

Analysis of the molecular size of tomato (*Lycopersicon esculentum* Mill) fruit polyuronides by gel filtration and low-speed sedimentation equilibrium

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The cell-wall structures of tomato (*Lycopersicon esculentum* Mill) and other fruit are intimately linked with the nature of their polyuronides. Cell-wall polyuronides from unripe and ripe tomato fruit were isolated and purified and their molecular size and molecular-size distributions were compared. It was demonstrated that there is a considerable decrease in the weight-average M_r upon ripening (from $160\,000 \pm 10\,000$ to $96\,000 \pm 4\,000$) and a corresponding increase in polydispersity, particularly at the low- M_r end of the distribution. The estimates of polyuronide molecular size and molecular-size distribution were obtained without the need for polyuronide standards of known M_r by using gel-filtration chromatography combined with the absolute method of low-speed sedimentation equilibrium.

INTRODUCTION

In tomato (*Lycopersicon esculentum* Mill) and many other fruit the alterations in texture which occur during ripening are thought to be brought about by changes in cell-wall structure. One of the most evident of these changes is an increase in the level of soluble polyuronide in the cell walls (Sawamura *et al.*, 1978; Gross & Wallner, 1979; Huber, 1983a). Studies with mutant tomato fruit suggest that there may be a close relationship between softening, polyuronide solubilization and the activity of the enzyme polygalacturonase (Tigchelaar *et al.*, 1978; Gross & Wallner, 1979; Tucker *et al.*, 1980). However, the exact nature of the relationship between these events is unclear.

An understanding of the characteristics of the polyuronide solubilized during fruit ripening should provide information on the mechanism by which it is solubilized and may increase our understanding of the biochemical basis of softening. A number of studies on cell-wall changes in tomato and other fruit during ripening have included determinations of the molecular size of various soluble polyuronide-containing fractions by gel-filtration chromatography (see Pressey *et al.*, 1971; Gross & Wallner, 1979; Ahmed & Labavitch, 1980; Pressey & Avants, 1982; Huber, 1983b). However, although significant changes in polymer molecular size have been observed during ripening, usually only very approximate estimates of polymer M_r are reported. This is because of difficulties in obtaining appropriate standards of similar conformation and known M_r for gel-filtration chromatography. In previous studies on polyuronides, columns have been calibrated with dextran standards, which may have a less extended conformation than the uronide polymers, thus making difficult even modest estimations of polymer M_r .

In the present study we use the combined approach of gel filtration and the technique of low-speed sedimentation equilibrium. This latter technique is an absolute

method that does not require the use of calibration standards, nor does it rely on any assumptions concerning macromolecular shape. Furthermore, simple procedures are available for meniscus concentration and M_r extraction (see, e.g., Creeth & Harding, 1982). It can be realistically applied to many polysaccharide systems, unlike laser-light-scattering techniques, which are fraught with problems of clarification of solutions and polydispersity.

MATERIALS AND METHODS

Isolation and purification

Polyuronides were extracted from acetone-insoluble solids of tomato fruit pericarp (Huber, 1983b; Seymour *et al.*, 1987). The acetone-insoluble material was prepared from mature green (35–40 days post anthesis) or red ripe tomato fruit (var. Ailsa Craig) and each acetone-dried powder was treated with phenol/acetic acid/water (2:1:1, by vol.) to remove endogenous enzyme activity. The polyuronides were extracted by incubating the acetone-insoluble solids at a concentration of ~ 6 or ~ 12 mg·ml⁻¹ for red and green fruit respectively in 50 mM-sodium acetate/40 mM EDTA, pH 4.5, with constant stirring for 4 h at room temperature. After incubation the slurry was centrifuged for 2 min at 12000 g and the resultant supernatant filtered through Whatman CF/A paper. Polyuronide extracts from red and green fruit were prepared in triplicate. After filtration through GF/A paper one sample was made up to a volume of 10 ml and analysed for overall sugar composition by using the *m*-hydroxydiphenyl method for uronic acids (Blumenkrantz & Asboe-Hansen, 1973) and the phenol/H₂SO₄ method for total sugars (Dubois *et al.*, 1956). The remaining samples were analysed by gel-filtration chromatography and used in the sedimentation-equilibrium study.

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Gel filtration

For gel-filtration chromatography the polyuronide extract was made up to a volume of 2 ml and applied to a column (1.6 cm × 89 cm) of Sephacryl S-400. Approx. 1 mg of sample was applied to the column, which was equilibrated and run in 0.1 M-sodium acetate/0.2 M-EDTA, pH 6.5, at a flow rate of 12 ml·h⁻¹·cm⁻². Fractions (2.2 ml) were assayed for uronic acids and total sugar as described above. Column recoveries were normally ~ 90–95%.

