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Polyuronides in Avocado (*Persea americana*) and Tomato (*Lycopersicon esculentum*) Fruits Exhibit Markedly Different Patterns of Molecular Weight Downshifts during Ripening[1]

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Avocado (*Persea americana*) fruit experience a rapid and extensive loss of firmness during ripening. In this study, we examined whether the chelator solubility and molecular weight of avocado polyuronides paralleled the accumulation of polygalacturonase (PG) activity and loss in fruit firmness. Polyuronides were derived from ethanolic precipitates of avocado mesocarp prepared using a procedure to rapidly inactivate endogenous enzymes. During ripening, chelator (cyclohexane-*trans*-1,2-diamine tetraacetic acid [CDTA])-soluble polyuronides increased from approximately 30 to 40 μg of galacturonic acid equivalents (mg alcohol-insoluble solids)$^{-1}$ in preripe fruit to 150 to 170 μg mg^{-1} in postclimacteric fruit. In preripe fruit, chelator-extractable polyuronides were of high molecular weight and were partially excluded from Sepharose CL-2B-300 gel filtration media. Avocado polyuronides exhibited marked downshifts in molecular weight during ripening. At the postclimacteric stage, nearly all chelator-extractable polyuronides, which constituted from 75 to 90% of total cell wall uronic acid content, eluted near the total volume of the filtration media. Rechromatography of low molecular weight polyuronides on Bio-Gel P-4 disclosed that oligomeric uronic acids are produced in vivo during avocado ripening. The gel filtration behavior and pattern of depolymerization of avocado polyuronides were not influenced by the polyuronide extraction protocol (imidazole versus CDTA) or by chromatographic conditions designed to minimize interpolymeric aggregation. Polyuronides from ripening tomato (*Lycopersicon esculentum*) fruit extracted and chromatographed under conditions identical with those used for avocado polyuronides exhibited markedly less rapid and less extensive downshifts in molecular weight during the transition from mature-green to fully ripe. Even during a 9-d period beyond the fully ripe stage, tomato fruit polyuronides exhibited limited additional depolymerization and did not include oligomeric species. A comparison of the data for the avocado and tomato fruit indicates that downshifts in polyuronide molecular weight are a prominent feature of avocado ripening and may also explain why molecular down-regulation of PG (EC 3.2.1.15) in tomato fruit has resulted in minimal effects on fruit performance until the terminal stages of ripening.

Avocado fruit soften extensively during ripening, with mesocarp firmness, measured in terms of resistance to penetration, exceeding 450 N at the preripe stage and decreasing several orders of magnitude during ripening (Pesis et al., 1978; Awad and Young, 1979; O'Donoghue and Huber, 1992). Although relatively few studies have addressed the contribution of specific cell wall polysaccharides to the loss of avocado fruit firmness (Colinas-Leon and Young, 1981; O'Donoghue and Huber, 1992), many researchers have attempted to establish that C_x-cellulase, a putative cell-wall enzyme that accumulates to very high activity levels during avocado ripening, may play an important role (Pesis et al., 1978; Hatfield and Nevins, 1986; O'Donoghue and Huber, 1992). Many details regarding the ripening-related gene regulation of avocado cellulase have been elucidated (Tucker et al., 1987; De Francesco et al., 1989; Cass et al., 1990); however, the native substrate for this enzyme remains an enigma. Recent studies (O'Donoghue and Huber, 1992) have not supported the idea that the avocado glucanase functions as a xyloglucanase (Hatfield and Nevins, 1986).

In the first study of avocado fruit cell-wall changes, Colinas-Leon and Young (1981) reported that ripening was accompanied by a nearly 70% decrease in cell-wall uronic acid content, indicating that polyuronide metabolism may be involved in the dramatic textural changes exhibited by this fruit. Although recent studies with tomato fruit have deemphasized the relationship between polyuronide metabolism, and in particular PG (EC 3.2.1.15)-mediated polyuronide depolymerization, and fruit ripening and softening (Giovannoni et al., 1989; DellaPenna et al., 1990; Smith et al., 1990), whether a similar situation holds true for other fruits containing PG or other polyuronide depolymerases remains to be established.

The purpose of this study was to determine whether the solubility and M_r features of avocado fruit polyuronides were consistent with the idea that these polymers contribute to the dramatic loss of texture that occurs during ripening. The protocol adopted for preparation and processing of ethanolic precipitates of avocado tissue appears to be superior to techniques used in studies of polyuronides in tomato fruit in facilitating a more rapid and complete inactivation of tissue enzymes, including PG. For this reason, and in view of the uncertainties regarding the role of polyuronide depolymerization in tomato fruit, the avocado data are compared with those obtained in parallel studies performed with tomato fruit.

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Abbreviations: AIS, alcohol-insoluble solid(s); CDTA, cyclohexane-*trans*-1,2-diamine tetraacetic acid; DP, degree of polymerization; N, newton; PG, polygalacturonase.

