and mobility of wall-bound PG. *In vitro* studies (28) employing enzymically active cell wall have demonstrated a quantitative relationship between the levels of wall-bound PG and the amount of pectin released. Preliminary studies (27) have also shown that the amount of pectin released from enzymically inactive cell wall is dependent on the dosage of added PG, indicating that the mobility and hydrolytic capacity of individual PG molecules are restricted to a spacially limited microenvironment. This situation would imply that the progressive softening occurring during ripening is dependent on the continual synthesis and deposition of new PG protein rather than on the repetitive action of individual PG molecules. Immunological assays have shown that PG synthesis persists throughout tomato ripening (37).

The objective of this study was to examine the relationship between wall-bound PG levels during ripening and the hydrolytic capacity of isolated cell wall. Of special concern was the influence of the level of bound PG on the molecular size characteristics of pectins released from enzymically active wall. We demonstrate that PG levels influence the quantity of pectin released from isolated cell wall but do not greatly influence the extent to which hydrolysis proceeds. The relationship of *in vitro* pectin hydrolysis and *in vivo* pectin degradation was also examined.

MATERIALS AND METHODS

Plant Material. Tomato plants (*Lycopersicon esculentum* Mill. cv Rutgers) were grown at the IFAS Gulf Coast Research and Education Center in Bradenton, FL. Fruit were harvested at selected developmental stages including mature-green (preclimacteric, no lycopene in locular gel), breaker (early climacteric, slight color blush at blossom end), turning, pink, and ripe (red). Fruit were surface-sterilized with sodium hypochlorite (100 ppm) and rinsed, and outer placental tissue was stored in sealed polyethylene bags at −20°C.

Cell Wall Preparation. Approximately 200 g partially thawed pericarp in 400 ml of cold Na-acetate buffer (40 mM, pH 4.5) were homogenized for 3 min in a Sorvall Omnimixer set at maximum speed. The homogenates were filtered through Miracloth and washed with 2 L of cold Na-acetate buffer (20 mM, pH 4.5) followed by 1 L of 95% ethanol. The wall was then transferred to 200 ml 100% acetone and filtered through a Buchner funnel (Whatman GF/C paper). An additional 1 L of acetone was applied under aspiration and the wall allowed to partially dry. The cell wall was transferred to Petri plates, oven-dried for 30 min at 40°C, and then placed in a desiccator. After 24 h, the wall was placed in 100 ml snap-top glass vials, which in turn were placed in sealed Mason jars containing desiccant. All wall isolates were stored at −20°C.

Other wall isolation protocols were designed to measure the influence of removing wall-bound PG on wall autolytic reactions. For this purpose, pericarp homogenates were adjusted to pH 6.5 with 0.1 N NaOH. The homogenates after standing (2 h, 2°C) were then filtered and washed with 2 L of buffer at pH 6.5. The Miracloth was gently squeezed to remove excess buffer, and the hydrated wall was weighed and then transferred to an equal

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[2] Abbreviations: PG, polygalacturonase; PAW, phenol-acetic acid-water; DP, degree of polymerization.

weight of Na-acetate buffer (20 mM, pH 5.6), containing 2.4 M NaCl. After adjusting to pH 6.5 with 0.1 N NaOH, the suspension was incubated at 4°C for 30 min with magnetic stirring. The suspension was filtered through Miracloth and then was washed with 200 ml of 1.2 M NaCl in Na-acetate buffer (20 mM, pH 5.6) adjusted to 6.5 with 0.1 N NaOH, followed by 2 L of the same buffer without NaCl. Ethanol/acetone procedures were as described above.