Sedimentation equilibrium

Sedimentation-equilibrium measurements were performed on a Beckman Model E analytical ultracentrifuge employing Rayleigh interference optics and an RTIC temperature-measurement system. All solutions had been dialysed for > 12 h against a standard phosphate/chloride buffer, pH 6.8 (*I* 0.10). The relevant proportions of Na₂HPO₄ and KH₂PO₄ were made up to a combined ionic strength of 0.05, and a further 0.05 M-NaCl was added as described by Green (1923). The buffer also contained 5 mM-EDTA.

The 'intermediate speed' method was employed (Creeth & Harding, 1982); in this method the speed is sufficiently low to allow adequate resolution of the fringes near the cell base. At equilibrium the concentration at the air/solution meniscus remains finite and is obtained by mathematical manipulation of the fringe data [the slope/intercept method of Creeth & Harding (1982)]. To minimize the effects of thermodynamic non-ideality and associative phenomena, all determinations were made in 30 mm-path-length cells at the lowest possible loading concentration, *c*₀ (~ 0.4 mg/ml).

Whole-cell weight-average *M*_r (*M*_{r,w}⁰) values were extracted by using the limiting value at the cell base of a particularly directly determinable point average [the 'star' average, *M*_r^{*} (Creeth & Harding, 1982)]; an independent estimate for the initial concentration was not required. Point weight-average *M*_r (*M*_{r,w}) values were obtained by using sliding-strip quadratic fits to the observed fringe data. 'Infinite-dilution' values [*M*_{r,w} (*J* → 0), where *J* is the absolute fringe concentration] were obtained by manual extrapolation to *J* = 0. Because of the low fringe increment near the meniscus, points less than 1.0 fringe were not used.

Partial specific volumes

Partial specific volumes (\bar{v}) were calculated from the values for overall sugar composition of the extracts by assuming the uronic acid component to be galacturonic acid and all non-uronic acid components to be neutral hexoses. The \bar{v} of hexose and galacturonic residues were calculated from the formula given by Gibbons (1972; see also Cohn & Edsall, 1943); values of 0.613 ml·g⁻¹ for the hexose and 0.479 ml·g⁻¹ for the galacturonic acid were obtained, the latter after correction for electrostriction. By using a galacturonic acid/hexose ratio of 781:219 for the polyuronide from green tomato and 823:177 for that from the red, values for the weight-average \bar{v} (Gibbons, 1972) for the green- and red-tomato pectin of 0.508 ml·g⁻¹ and 0.500 ml·g⁻¹ were calculated. The total level of neutral sugar in polyuronide extracts, although low, appears to vary between samples of fruit at similar stages of ripeness (G. B. Seymour, unpublished work), and calculations taking this variation into account

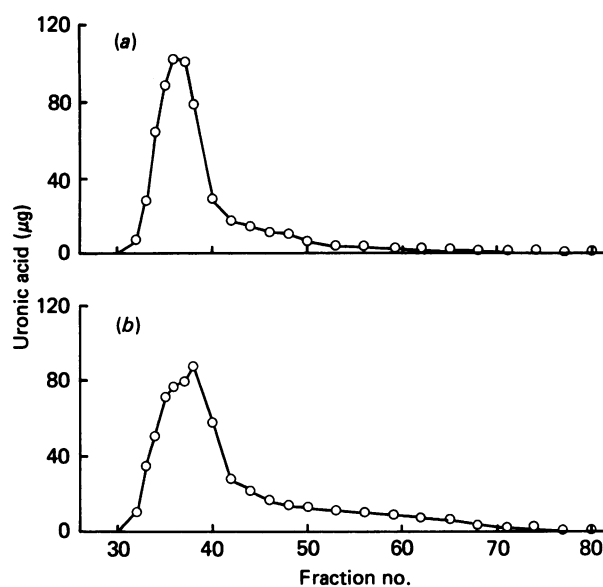


Fig. 1 Sphacryl S-400 profile of soluble polyuronides extractable from unripe (a) and ripe (b) tomato fruit

Column *V*₀ and *V*_t values correspond to fractions 33 and 81 respectively.