MATERIALS AND METHODS

Plant Material

Avocado (*Persea americana*) fruits were obtained from a commercial source in California (cv Hass) or Florida (cv Lula). Tomato (*Lycopersicon esculentum* L. Mill, cv Sunny) fruit were obtained from a commercial source in Lantana, FL. All fruits were received within 48 h of harvest. Avocado (cv Hass) fruit were ripened at 25°C and selected at three developmental stages based on subjective firmness determinations: stage A, fruit firm, mesocarp exhibiting no yield to finger force; stage B, 3 d after stage A, near edibly soft; stage C, 5 d after stage A, mesocarp exhibiting minimal resistance to deformation. Avocado (cv Lula) fruit were selected at three developmental stages during a 5-d period based on ethylene production and objective firmness measurements (O'Donoghue and Huber, 1992). For both avocado cultivars, stages A through C correspond approximately to the preclimacteric (preripe), climacteric (ripening), and postclimacteric (ripe) periods of development, respectively. At each sampling period, mesocarp derived from five avocado fruit was excised, weighed, and immersed immediately in 95% ethanol. The final suspension was adjusted to 80% ethanol and homogenized using a Polytron. The slurry was stored at −30°C. Tomato (cv Sunny) fruit were ripened at 25°C and selected based on color/time criteria: stage A, mature green; stage B, 5 d after stage A, pink; stage C, 9 d after stage A, fully ripe; stage D, 18 d after stage A, overripe. For each developmental stage, outer pericarp tissue derived from six tomato fruit was excised, weighed, and processed into 80% ethanol homogenates as described for the avocado mesocarp.

Preparation of AIS

AIS were prepared as described previously (Huber, 1992) with some modification. Volumes (typically 40 mL) of the 80% ethanol suspensions containing 8 g tissue fresh weight were poured directly onto Miracloth (Biochemical Corp., La Jolla, CA) filtration cloth in a Buchner funnel and washed with 100 mL of 80% ethanol. The residue was removed from the filter and immediately transferred to 40 mL of Tris-buffered phenol, pH 7.0 (Huber, 1991), and incubated for 30 min at 23°C. Afterward, the suspension was readjusted to 80% ethanol and maintained at −20°C for 1 h to facilitate reprecipitation. The suspension was filtered through GF/C filter paper (Whatman) and washed with 200 mL of 95% ethanol. The AIS were transferred to 100 mL of chloroform:methanol (1:1, v/v) and stirred for 30 min at 23°C. The suspension was filtered through a GF/C filter, washed with 200 mL of acetone, and partially dried by applying a mild vacuum. The AIS were transferred to glass containers and incubated in an oven at 34°C for 12 h and stored in a dessicator at 23°C. Other AIS samples were prepared as above but without the use of Tris-buffered phenol.

Extraction of Chelator-Soluble Polyuronides

Total uronic acid content of AIS preparations was measured as described by Ahmed and Labavitch (1977). Chelator-soluble pectins were extracted as described by Jarvis (1982). Approximately 30 mg of AIS in 10 to 15 mL of 50 mM Na-acetate, 50 mM CDTA, pH 6.5, were incubated for 6 h at 23°C. The suspensions were filtered through GF/C filters, and the filtrates were measured for soluble uronic acids (Blumenkrantz and Asboe-Hansen, 1973). As an alternative to the use of CDTA, 500 mM imidazole, pH 7.0, was also used to extract chelator-soluble polyuronides (Mort et al., 1991) from AIS. In some experiments, AIS following extraction in CDTA-containing buffers were transferred to Na_2CO_3 and maintained for 6 h at 4°C (Jarvis, 1982). The suspensions were filtered and analyzed for uronic acid sugars.

Gel Chromatography of Polyuronides

Polyuronides (0.3–0.5 mg of galacturonic acid equivalents) in 1 mL of extraction buffer were applied to a column (31 cm high, 1.5 cm wide) of Sepharose CL-2B-300 operated in 200 mM ammonium acetate, pH 5.0. Similar buffer conditions have been shown to minimize intermolecular aggregation and to yield more uniform behavior of polyuronides on gel filtration matrices (Mort et al., 1991). Two-milliliter fractions were collected at a flow rate of 15 mL h^{-1}, and 0.5-mL aliquots were assayed for uronic acid content. In other experiments, selected polyuronide fractions derived from the Sepharose profiles were rechromatographed on a column (64 cm high, 1 cm wide) of Bio-Gel P-4 (particle size <45 μm) operated in 200 mM ammonium acetate, pH 5.0. One-milliliter fractions were collected at a flow rate of 6 mL h^{-1}.

The P-4 column was calibrated with an oligouronide mixture and the purified trimer, both recovered from a partial digest of pectic acid with a purified PG, and galacturonic acid. The oligomers were generously provided by Dr. Andrew Mort and Dr. Niels Maness of Oklahoma State University.

