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POLYURONIDE SOLUBILIZATION DURING RIPENING OF NORMAL AND MUTANT TOMATO FRUIT

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Abstract—The amount and molecular size of soluble polyuronide extractable from ripening tomatoes is markedly affected by residual enzyme activity. The efficacy of phenol–acetic acid–water treatment to remove this residual activity is demonstrated. Data obtained using treated wall preparations confirms that there is an increase in soluble polyuronide during normal ripening and that this also occurs in the 'Never-ripe' mutant, and to a lesser degree in the 'ripening-inhibitor' mutant. However, changes in the molecular size of this polyuronide during normal ripening were not as extensive as previously reported and few changes were apparent in either of the mutants.

Measurements were also made of polygalacturonase (EC 3.2.1.15) and pectinesterase (EC 3.1.1.11) activity during ripening. The level of polygalacturonase activity does not appear to correlate with the amount of soluble polyuronide released, but may be related to the extent of depolymerisation. No relationship was apparent between the level of pectinesterase and either soluble polyuronide released or depolymerization.

INTRODUCTION

During the ripening of tomato fruit there are significant changes in cell wall structure. The most apparent of these changes are an increase in soluble polyuronide and a loss of galactose and arabinose from the walls [1–3]. However, the mechanism by which these changes are accomplished and their relationship to fruit softening is not fully understood.

Polygalacturonase (PG) activity is thought to be involved in polyuronide solubilization in tomato fruit. An increase in the activity of this enzyme is observed which coincides with the increase in soluble polyuronide during ripening [2], and purified PG can release uronides from cell wall material *in vitro* [4]. A key role for PG during the softening of tomato fruit is suggested by results using mutant varieties. The 'Never-ripe' (Nr) mutant has reduced levels of PG compared to normal fruit and softens comparatively slowly, while the 'ripening inhibitor' (rin) mutant has no, or very little, PG activity and does not soften to any great extent [5, 6]. The rin mutant has also been shown to contain very little soluble pectin compared to normal fruit [2].

Although there appears to be a relationship between PG activity, polyuronide solubilization and softening, comparatively little information is available on *in vivo* changes in polyuronide structure during ripening; such data might be expected to yield important information on the mechanism by which polyuronide is solubilized. Several workers have documented compositional changes in cell wall material that accompany ripening and studies have been undertaken on various uronide containing wall fractions from tomato fruit [1–3, 7]. However, the range of techniques used to prepare the wall material, and related fractions, makes it difficult to compare directly the results from these various studies. There are problems also in relating many of these studies to *in vivo* changes in polyuronide structure. Many of the classical methods for the isolation of cell wall material involve either homogenisation in water or aqueous buffers, this could result in the loss of much soluble polyuronide [8]. Also in several previous studies no attempts have been made to inactivate endogenous enzymes during the wall preparation. These enzymes could significantly alter the structure of the wall components during isolation. The extraction, into aqueous buffer, of pectic polymers from acetone insoluble material, prepared so as to remove any endogenous enzyme activity, could provide a solution to these problems. Huber [9] used this technique to investigate changes in the degree of polymerisation of pectic polymers during tomato ripening. The polyuronides isolated from green fruit were shown to have a relatively high M_r, but to undergo extensive degradation during ripening. In the present study a more detailed investigation was undertaken of changes in polyuronide molecular size during ripening in both normal and mutant fruit, and to relate changes in levels of polyuronide solubilization and pectolytic activity. The data obtained indicates that alterations in polyuronide molecular weight during ripening are much less extensive than previously thought and that the extent of polyuronide solubilization is not closely related to levels of either PG or pectinesterase.

RESULTS AND DISCUSSION

Effect of endogenous enzyme activity on soluble polyuronide

Acetone insoluble solids were prepared from red fruit with or without a phenol–acetic acid–water (PAW) treatment. Following the incubation of these powders in acetate–EDTA buffer the amount and molecular size distribution (Fig. 1) of the soluble polyuronide released, were determined. The amount of polyuronide released from the untreated preparation was higher than that from