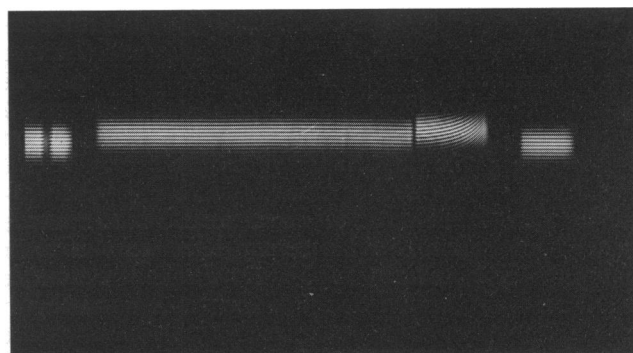


Fig. 2. Rayleigh interference fringes from cell-wall polyuronide (ripe fruit)

The solution was at pH 7.0 (*I* 0.10). The rotor speed was 9341 rev./min and the temperature was 20.0 °C. *c*₀ was ~ 0.4 mg/ml.

indicate errors in \bar{v} of no more than 4% at worst; this would lead to errors in *M*_r of a similar order.

RESULTS AND DISCUSSION

The amount of EDTA/acetate (pH 4.5)-soluble polyuronide extractable from acetone-insoluble preparations of tomato increases during ripening from ~ 50 µg/mg in unripe fruit to ~ 100 µg/mg in ripe fruit, this representing 50% of the total polyuronide from ripe fruit.

The apparent molecular-size distributions of the soluble polyuronide from unripe and ripe tomato fruit on Sephacryl S-400 are shown in Fig. 1. Most of the polymers from unripe fruit were just included on the column, which would indicate an apparent *M*_r (based on the manufacturer's specifications for dextran standards) of ~ (1–2) × 10⁶. The ripe extract also contained many polymers of high *M*_r, but there appeared to be a greater

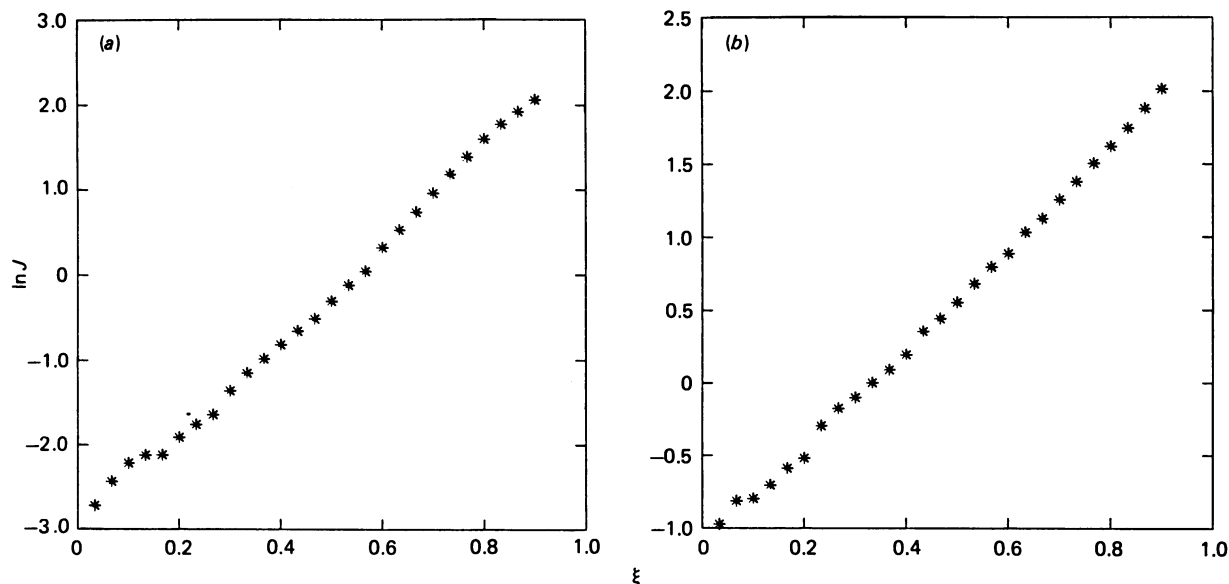


Fig. 3. Comparison of log(fringe concentration)-versus- ξ (radial-displacement parameter) plots for cell-wall polyuronides from unripe (a) and ripe fruit (b)

The rotor speed, temperature and concentrations were as for Fig. 2.