Extraction and Assay of PG Activity

Fifteen milliliters of the 80% ethanol homogenates of cv Hass and cv Lula avocado mesocarp and cv Sunny pericarp tissues were centrifuged at 15,000*g* for 20 min at 4°C in a Beckman J-20 rotor. The supernatant was discarded, and the pellet was resuspended in 25 mL of cold 80% ethanol. The suspension was again centrifuged, and the pellet was redissolved in 15 mL of 50 mM Tris, pH 7.0, 1.2 M NaCl. The slurry was maintained on ice for 30 min and centrifuged at 15,000*g*. The supernatant was filtered through Miracloth and maintained on ice until assayed for PG activity. PG was assayed using polygalacturonic acid (Sigma) dissolved in 40 mM Na-acetate, pH 4.5 (tomato) or 5.5 (avocado). One-half milliliter of substrate (1 mg of polygalacturonic acid) and 100 μL of the enzyme extract were incubated for 60 min at 34°C. Heat-denatured samples were used as controls. Reactions were terminated by the addition of copper reagent (Somogyi, 1952) as reformulated for the specific determination of uronic acid reducing groups (Milner and Avigad, 1967). Enzyme activity is expressed as μmol of galacturonic reducing equivalents (g tissue fresh weight)$^{-1}$ h^{-1}.

RESULTS AND DISCUSSION

Preparation of Enzymically Inactive AIS

In our analyses of the solubility and M_r properties of avocado and tomato polyuronides, our first objective was to

ensure rapid and complete denaturation of tissue PG activity. Studies with tomato fruit have demonstrated that PG associated with cell-wall/AIS preparations causes much more extensive depolymerization than occurs in vivo (Seymour et al., 1987; Huber and Lee, 1988). To minimize the occurrence of polyuronide hydrolysis by endogenous enzymes, avocado mesocarp and tomato pericarp were excised and immediately processed into 80% ethanol suspensions. Koch and Nevins (1989) have shown that tomato PG is catalytically inactive in ethanol at concentrations of 50% and higher. Because the inactivation of PG by ethanol is completely reversible (Koch and Nevins, 1989), further processing of AIS involved direct transfer of the ethanolic precipitates into Tris-buffered phenol, a solvent that appears to be completely effective at irreversibly denaturating PG activity (Huber, 1992). In a study of pectin characteristics of kiwifruit, Redgwell et al. (1992) avoided the problem of endogenous enzyme activity by direct homogenization of tissue in phenol-acetic acid-water. Although phenol-acetic acid-water is a powerful protein solvent/denaturant (Selvendran, 1975) that may be adequate for inactivating enzymes in most fruit tissues, tomato cell walls treated with this solvent show substantially reduced calcium levels (Huber, 1991) and exhibit a residual, apparently enzymic capacity for polyuronide depolymerization (Huber, 1992). Criteria used in studies of tomato cell-wall/ AIS preparations (Huber, 1992) disclosed no residual PG activity in avocado AIS prepared with Tris-buffered phenol.

Uronic Acid Content and Solubility in AIS Derived from Avocado and Tomato Fruit AIS

Total uronic acid values and polyuronide solubility in 50 mM Na-acetate, 50 mM CDTA, pH 6.5, for the avocado and tomato AIS preparations are summarized in Table I. Total uronic acid content (μg of galacturonic acid equivalents mg^{-1} of AIS) differed between the two avocado cultivars, with levels in Lula averaging about 25% higher than values for Hass. With the exception of the mature-green stage (A), total uronic acid content in Sunny pericarp AIS exceeded that of both avocado cultivars. The inclusion of sulfamic acid in the *m*-hydroxydiphenyl assay exerted no effect on the total uronic acid values, indicating that neutral sugar interference was not a factor in the uronic acid measurements (Tullia et al., 1991). Differences in polyuronide solubility were apparent between the avocado and tomato fruits. In preripe (stage A) fruit of both avocado cultivars, CDTA-soluble uronic acids constituted less than 15% of the total uronic acid content. Ripening was accompanied by a nearly 5-fold increase in polyuronide solubilization in both cv Hass and cv Lula avocado fruits. At the final developmental stage sampled, CDTA-soluble uronic acids made up approximately 90% (Hass) and 70% (Lula) of total AIS uronic acids. Although little change in total uronic acid content was observed (Table I), presumably due to complete recovery of tissue polyuronides in AIS, the subsequent solubilization of uronic acids in CDTA is consistent with the trend of decreasing cell-wall uronic acid reported for ripening Hass avocado fruit (Colinas-Leon and Young, 1981).

The pattern of polyuronide solubilization in Sunny tomato fruit differed from that observed for the avocado fruits. In preripe Sunny tomato fruit, CDTA-soluble polyuronides as a percentage of total polyuronides were proportionately higher (40%) than values observed for the preripe avocado fruits. After an initial increase (20%) in polyuronide solubilization at stage B (pink), little further increase in CDTA-soluble polyuronides occurred, even during a 9-d period beyond the fully ripe stage (Table I). Previous investigators have attempted to explain polyuronide solubility changes in tomato fruit relative to levels of extractable PG. Polyuronide solubilization in AIS derived from the *rin* tomato mutant (which normally contains very low levels of PG) transformed with the PG structural gene led DellaPenna et al. (1990) to conclude that the chelator (EDTA) solubility of tomato polyuronides was PG dependent. Smith et al. (1990) observed that AIS derived from tomato fruit transformed with the PG antisense gene exhibited no reduction in chelator-soluble polyuronides, indicating a mechanism for solubilization independent of the enzyme. Our data support the latter view. The quantity of CDTA-soluble polyuronides recovered from mature-green tomato fruit (which contain negligible levels of PG) was relatively high (40% of total AIS levels) and, as a percentage of total polyuronides, increased little during ripening (Table I). Hobson (1964) observed that extractable levels of PG increased during ripening and continued to increase throughout a 10-d period beyond the fully ripe stage. The polyuronide solubility data reported here clearly do not parallel the anticipated increase in PG activity.