Autolysis Experiments. Cell wall (50–200 mg, dry weight) was hydrated in cold (2°C) Na-acetate buffer (30 mM, pH 4.5) (buffer:cell wall ratio, 2 ml:50 mg) containing 150 mM NaCl and 0.01% Thimerosal. The suspensions were incubated at 34°C in a shaking water bath for 1.5 h unless otherwise stated. The reaction mixtures were then filtered (Miracloth) and washed, and the combined filtrate-washing was filtered through glass fiber filter paper (Whatman GF/C). The filtrates were adjusted to 80% ethanol, refluxed (30 min, 84°C), and then concentrated *in vacuo* to remove ethanol. The ethanol-reflux step was employed as a precautionary measure to ensure the inactivation of any polygalacturonase released during autolytic reactions. Although refluxing in 80% ethanol has been reported to not completely inactivate PG bound to alcohol-insoluble isolates (32), we found that the reflux step is effective at inactivating added soluble PG. At any rate, no PG activity could be detected in the postautolysis filtrates. Upon removal of the ethanol, precipitated autolysis products readily resolubilized. The samples were analyzed for total uronic acid content using the procedure of Blumenkrantz and Asboe-Hansen (1).

Isolation and Purification of Water-Soluble Pectins from Tomato Pericarp Tissue. Partially thawed ripe pericarp (40 g) was homogenized in 40 ml cold (2°C) deionized water for 3 min in a Sorvall Omnimixer set at maximum speed. The homogenate was centrifuged at 2°C for 10 min at 8000*g* in a J20 rotor, and the supernatant was decanted and brought to 80% with ethanol. The samples were refluxed at 84°C for 20 min and then evaporated under vacuum to approximately 10 ml. The samples were applied to a bed (17 cm high, 2.5 cm wide) of DEAE-Sephadex (A 25–120, Sigma) which had been equilibrated in Na-phosphate buffer (4 mM, pH 6.8). After sample application, 250 ml of the phosphate buffer were passed through the column to elute neutral sugars. The column was then step-eluted with the phosphate buffer containing 600 mM NaCl. Seven-ml fractions were collected at a flow rate of 10 $ml \cdot cm^{-2} \cdot hr^{-1}$, and 0.5 ml fractions were analyzed for total uronic acids (1). Pectins were recovered within the elution volume of 70 to 120 ml. No additional pectins were eluted using higher NaCl concentrations. The fractions containing pectin were pooled, concentrated, and applied to a Bio-Gel P-2 column as described below.

Gel Chromatography. Approximately 1 mg product from autolytic reactions or from pericarp isolates in a volume of 2 ml was applied to a water-jacketed bed (60 × 1.5 cm) of Bio-Gel P-2 (−400 mesh) operated at 44°C. One-ml fractions were collected at a flow rate of 10 $ml \cdot cm^{-2} \cdot h^{-1}$. Aliquots were analyzed for total uronic acid content.

Isolation of Tomato Polygalacturonase and Preparation of Artificial Autolytically Active Cell Wall. Ripe pericarp tissue (40 g) was homogenized in 40 ml of Na-acetate buffer (40 mM, pH 5.0) containing 2.4 M NaCl for 2 min in a Sorvall Omnimixer. The homogenate, after standing for 2 h at 4°C, was centrifuged for 10 min at 25,000*g*, and the supernatant was adjusted to 80% saturation with solid ammonium sulfate. After centrifugation (25,000*g*), the pellets were dissolved in Na-acetate buffer (20 mM, pH 4.5) containing 150 mM NaCl and desalted on a bed (14 cm high, 1.4 cm wide) of Sephadex G-25 operated in the same buffer. Fractions active toward polygalacturonic acid were pooled and used in the preparation of artificial autolytically active wall. In reductometric tests specific for uronic acids (18), the desalted wall protein exhibited a specific activity of about 3.0 μmol galacturonic acid equivalents·mg $protein^{-1} \cdot min^{-1}$. Artificially active cell wall was prepared by incubating enzymically inactive (*i.e.* nonautolytic) cell wall from mature-green fruit with the PG prepared from ripe fruit. Approximately 50 mg wall from mature-green fruit (equivalent to about 4 g fresh weight mature-green pericarp) were placed in 50 ml centrifuge tubes containing 15 ml Na-acetate buffer (20 mM, pH 4.5) at 2°C. While the wall suspension was stirring, 200 μl of the desalted protein preparation (42 μg protein, Bradford method [2]) containing polygalacturonase activity diluted to 2 ml with the Na-acetate buffer were added in a dropwise fashion. The added protein was equivalent to that recovered from 0.5 g fresh weight of ripe pericarp. After 30 min at 1°C, the suspension was filtered through Miracloth, and the wall was washed with 200 ml of cold 100 mM NaCl followed by 200 ml deionized H_2O. The walls were then subjected to standard autolysis reaction conditions as described above.