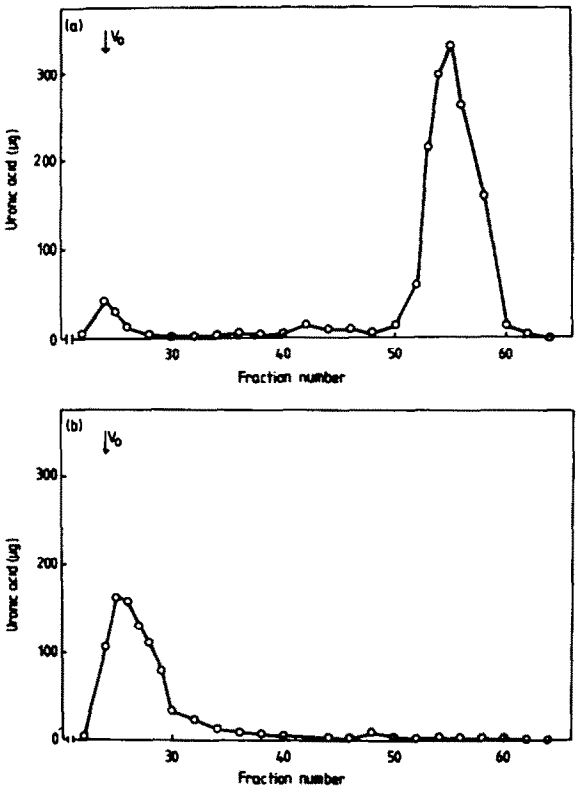

Fig. 1. Sephacryl S-200 profile of soluble polyuronides extractable from ripe tomato fruit acetone-insoluble material prepared (a) without and (b) with a PAW treatment.

the treated sample, also PAW treatment has a marked effect on the molecular size of the polyuronide extractable from ripe fruit (Fig. 1). In treated material the majority of the polyuronide released was in a high form, eluting at or around the void volume of a Sephacryl S-200 column. This would suggest (using the manufacturer's specifications for dextran standards) an apparent M_r *ca*, or in excess of, 80 000. However, most of the polyuronide from untreated material was of a much lower molecular size and examination of Trisacryl GF05 indicated uronide oligomers with apparent M_rs equivalent to degrees of polymerisation of approximately 6, 4 and 1 (Seymour *et al.*, unpublished). The presence of monomer was confirmed using paper chromatography.

These results indicate that there are appreciable levels of uronide degrading activity in the acetone insoluble material, and that a PAW treatment will significantly reduce this activity. Although these data support earlier claims that a PAW treatment will inactivate endogenous enzymes [10], Rushing and Huber [8] have reported this treatment to be only partially effective. It is therefore possible that even the above result for PAW treated material represents, at least partly, the action of endogenous enzymes. Experiments were undertaken to examine this possibility.

The two main pectolytic enzymes associated with tomato cell walls are polygalacturonase (PG) and pectinesterase. Acetone insoluble material prepared either with or without a PAW treatment was incubated in 1M NaCl, a procedure which will solubilize both pectolytic enzymes, and the resultant extract assayed for both activities (Table 1). Untreated material contained, as expected, appreciable levels of both PG and pectinesterase activity. In comparison, PAW treated material had no detectable activity. The ability of the PAW treatment to remove residual pectolytic activity from the acetone insoluble material was further demonstrated by mixing experiments. These involved mixing PAW treated material from unripe green fruit with that from red ripe fruit; the acetone insoluble material from red fruit having been prepared either with or without treatment with PAW. Following incubation in acetate–EDTA buffer for 4 hr at room temperature the amount of soluble polyuronide in each case was then determined. Taking the sum of the polyuronides released by each component in a particular mixture when incubated alone as 100% the mixture containing the untreated ripe sample released 180% of the expected yield of soluble polyuronide. Therefore in this case the residual enzyme activity in the ripe material was still capable of solubilizing polyuronide from the green material, even when the latter had been treated with PAW. However, on mixing PAW treated material from unripe fruit with PAW treated material from ripe fruit the amount of soluble polyuronide released was only 2% greater than that expected from the sum of each individual component in the mixture incubated alone. This indicated that the PAW treatment had removed most, if not all, the residual uronide solubilizing activity in the acetone insoluble material.

Changes in soluble polyuronide and pectolytic enzymes during ripening of normal and mutant fruit

Tomato fruit were harvested at the mature green stage and monitored for onset of ripening. For normal and Never-ripe (Nr) fruit this was taken as the time when ethylene production of an individual fruit exceeded 0.1 nl/g/hr. For ripening inhibitor (rin) fruit the stage of maturity was determined by colour change and time from harvest. At various stages of ripening individual fruit were selected for analysis. Each fruit was cut in half and one half used to prepare soluble polyuronides from enzymatically inactive PAW treated acetone insoluble material. Cell wall bound proteins were isolated from the second half and the levels of activity of the two putative polyuronide degrading enzymes PG and pectinesterase were assayed.

The results of this experiment are shown in Fig. 2. There was a large increase in the level of soluble polyuronide extractable from both normal and Nr fruit during ripening (Fig. 2a). In both cases this increase in solubility reached a maximum of around 100 μg/mg after 7–9 days.