Polyuronide solubility was also examined using 500 mM imidazole, pH 7.0 (Mort et al., 1991). As summarized in Table I, yields of imidazole-extractable polyuronide from Hass avocado fruit AIS approached values obtained with CDTA. With tomato AIS, however, recoveries were markedly lower

Table I. *Total uronic acid content and chelator-soluble polyuronides in AIS derived from Hass and LuLa avocado fruit and Sunny tomato fruit*[a]

Stage	Hass			Lula		Sunny		
	Total	CDTA	Imidazole	Total	CDTA	Total	CDTA	Imidazole
A	192.9[b]	33.3	32.6	260.3	35.5	262.7	105.7	5.5
B	197.3	114.4	109.2	249.4	85.4	293.6	139.8	34.7
C	192.1	173.2	155.4	263.5	189.4	303.7	156.1	47.8
D						306.1	160.8	43.4

[a] All values presented as μg of galacturonic acid equivalents (mg of AIS)$^{-1}$. [b] Means of values obtained from three separate AIS preparations.

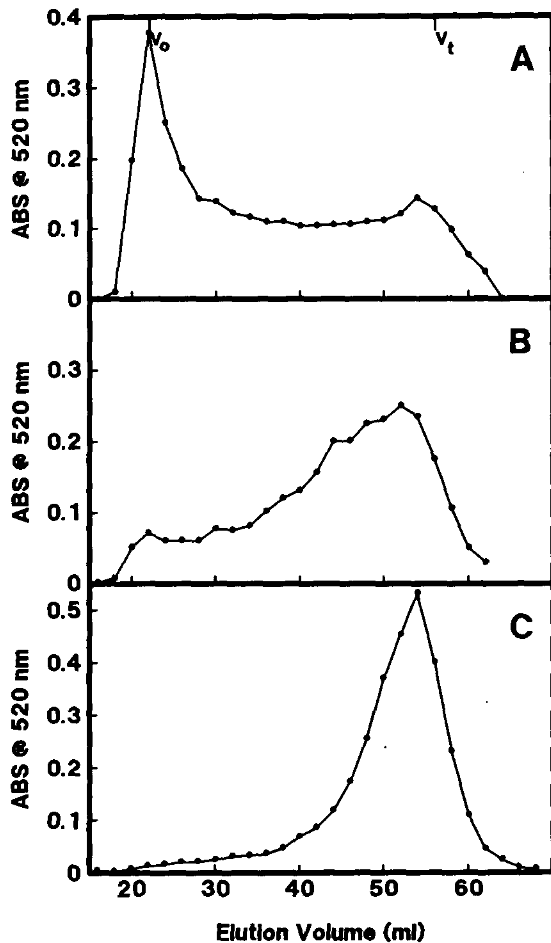

Figure 1. Sepharose CL-2B-300 profiles of CDTA-soluble polyuronides derived from cv Hass avocado mesocarp tissue. Polyuronides (0.3–0.5 mg of galacturonic acid equivalents) in 1.5 mL of extraction buffer (50 mM CDTA, 50 mM Na-acetate, pH 6.5) were applied to the Sepharose 2B-300 column operated in 200 mM ammonium acetate, pH 5.0. Two-milliliter fractions were collected at a flow rate of 15 mL h^{-1}. Fractions were assayed for uronic acid using the *m*-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973). Polyuronides from avocado fruit at: A, stage A; B, stage B; C, stage C. V_0, Void volume; V_t, total volume; ABS, *A*.

in imidazole and were not significantly increased with prolonged incubation periods. For this reason, polyuronide extraction was routinely performed in buffer containing CDTA.

M_r Analysis of CDTA-Soluble Polyuronides from Avocado Fruit and Tomato Fruit

After we examined a number of gel filtration media, Sepharose CL-2B-300 (exclusion limit for dextran standards, 20×10^6) was found to serve as the most appropriate medium for the comparative analyses of ripening-related alterations in avocado and tomato polyuronides. Tomato polyuronides in particular were nearly entirely excluded from Sepharose 4B-200 (5×10^6), even at the overripe stage (stage D) of development (profiles not shown). We previously found 4B-200 to be suitable for monitoring ripening-related changes in Sunny tomato fruit polyuronides (Huber, 1992). The higher M_r values observed in the present study is likely explained by the use of protocols designed to achieve more rapid inactivation and denaturation of tissue enzymes. Chromatography conditions used (ionic strength, pH) were based on those reported to minimize intermolecular aggregates and to yield highly reproducible elution behavior (Fishman et al., 1989; Mort et al., 1991).