Neutral Sugar Analysis. Products (approximately 8 mg) recovered from autolytic reactions were concentrated to a volume of 5 ml and subjected to DEAE chromatography as described above for the aqueous isolates of pericarp tissue. The salt-eluted pectins were concentrated to a volume of 2 ml and then fractionated on Bio-Gel P-2. Specific fractions were pooled and concentrated, and volumes equivalent to 1 mg uronic acid equivalents were air-dried in Reacti-Vials (Pierce Chemical Co.). Hydrolysis and acetylation were performed as previously described (10).

RESULTS AND DISCUSSION

The enzymic nature of pectin release from isolated tomato fruit cell wall was first reported by Rushing and Huber, who demonstrated that the capacity of isolated cell wall to release pectin increased with ripening (28) and was strongly influenced by NaCl, Ca^{2+}, and pH (29), factors known to influence the activity of isolated polygalacturonase. An unknown aspect of cell wall autolysis reactions and pectin degradation in general is the extent of mobility of bound PG, a factor which may be influential in regulating the manner by and the extent to which pectin is modified by the enzyme. A number of observations indicate that the mobility of wall-bound PG in autolytic reactions is quite limited. First, the quantity of pectin released by PG from cell wall from mature-green fruit is proportional to the amount of enzyme provided (27). Second, during autolytic reactions of up to 5 h at pH 4.5, no PG activity is detected in the wall-free filtrate (DJ Huber, unpublished data). Although we cannot preclude the possibility that PG is released from cell wall in an inactive form, *in vitro* autolysis apparently represents a solid-state enzymic process, with pectin hydrolysis being restricted to the immediate environment of the bound enzyme. Figure 1 illustrates representative Bio-Gel P-2 profiles of uronic acids released from autolytically active cell wall from fruit at selected stages of ripening. Quantitatively, net pectin yields ranged from 1.6 to 1.8 μg galacturonic equivalents·mg $cell\ wall^{-1}$ from breaker fruit, 7.4 to 8.7 for turning, 35 to 40 for pink, and 55 to 65 for ripe. Wall from mature-green fruit released only trace quantities of uronic acids (0.20–0.30 $\mu g \cdot mg^{-1}$), and these values did not exceed quantities recovered from hot-ethanol-inactivated wall. For all other fruit, wall treated in boiling 80% ethanol (84°C) released products equivalent to less than 10% of those recovered from active walls. Seymour *et al.* (32) have recently reported that hot ethanol is not totally effective at inactivating bound PG and recommend the use of PAW (30) as an alternative procedure for eliminating bound PG. In our experience, the use of PAW completely negated the capacity of isolated walls to liberate oligomeric and monomeric products, but a substantial polymeric (P-2 void) component accounting for 20 to 30 μg·mg $wall\ weight^{-1}$ was consistently obtained. In the reaction periods employed in this study, hot-ethanol-treated wall liberated no oligo-