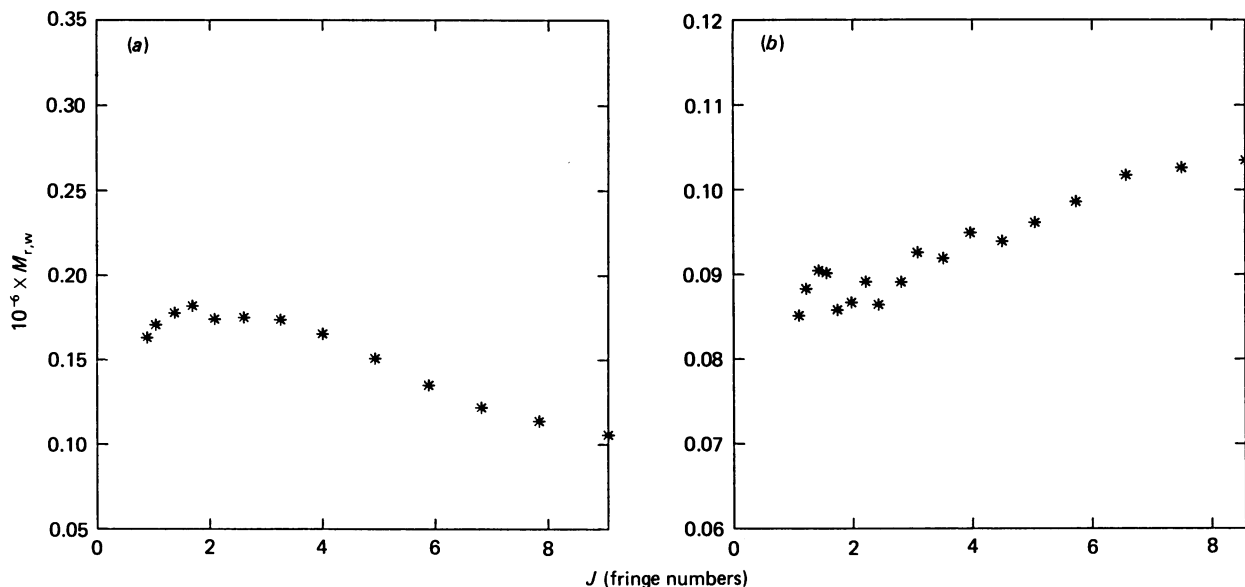


Fig. 4. Comparison of point-weight-average- M_r ($M_{r,w}$)-versus-(fringe)-concentration (J) plots for cell-wall polyuronides from unripe (a) and ripe fruits (b)

Other details are as for Fig. 2.

degree of polydispersity among the polyuronides in the extract, particularly with regard to the low- M_r tail (Fig. 1b). Low levels of neutral sugars were apparent in column fractions from both red and green fruit samples, as might be expected from determinations of overall sugar composition (see the Materials and methods section). However, the low levels of neutral sugars in the column fractions made quantitative determinations difficult, and therefore only values for uronic acid are presented in Fig. 1. The very high apparent M_r values for some of the tomato fruit polyuronides in the present study are similar to those given by other workers using

gel filtration (Pressey *et al.*, 1971; Ahmed & Labavitch, 1980; Huber, 1983b). However, it is likely that the extended conformation of the polymer chains gives an exaggerated impression of the M_r when compared with 'standards' such as dextrans. Two conclusions can be drawn from the chromatography results alone: upon ripening (green fruit → red fruit) (1) the mode and mean (apparent) M_r values appear to be decreased and (2) there appears to be greater polydispersity, particularly in the lower- M_r tail region (Fig. 1b).

It is unlikely that differential extractability could account for these M_r -distribution differences. The use of

Table 1. M_r values of polyuronides from low-speed sedimentation-equilibrium analysis

Source of polyuronide	Polyuronide M_r	
	Whole-cell weight-average value ($M_{r,w}^0$)	Infinite-dilution value [$M_{r,w}(J \rightarrow 0)$]
Green unripe tomato	160 000 ± 10 000	170 000 ± 15 000
Red ripe tomato	96 000 ± 4 000	88 000 ± 8 000

CDTA (*trans*-1,2-diaminocyclohexane-*NNN'*-tetraacetic acid) (a stronger chelator than EDTA) extracts ~ 120 µg of uronic acid/mg from both unripe and ripe fruit. Polyuronides extracted in this way show similar differences in size between unripe and ripe fruit to those extracted with EDTA/acetate.

To confirm the apparent changes in molecular size observed above and to quantify these observations in terms of actual M_r values, we need to use the results from low-speed sedimentation equilibrium. Fringe profiles for the polyuronides from the red fruit are shown in Fig. 2, illustrating clearly that meniscus-depletion conditions are not fulfilled.