Sepharose CL-2B-300 profiles of CDTA-soluble polyuronides from Hass avocado fruit are illustrated in Figure 1. Polyuronides from stage A fruit consisted of high M_r polymers excluded from 2B-300 and polydisperse polymers eluting throughout the fractionation range of the gel (Fig. 1A). As ripening proceeded, striking downshifts in polyuronide M_r were observed. At stage C of Hass avocado ripening (Fig. 1C), nearly all CDTA-soluble polyuronides (approximately 90% of total AIS uronic acid content) eluted as a relatively symmetrical population near the total volume of the gel.

Polyuronides from Lula exhibited a similar trend of depolymerization during ripening (Fig. 2), although the downshift observed at stage B was not as marked as was observed for

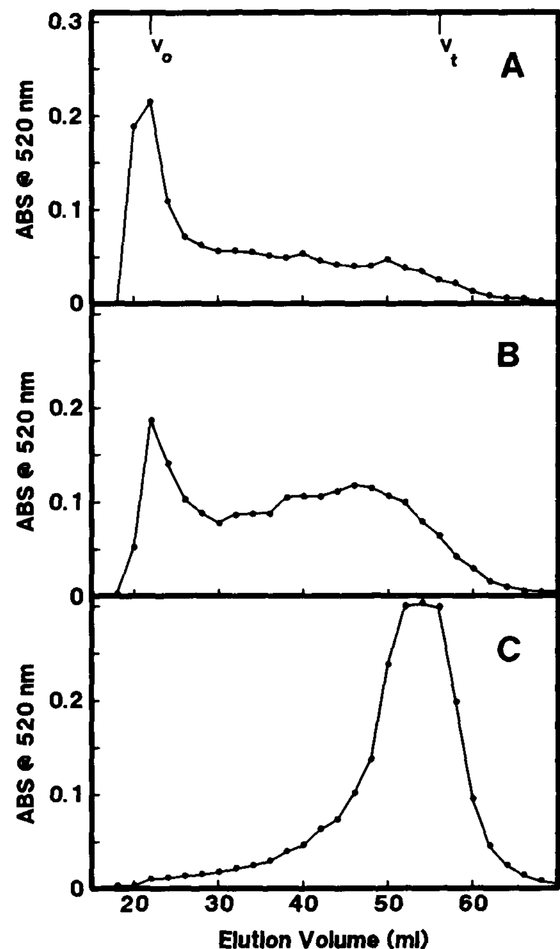

Figure 2. Sepharose CL-2B-300 profiles of CDTA-soluble polyuronides derived from cv Lula avocado AIS. Details are as described for Figure 1.

stage B Hass. We attribute the more extensive depolymerization evident in stage B Hass relative to stage B Lula to slight chronological differences. The firmness of Lula mesocarp tissue, as measured using Instron methods, decreased from about 500 N at stage A to 260 N at stage B (O'Donoghue and Huber, 1992), a change that is minimally detectable using the subjective methods used for selecting the developmental stages of Hass fruit. Nevertheless, the overall magnitude of the ripening-related downshift in polyuronide M_r is quite similar for the two cultivars. Bio-Gel P-4 chromatography of polyuronides from avocado fruit at stage C (only Hass shown) disclosed uronic acids with elution behavior similar to that of oligomers of galacturonic acid (Fig. 3). Although polyuronides from both stage A and B Hass avocado fruit included some components eluting near the total volume (56 mL) of the 2B-300 column, uronic acids retained on Bio-Gel P-4 were not observed at these developmental stages (profiles not shown).

The dramatic increase in CDTA-soluble polyuronides during avocado ripening (Table I) raises the possibility that the M_r downshifts are due, in part, to the solubilization of inherently lower M_r polymers. Redgwell et al. (1992) considered this possibility when interpreting the M_r downshifts occurring in CDTA-soluble polyuronides from ethylene-treated kiwifruit. Subsequent extraction of stage A avocado AIS with Na_2CO_3 solubilized an additional 50 (Hass) and 30 (Lula) μg of galacturonic acid equivalents (mg of AIS)$^{-1}$. Sepharose 2B-300 chromatography (Fig. 4) illustrates that Na_2CO_3-soluble polyuronides behave as lower M_r polymers than those extracted with CDTA; however, these polymers collectively are of considerably higher M_r than CDTA-soluble polyuronides derived from fruit at more advanced stages of ripening (Figs. 1C and 2C).

Sepharose CL-2B-300 profiles of CDTA-soluble polyuronides from ripening tomato fruit are shown in Figure 5. Although a pattern of decreasing M_r is evident, the downshift during ripening is much less extensive than that observed for the avocado fruit. Throughout an additional 9-d period beyond stage C (fully ripe, Fig. 5C), significant downshifts in M_r were observed in the excluded polyuronides but without a concomitant change in the retained polymers (Fig. 5D). Consistent with this trend, uronic acids in the CDTA-soluble extracts of stage D tomato fruit were entirely excluded from Bio-Gel P-4 (profile not shown). The fact that characteristic oligouronides are apparently not produced in tomato fruit and that oligouronides appear only during the latter period of avocado ripening is not consistent with the idea that low-DP ($\leq$10) pectic oligomers play a regulatory role in fruit ripening (Brecht and Huber, 1988; Campbell and Labavitch, 1991). We cannot preclude the possibility that higher-DP oligomers (outside the fractionation range of P-4) are present or that oligouronides fail to accumulate in vivo because of rapid turnover; however, the production of transient species is inconsistent with the observation that oligogalacturonides generated from enzymically active cell wall from tomato fruit are quite persistent (Huber and Lee, 1988).