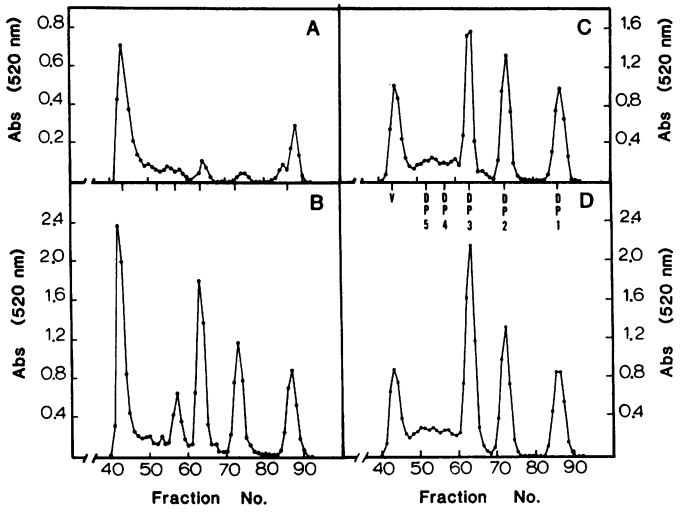

FIG. 1. Bio-Gel P-2 profiles of uronic acids released from autolytically active wall during a 90 min incubation at 34°C. Fractions (1 ml) were analyzed for total uronic acids (A_{520}). Products released from wall from A, breaker fruit; B, turning fruit; C, pink fruit; D, ripe fruit. Vertical markers in D refer to the excluded volume (V) and the elution positions (DP5-DP1) of oligouronides recovered from an *A. niger* pectinase digest of polygalacturonic acid.

meric or monomeric products, and yields of the P-2 void component were substantially less than with PAW-treated wall.

Although differences were readily apparent in the quantities of pectins released from wall derived from fruit at progressive stages of ripening, a notable observation was the similarity in the size characteristics of the products recovered. Even in wall derived from fruit at the breaker stage of development, at which time PG activity and protein are first apparent but quite low (37), characteristic oligomeric and monomeric products were observed (Fig. 1A). Only in wall from mature-green fruit were oligomeric and monomeric products not observed (P-2 profile not shown), consistent with reports that PG is absent from mature-green, preclimacteric fruit (5, 37). The vertical markers in Figure 1D correspond to the elution positions of oligouronides recovered from an *Aspergillus niger* pectinase (Sigma P5146) digest of ethanol-purified polygalacturonic acid. The *A. niger* enzyme (25), as well as isolated tomato and avocado PGs (16), were shown to generate from pectin substrates a series of oligouronides which were ultimately converted to galacturonic acid. Patel and Phaff (20) demonstrated for tomato PG that the hydrolysis of digalacturonic acid represented the slowest reaction. Consistent with their observation, products recovered from autolytic reactions permitted to proceed for 20 h were composed largely of dimer/monomer products (not shown). Even in these long reaction periods, however, pectins excluded from Bio-Gel P-2 were consistently obtained and were resistant to further degradation as indicated by the inability of isolated PG to generate from these polymers products retained by Bio-Gel P-2. A possible explanation for the resistance of the excluded product to further PG action resides in the high neutral sugar content of this polymer. It is known that neutral sugars, present as side chains and as intramolecular substituents (rhamnose), suppress the degree of enzymic hydrolysis of pectins (7, 36). Table I summarizes the neutral sugar composition of uronic acid products released in a 1 h reaction period from wall from ripe fruit (Fig. 2). The low DP products contained negligible quantities of neutral sugars, undoubtedly a factor responsible for the ability of PG to affect extensive hydrolysis of the parent polymer. The products excluded from the P-2 gel were rich in the neutral sugars arabinose (53%), galactose (32%), and rhamnose (11.6%). The mole ratio of neutral sugar/uronic acid (0.82) of these products was greater than 10-fold higher than that of the largest products retained by the gel. Omitting the ion-exchange step resulted in a different neutral sugar profile (higher xylose levels), indicating that quantities of nonpectin products were released during autolytic reactions. It was not determined whether these were released enzymically or passively over the course of the incubation period. Other factors, including the presence of acetyl (36) and methyl ester groups (14), may also be involved in the resistance to enzymic hydrolysis of the high neutral sugar pectin. It is not known whether the polymeric, high neutral sugar product released from cell wall originated from the limited hydrolysis of a distinct, high neutral sugar pectin or passively as a consequence of the hydrolysis of pectins possessing both hairy and smooth (homogalacturonan) regions (7). Evidence that the neutral sugars are in fact structural components of the pectins was the observation that nearly all autolytically released products were strongly retained by anion-exchange (DEAE) gels. Although specific DP values of the larger polymers were not investigated here, Bio-Gel P-10 chromatography revealed considerable size heterogeneity of the excluded P-2 component (not shown). Thibault (35) reported that oligouronides of a DP > 7 to 8 were excluded from Bio-Gel P-2 operated in Na-acetate buffer at pH 4.5.