In Fig. 3 we have compared plots of the absolute fringe concentrations (J) versus the radial-displacement parameter, ξ [$= (r^2 - a^2)/(b^2 - a^2)$, where r is the radial displacement of a given point in the cell from the centre of the rotor and a and b the corresponding positions of the meniscus and cell base respectively], for the polyuronides from the unripe (Fig. 3a) and ripe fruit (Fig. 3b). The downward curvature near the cell base is indicative of the presence of considerable thermodynamic non-ideality (presumably because of high solvation effects and also a charge contribution, even in the presence of 10.1 buffer). Such effects will tend to mask the effects of any polydispersity. However, for the polyuronides from the ripe tomato, the polydispersity appears to be considerably greater (or the non-ideality considerably lower), resulting in upward curvature of $\ln J$ near the cell base; these observations are consistent with the findings from chromatography (Fig. 1). The corresponding values for the $M_{r,w}^0$ are given in Table 1. These values may be lower than the true 'ideal' M_r values because of the non-ideality, even at the low cell-loading concentrations used ($c \sim 0.4$ mg · ml⁻¹). However, extrapolation of the point weight-average M_r values (Fig. 4) to zero (fringe) concentration yields a weight-average M_r [$M_{r,w}(J \rightarrow 0)$] that is less precise than the $M_{r,w}^0$ values, but not affected by non-ideality or self-association phenomena (but will still be affected by the polydispersity;

i.e. the non-interacting components of different M_r and/or \bar{v}). Such values are also given in Table 1. Values for $M_{r,w}$ and $M_{r,w}(J \rightarrow 0)$ are also consistent with the observations of Fig. 1, concerning the apparent decrease in the average M_r upon ripening. The $M_{r,w}$ -versus- J -plots of Fig. 4 also reveal the greater apparent polydispersity of the ripe-tomato polyuronides, assuming that the non-ideality is not considerably less and that self-association phenomena are absent. A further experiment with a different preparation of ripe-tomato polyuronides gave virtually identical elution profiles and identical sedimentation-equilibrium results: $M_{r,w}^0 = 98 000 \pm 4 000$; $M_{r,w}(J \rightarrow 0) = 93 000 \pm 6 000$.

The results that we have obtained from gel filtration (which is useful for illustrating size distribution qualitatively) and sedimentation equilibrium (which is arguably the most powerful technique for obtaining average M_r values of polysaccharides) are strongly indicative that, when tomatoes ripen, their cell-wall polyuronides show a decrease in M_r and an increase in polydispersity, particularly at the low- M_r end of the distribution.

We thank Dr. G. Tucker and Dr. J. M. Creeth for their helpful comments and suggestions, and Mr. M. S. Ramzan for expert technical assistance.

REFERENCES

- Ahmed, A. E. & Labavitch, J. M. (1980) *Plant Physiol.* **65**, 1009–1013
- Blumenkranz, N. & Asboe-Hansen, G. (1973) *Anal. Biochem.* **54**, 484–489
- Cohn, E. J. & Edsall, J. T. (1943) *Proteins, Amino Acids and Peptides*, chapter 16, Reinhold, New York
- Creeth, J. M. & Harding, S. E. (1982) *J. Biochem. Biophys. Methods* **7**, 25–34
- Dubois, M., Gillies, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* **28**, 350–356
- Gibbons, R. A. (1972) in *Glycoproteins: Their Composition, Structure and Function* (Gottschalk, A., ed.), vol. 5, part A, pp. 31–157, Elsevier, Amsterdam
- Green, A. A. (1923) *J. Am. Chem. Soc.* **55**, 2331–2336
- Gross, K. C. & Wallner, S. J. (1979) *Plant Physiol.* **63**, 117–120
- Huber, D. J. (1983a) *Hortic. Rev.* **5**, 169–219
- Huber, D. J. (1983b) *J. Am. Soc. Hortic. Sci.* **108**, 405–409
- Pressey, R. & Avants, J. K. (1982) *J. Food Biochem.* **6**, 57–74
- Pressey, R., Hinton, D. M. & Avants, J. K. (1971) *J. Food Sci.* **36**, 1070–1073
- Sawamura, M., Knegt, E. & Bruinsma, J. (1978) *Plant Cell Physiol.* **19**, 1061–1069
- Seymour, G. B., Harding, S. E., Taylor, A. J., Hobson, G. E. & Tucker, G. A. (1987) *Phytochemistry*, in the press
- Tigheelaar, E. C., McGlasson, W. B. & Buescher, R. W. (1978) *HortScience* **13**, 508–513
- Tucker, G. A., Robertson, N. G. & Grierson, D. (1980) *Eur. J. Biochem.* **112**, 119–124