The data indicate that polyuronide depolymerization in avocado fruit is a much more prominent feature of ripening

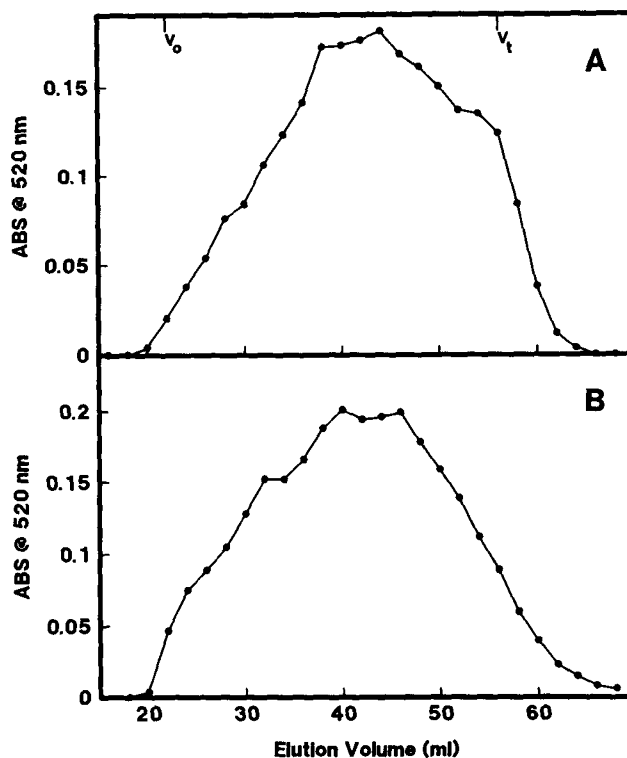

Figure 4. Sepharose CL-2B-300 profiles of Na_2CO_3-soluble polyuronides derived from stage A Hass and Lula avocado fruit AIS. Stage A avocado AIS following extraction with 50 mM Na-acetate, 50 mM CDTA, pH 6.5, were transferred to 50 mM Na_2CO_3 for 6 h at 4°C. One-half milligram of polyuronides (galacturonic acid equivalents) was applied to Sepharose 2B-300. Conditions were as described for Figure 1. A, Na_2CO_3-soluble polyuronides from stage A Hass avocado; B, stage A Lula avocado fruit. V_0, Void volume; V_t, total volume.

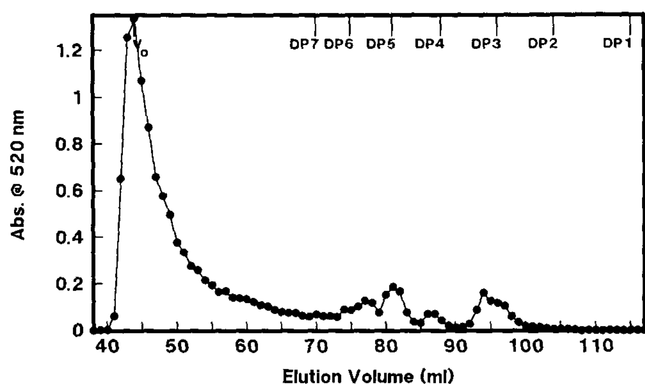

Figure 3. Bio-Gel P-4 profile of polyuronides derived from stage C cv Hass avocado fruit AIS. Polyuronides derived from elution volume 56 to 66 mL of the Sepharose 2B-300 profiles from stage C Hass avocado AIS (Fig. 1C) were concentrated to a volume of 1.5 mL and applied to Bio-Gel P-4. One-milliliter fractions were collected at a flow rate of 10 mL h^{-1}. Fractions were analyzed for uronic acid sugars. V_0, Void volume; DP7 through DP1 index the elution positions of oligouronides and galacturonic acid used for column calibration. Abs., *A*.