The capacity of enzymically active cell wall to generate oligomeric and monomeric uronides is not in agreement with studies showing that isolated PG generated predominately large DP products from isolated cell wall from mature-green pericarp. Wallner and Bloom (40) reported that cell wall from mature-green fruit, when treated with a PG isolate from ripe fruit, liberated only trace quantities of material retained by Bio-Gel P-2. They concluded that PG functioned in liberating primarily high mol wt pectins. In contrast, Figure 3 illustrates that PG bound to cell wall from mature-green fruit (*i.e.* artificial autolytically active wall) generated products not unlike those recovered from native autolytically active cell wall from ripe fruit. The failure of earlier researchers (40) to detect the range of products observed here is likely attributable to their use of distilled water as a column eluant. Thibault (35) demonstrated that oligouronides were ionically excluded when chromatographed on Bio-Gel P-2 eluted with distilled water. Consistent with his findings, we observed that products similar to those shown in Figure 3 eluted almost entirely as void components when chromatographed on Bio-Gel P-2 operated in distilled water. It is possible that enzymes other than PG contributed to the release of pectin from both artificial and native enzymically active wall. Themmen *et al.* (34) concluded from *in vitro* wall degradation studies, monitored by the reductometric assessment of both neutral and uronic acid products, that purified PG was quantitatively as effective as total wall protein preparations. However, other studies have demonstrated a promotive effect of pectinmethylesterase on PG-mediated wall degradation (23, 33).

These studies demonstrate that enzyme quantity is of importance in regulating the amount of pectin released in wall autolytic reactions. However, the similarity of product profiles generated from walls prepared from fruit at progressive stages of ripening and therefore containing increasing quantities of PG indicates that the extent to which hydrolysis proceeds is relatively independent of the quantity of enzyme. To further test these ideas, wall was subjected to treatments reported to remove bound PG protein. Pressey (22) reported that a combination of pH (6.5) and high ionic strength (1.2 M NaCl) afforded maximum dissociation of wall-bound PG. However, wall subjected to these conditions showed no attenuation in subsequent autolytic activity, and yields were actually enhanced by about 30% over samples not subjected to the pretreatments. Salt-treated wall subjected to hot-ethanol pretreatment also released more pectin; however, the

Table I. *Neutral Sugar Composition of Pectins Autolytically Released from Cell Wall from Ripe Fruit (see Fig. 2)*

P-2 Fractions	Mole Ratio (Neutral/Uronic Acid)	Rham	Ara	Xyl	Gal	Glu
			% total neutral sugars			
Void (41–47)	0.821	11.6	53.2	1.4	32.4	1.4
Midrange (49–59)	0.069	6.3	56.8	2.7	30.7	3.5
DP 3 (60–65)	0.020		98.2		0.8	
DP 2 (68–72)	0.018		99.4		0.6	
DP 1 (82–86)	0.021		99.5		0.5	

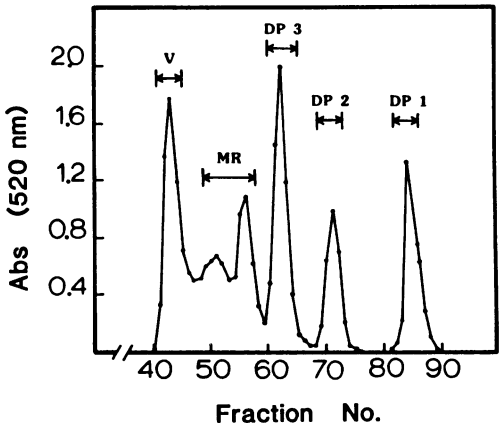

FIG. 2. Bio-Gel P-2 profile of uronic acid products released from autolytically active wall from ripe fruit during a 60 min incubation at 34°C. Prior to P-2 chromatography, autolysis products were first subjected to DEAE chromatography. Regions of the profile designated V (41–47 ml), MR (midrange, 49–59), DP 3 (60–65), DP 2 (68–72), and DP 1 (82–86) were individually pooled and subjected to neutral sugar analysis (see Table I).

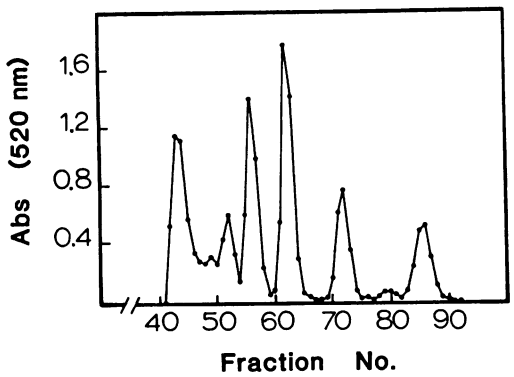

FIG. 3. Bio-Gel P-2 profile of uronic acid products released from artificial autolytically active wall during a 90 min incubation at 34°C. Wall was prepared by incubating cell wall from mature-green fruit with a PG isolate from ripe fruit prior to the autolytic experiment.

net difference between active and heat-treated cell wall was similar to net release from cell wall not subjected to the salt pretreatment. The increased pectin release from wall pretreated with 1.2 M NaCl may be due, in part, to the known capacity of this and other salts to displace calcium from pectin (38, 39). The products generated from salt-treated wall were composed almost entirely of those which voided the P-2 gel (Fig. 4A). Treatment of this component with a PG isolate yielded products (Fig. 4B) similar to those obtained from a direct autolytic digest of wall not treated with salt (*e.g.* Fig. 1D). This indicates that the salt treatment does not have a major influence on the qualitative

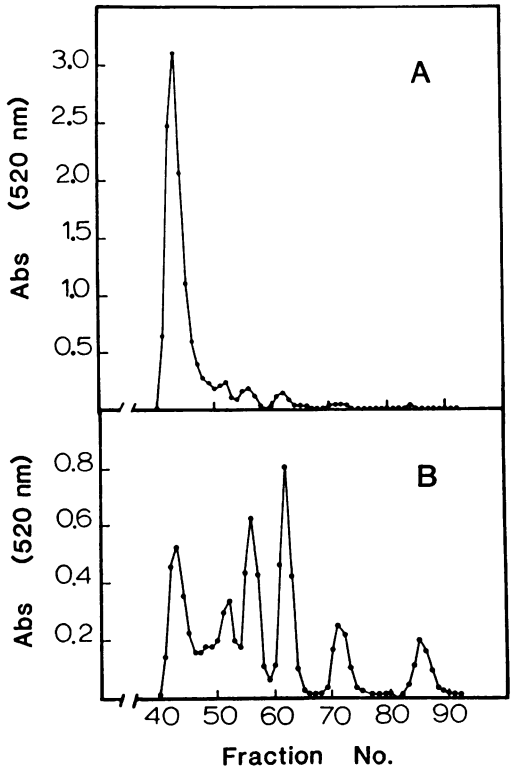

FIG. 4. Bio-Gel P-2 profiles of uronic acid products released from autolytically active wall isolated at pH 6.5 and subjected to a high salt (1.2 M NaCl) pretreatment. A, Products autolytically released from salt-treated wall incubated in pH 4.5 buffer for 90 min at 34°C; B, P-2 profile of products obtained from incubation of the P-2 excluded component generated from salt-treated wall (A, fractions 41–48) with a PG isolate from ripe fruit. Reaction mixture was incubated for 60 min at 34°C.

nature of the pectin released in autolytic reactions. Interestingly, wall treated with slightly lower concentrations of NaCl (1.0 M) did not exhibit a measurable reduction in their capacity to generate oligomeric and monomeric uronic acids (not shown).