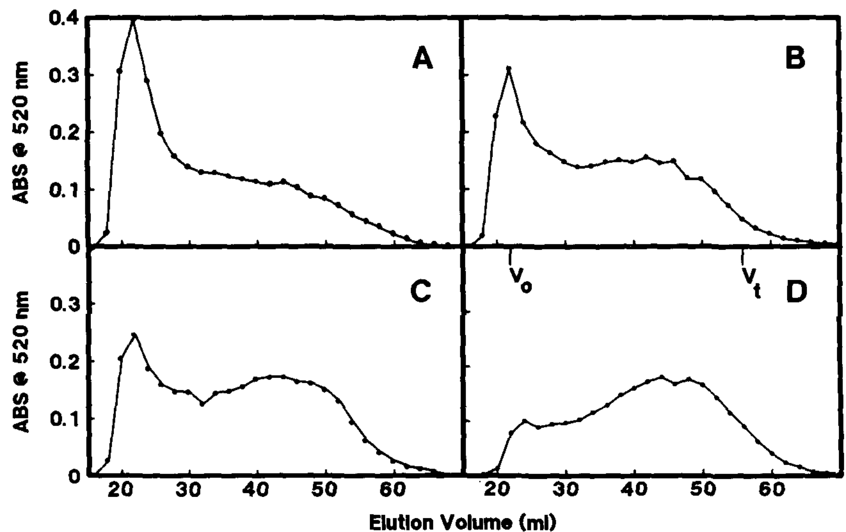

Figure 5. Sepharose CL-2B-300 profiles of CDTA-soluble polyuronides derived from Sunny tomato fruit AIS. Conditions were as described for Figure 1. Polyuronides from tomato fruit at: A, stage A (mature green); B, stage B (pink); C, stage C (fully ripe); D, stage D (overripe). ABS, *A*; V_0, void volume; V_t, total volume.

than is the case with tomato fruit, and it raises the question of whether polyuronide changes represent a more influential factor in avocado fruit ripening and softening. The marked downshifts in polyuronide M_r were apparent in both Hass and Lula, Mexican and Mexican/Guatamalen strains, respectively (Ryall and Pentzer, 1982). The limited downshift in M_r of polyuronides from Sunny tomato fruit is consistent with data for other tomato cultivars, including Rutgers (DellaPenna et al., 1990), an inherently softer cultivar than Sunny (Ahrens and Huber, 1990). Similarly, Smith et al. (1990) observed an approximately 50% reduction in weight-average M_r of polyuronides during ripening of Ailsa Craig tomato fruit. Moreover, there is reason to suspect that the data reported by DellaPenna et al. (1990) and Smith et al. (1990) may overestimate the extent of in vivo polyuronide depolymerization occurring during tomato ripening. In both studies, phenol-acetic acid-water was used to inactivate tissue enzymes before polyuronide extraction and M_r analyses, a treatment we have found to be ineffective at completely eliminating polyuronide depolymerization in cell-wall/AIS preparations (Huber, 1992).

Analysis of Avocado and Tomato Fruit PG Activity

Although uncertainties exist regarding the influence of PG on tomato fruit firmness and polyuronide solubilization, studies with transgenic tomato fruit exhibiting up- or down-regulated levels of the enzyme have established an apparent relationship between the protein and polyuronide depolymerization (DellaPenna et al., 1990; Smith et al., 1990). Thus, the dramatic downshift in polyuronide M_r in avocado fruit was somewhat unanticipated in view of the report of Hobson (1962) that tomato fruit (cv Potentate) PG levels were nearly 15-fold higher (fresh weight basis) than levels found in avocado (*Persea gratissima*). Although rigorous purification studies have not been reported for avocado PG, data from early comparative studies of partially purified tomato and avocado fruit PGs (McCready et al., 1955; Reymond and Phaff, 1965) indicate that the enzymes are catalytically similar, with the exception of in vitro pH optima (5.5 for avocado, 4.5 for tomato) (Reymond and Phaff, 1965). We determined the relative levels of PG activity in extracts derived from the avocado and tomato fruits.

PG activity was not detected in preripe tissues of either fruit type. In stage B Hass avocado fruit, relatively high (15.7 μmol of reducing equivalents [grams fresh weight]$^{-1}$ h^{-1}) activity was detected, followed by an additional 2-fold increase at stage C (31 μmol). The activity present in stage C Hass avocado is quite similar to PG activity (approximately 35 μmol of reducing equivalents [grams fresh weight]$^{-1}$ h^{-1}) in unpurified extracts of Fuerte avocado (McCready and McComb, 1954). Lower activity was apparent in Lula avocado: 3.5 μmol at stage B and 23.6 μmol at stage C. In Sunny tomato fruit, activity was first present at stage B (pink, 1.5 μmol) and showed additional increases with further ripening (ripe, 10.4 μmol; overripe, 16 μmol), a rather typical trend for tomato fruit (Speirs et al., 1989).

The differences in PG activity between the tomato and avocado fruits are not dramatic and suggest that factors other than absolute activity levels are responsible for the trends of M_r downshifts in polyuronides. This idea is supported by M_r analysis of polyuronides from Rutgers tomato fruit (DellaPenna et al., 1990), which contain PG at levels 2- to 3-fold higher than Sunny (Ahrens and Huber 1990) and thus comparable to levels reported here for the avocado fruits. Even though AIS preparations were probably not totally enzymically inactive, nearly 50% of the CDTA-soluble polyuronides derived from ripe Rutgers tomato fruit AIS were excluded from Sepharose 4B-200 (exclusion limit for dextrans, 5×10^6) (DellaPenna et al., 1990). Although structural features of the polyuronides in the different fruit types may contribute to the disparity in ripening-related depolymerization, tomato fruit clearly possess the capacity for more extensive downshifts in polymer M_r. Polyuronides from tomato AIS not treated with phenol (enzymically active AIS) are degraded rapidly in vitro, yielding products with elution characteristics on Sepharose 2B-300 (Fig. 6) similar to those of CDTA-extractable polyuronides from stage C avocado fruit (Figs. 1C and 2C). The evidence collectively suggests that polyuronide depolymerization in tomato fruit is normally restricted in