The absence of oligomeric and monomeric autolysis products from salt-treated (and, presumably, low PG) wall would appear to be in disagreement with the observation that other wall isolates low in PG, for example those from fruit at the breaker and turning stages of development, generated a typical distribution of low DP products. This apparent discrepancy can be explained by considering the nonuniform ripening pattern of the tomato fruit. In the tomato fruit, ripening is initiated internally in the placental tissue (3, 12), is first apparent externally at the distal end of the fruit, and progresses peripherally toward the proximal (stem) end. Fruit designated breaker (color break at distal or blossom portion) and turning would include tissues at markedly different stages of ripening. A consequence of this would be an

unequal distribution of PG within tissues derived from these fruit. Evidence for a nonuniform distribution of PG within tomato fruit was demonstrated by the application of an *in vivo* assay for measuring the enzyme (13). Discs (1 cm) prepared from tomato fruit and incubated in pH 4.5 buffer released products indistinguishable in size characteristics from those released from isolated cell wall, providing evidence for the role of PG in the release of uronic acids from the disc tissue. Furthermore, the quantity of pectin released was greatly dependent on fruit portion, with activity increasing progressively in discs removed from the proximal (stem end), equatorial, and distal (blossom) portions. As a consequence of this differential distribution, PG in cell wall prepared from partially ripe fruit would not be uniformly distributed, and the autolytic release of pectins would reflect the action of a very localized population of PG protein in wall originating from the ripening tissues. Fully ripe fruit would be expected to have a more uniform although perhaps somewhat skewed distribution of PG. Given the limited mobility of bound PG, the generation of oligomeric and monomeric products would arise as a consequence of pectin diffusion following initial hydrolysis by PG. This scenario would explain the fact that walls derived from fruit at the early stages of ripening (breaker) released a higher proportion of polymeric pectin. This would result from the diffusion of initial products into the relatively high proportion of wall not containing PG. The effect of ionic strength might be to accentuate this condition by greatly reducing the overall distribution density of the enzyme protein, reducing the frequency of enzyme-substrate encounters. Alternatively, the PG removed by high concentrations of salt may reside in a microenvironment qualitatively different from that in which the salt-resistant PG resides. Finally, in view of the observation that the *in vitro* degradation of isolated tomato fruit cell wall by PG is enhanced by the addition of pectinmethylesterase (23, 33), it is possible that the high salt removes a critical portion of the latter enzyme. Seymour *et al.* (33) demonstrated that the addition of PG (isozyme 2) to enzymically inactive wall generated significant quantities of oligomeric products only when added in combination with PME. Although it is difficult to ascertain how much PG protein remains in the wall following salt treatment, it would appear that the quantitative features of *in vitro* autolysis do not require the full complement of wall-bound PG.

In an effort to investigate the *in vivo* significance of the reaction characteristics of enzymically active wall isolates, we examined the water-soluble pectins in pericarp homogenates. Previous studies of water-soluble pectins have been limited to an examination of those extracted by water from precipitates of 80% alcohol or acetone homogenates of fruit tissues. This approach would preclude the ability to detect smaller, ethanol-soluble uronic acids. To circumvent this problem, we examined the soluble pectin components, and, presumably, those generated by *in vivo* action of PG, present in aqueous extracts of pericarp tissue. Direct assay for uronic acids in aqueous extracts was not possible due to the high quantities of neutral sugars (primarily glucose and fructose). This problem was alleviated by DEAE-Sephadex purification of the water-soluble extracts. After complete elution of neutral sugars from the DEAE column, a step elution with 600 mM NaCl allowed complete recovery of the bound uronic acids (not shown). The Bio-Gel P-2 profile of these components is shown in Figure 5. In addition to the void component, the only major product consistently recovered was monomer, which accounted for nearly 50% of the water-soluble pectins. Trace quantities of higher oligomers were also observed.

A number of observations offer evidence that the galacturonic acid recovered from the aqueous extracts originated as a result of *in vivo* PG action and that PG activity during the homogenization and isolation steps was not a contributing factor. First, aqueous isolates of mature-green fruit, which contain no PG,

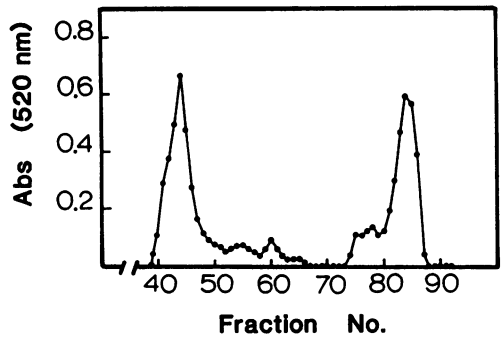

FIG. 5. Bio-Gel P-2 profiles of DEAE-purified water-soluble pectins from ripe fruit.

yielded no monomeric uronic acids (not shown). Second, homogenates of mature-green fruit to which was added an extract containing PG yielded neither monomers nor increased quantities of soluble pectins but did so when homogenates were permitted to stand at room temperature (23°C). These observations indicate that the presence of monomers in extracts from ripe fruit was not a consequence of PG activity during pectin isolation. Evidence that the monomeric uronic acid originates from *in vivo* pectin degradation and, presumably, PG action, is the observation that quantities of monomer recovered were increased from pericarp tissue provided (via injection) with polymeric pectin (DJ Huber, unpublished data). It is possible that the production of galacturonic acid *in vivo* and also in *in vitro* autolytic reactions is facilitated by the presence of an exopolygalacturonase (EC 3.2.1.67) that has been detected (8) and recently purified from tomato fruit (24). Some evidence that exo-PG may in fact participate in autolysis was the observation that proportionally higher quantities of monomer were recovered from cell wall from breaker fruit (Fig. 1A). Since exo-PG is present throughout ripening, including the mature-green stage (24), the very low levels of endo-PG present at the early, breaker stage of development may result in a proportionally higher contribution of the exoenzyme.

The lack of congruency between the products recovered from autolytic reactions in ripe cell wall and those recovered from tissue homogenates of ripe pericarp is possibly explained by a concentration effect *in vivo*. Products generated *in vitro* would possess greater diffusional freedom, resulting in reduced contact frequency with enzyme and decreased probability of further degradation. *In vivo*, however, initial degradation products would be restricted in their diffusion environment to the apoplast, greatly increasing the probability of further encounters with enzyme. Since only trace quantities of neutral sugars were associated with the low-DP products generated in autolytic reactions, it seems likely that they, as well as the monomer recovered from pericarp tissue, originate from the degradation of relatively smooth (7) regions of pectin. If these regions are initially released as larger DP products, it is possible that they might exert transient elicitor activity. Preliminary studies have provided some evidence for this possibility. Vacuum infiltration of autolytically generated pectins into intact, prepripe tomato fruit resulted in enhanced ethylene biosynthesis and an advancement in the onset of ripening (4). The response was fraction-specific in that only the P-2 void component was effective. This is consistent with reports that elicitors of pectin origin generally have DPs in the range of 12 to 13 (15, 19).

Although products recovered from *in vitro* autolysis reactions display some similarity to those generated *in vivo*, it is clear that the *in vitro* reaction is greatly accelerated relative to *in vivo* pectin degradation. Calcium may be an influential factor *in vivo* (6), and undoubtedly some proportion of wall calcium is lost during wall isolation. However, while *in vitro* pectin release is in fact

greatly attenuated by the addition of calcium (29), the ion does not completely suppress the generation of oligomeric and monomeric products (11).

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