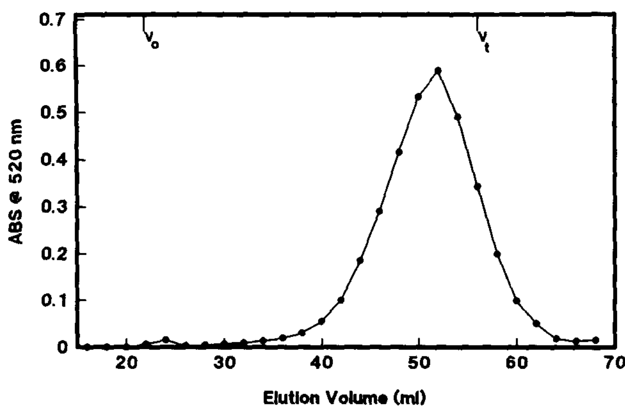

Figure 6. Sepharose CL-2B-300 profile of CDTA-soluble polyuronides derived from enzymically active (non-phenol treated) AIS from stage D Sunny tomato fruit. V_0, Void volume; V_t, total volume.

vivo and that extensive M_r downshifts occur only under in vitro conditions. The extensive M_r downshifts in CDTA-soluble polyuronides from ethylene-treated kiwifruit (Redgwell et al., 1992), which contain very low levels of PG (Wegrzyn and MacRae, 1992), provide further evidence that the extent of polyuronide depolymerization is not always an indicator of the levels of extractable PG.

Possible Role of Polyuronide Depolymerization and PG in Ripening Fruit

The comparatively restricted in vivo depolymerization of polyuronide in tomato fruit may help explain the effects of genetic down-regulated levels of PG on tomato fruit development. With one exception (Kramer et al., 1992), introduction of PG antisense constructs into various tomato lines has had little effect on fruit characteristics until the late stages of ripening (Kramer et al., 1990; Schuch et al., 1991). Schuch et al. (1991) observed that the reduced PG levels (>99% reduction) in tomato fruit containing the PG antisense gene, although not influencing firmness as assessed by whole fruit compression, resulted in a much reduced susceptibility to cracking, decay, and other damage, particularly during simulated transport studies. Similarly, Kramer et al. (1990) concluded, based on an analysis of tomato lines containing a PG antisense construct, that the major role of the enzyme is the deterioration of fruit during the overripe stage. We suggest that the divergence in quality between the normal and transgenic tomato fruit during late development or during transport and handling studies is a consequence of developmental or damage-induced modulations in the apoplastic environment. Cellular leakage resulting from impact or compression bruising or as occurs normally due to membrane dysfunction during late fruit development (Legge et al., 1986; Güčlü et al., 1989) would alter the apoplastic ionic composition, possibly providing conditions more conducive to expression of maximum polyuronide depolymerization potential and thus rapid deterioration of fruit with normal levels of PG. These interpretations are consistent with observations that the depolymerization of polyuronides in isolated cell wall (i.e. measured under favorable pH and ionic conditions) from tomato fruit greatly exceeds that occurring in vivo (Rushing and Huber 1984; Huber and Lee 1988). Additionally, the extensive depolymerization and release of uronic acids, in predominately oligomeric form, from tomato pericarp discs is largely a cut-surface (wound) phenomenon and is dependent on the presence of buffers of suitable pH and ionic strength (Huber and Lee, 1989). Several investigators have reported that juice from PG antisense fruit was of significantly higher viscosity than juice from the untransformed fruit (Kramer et al., 1990; Schuch et al., 1991; Kramer et al., 1992). These observations indicate that the environment provided by the release of endogenous cellular fluids is favorable for enhancing polyuronide depolymerization and, therefore, accentuating differences in the PG antisense versus normal tomato lines. In addition to stimulating enzymic hydrolysis of polyuronides, altered ionic strength could have the effect of causing nonenzymic dissociation of noncovalently associated polyuronide aggregates into smaller subunits (Fishman et al., 1989; Mort et al., 1991).

The extensive in vivo depolymerization of polyuronides occurring in avocado fruit suggests that apoplastic conditions are inherently more conducive to enzymic hydrolysis. Calcium levels and binding characteristics may also be involved, as indicated by the fact that imidazole, a monodentate chelator, was much less effective than CDTA at solubilizing polyuronides from AIS of tomato but not of avocado. There is evidence that enzymes other than PG may contribute to decreases in polyuronide M_r. For example, purified preparations of β-galactosidase from avocado (de Veau et al., 1993) and muskmelon (Ranwala et al., 1992) fruits have been shown to mediate PG-independent M_r downshifts in branched polyuronides via degalactosidation. The possibility that this mechanism may participate in the M_r downshift in avocado polyuronides is supported by the observation that Gal levels in avocado CDTA-soluble polyuronides decrease nearly 35% during ripening (our unpublished data).

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