Table 1. Protein content, polygalacturonase and pectinesterase activity of 1M NaCl extracts from ripe tomato cell walls prepared with or without a PAW treatment

PAW	Protein (μg/mg)	Polygalacturonase activity (nmol/min/mg)	Pectinesterase activity (μ equiv/min/mg)
–	16.9	6.47	0.63
+	0.33	0.00	0.00

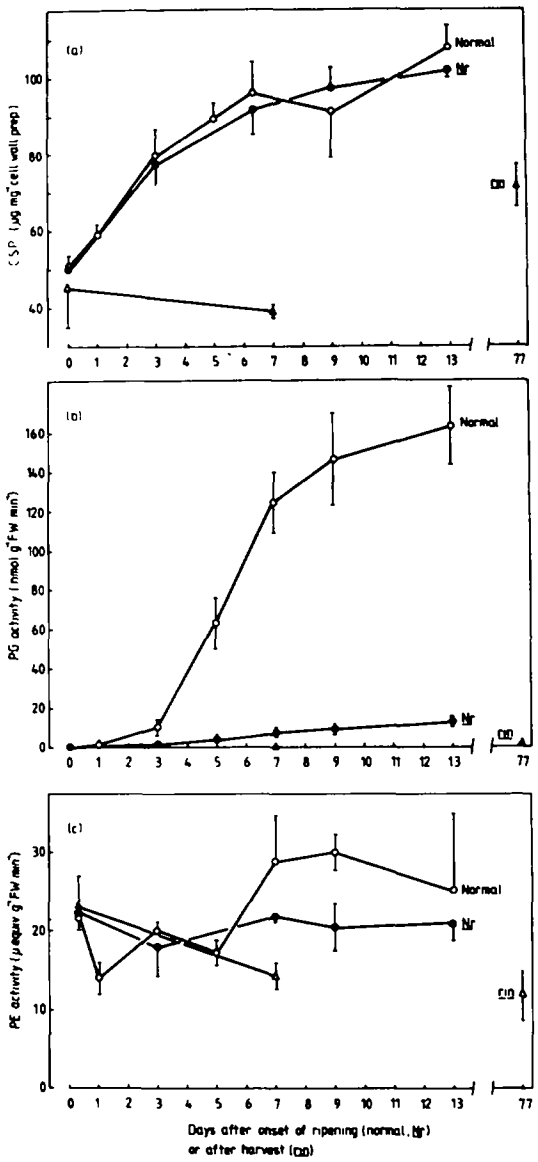

Fig. 2. Changes in (a) soluble polyuronide (SP), (b) polygalacturonase (PG) activity and (c) pectinesterase (PE) activity in relation to the onset of ripening in normal (○) and Nr (●) and time after harvest, in rin (△). Each point represents the mean of three fruit.

In rin an increase in soluble polyuronide was also apparent, but was only detectable several weeks after harvest when the fruit were fully yellow.

Although it has been reported that there is an increase in water soluble pectin during ripening [2], Gross [3] has demonstrated only a minor increase in chelator soluble pectin during ripening. Our experiments using enzymatically inactive preparations have confirmed these observations (unpublished). The extraction method used in this study, and by Huber [9], employs a buffer with a weak chelating capacity, and the results shown in Fig. 2(a) are, therefore, essentially the same as when acetate buffer alone was used except that slightly more polyuronide was released at all stages of ripeness.

One mechanism suggested for the increases in soluble polyuronide is the appearance of PG activity [2]. This enzyme is absent from green fruit and increases during ripening of both normal and Nr fruit [6, 11]. It can be seen (Fig. 2b) that the level of PG activity in Nr was much lower than that in normal fruit. The rin fruit contained no detectable PG activity until several weeks after harvest, at which stage very low levels of enzyme could be observed. These findings show that for normal, Nr and rin fruit the increase in soluble polyuronide was accompanied by a rise in PG activity. However, the magnitude of the increase in soluble polyuronide did not appear to be directly correlated with the levels of PG activity found in these fruit. Therefore, although these observations suggest that the increase in soluble polyuronide during ripening required some PG activity, they may also indicate that other factors are operating to determine the rate and extent of polyuronide solubilization. These factors may include the synthesis of soluble polyuronides [12], changes in pectinesterase activity or the removal of any neutral sugar side chains attached to the uronide polymers.

During the present study the levels of pectinesterase activity in normal and mutant fruit were determined (Fig. 2c). In normal fruit there was comparatively little change in activity compared with PG, although there appears to be some increase in pectinesterase activity about 7 days after the onset of ripening. In Nr the level of pectinesterase activity remained approximately constant, but declined in rin. The magnitude of these changes in pectinesterase activity are similar to those reported by Tucker *et al.* [13] and the findings suggest that gross changes in pectinesterase activity may not be important in polyuronide solubilization.

Soluble polyuronides from unripe and ripe normal fruit were fractionated on Sephacryl S-400 (Fig. 3). The polymers from green normal fruit were almost entirely of a high M_r form (Fig. 3a). Since these were only just included on the S-400 column, this would indicate an apparent M_r (based on the manufacturer's specifications for dextran standards) of around $1–2 \times 10^6$. The polymers from ripe normal fruit (Fig. 3b) showed a similar peak of

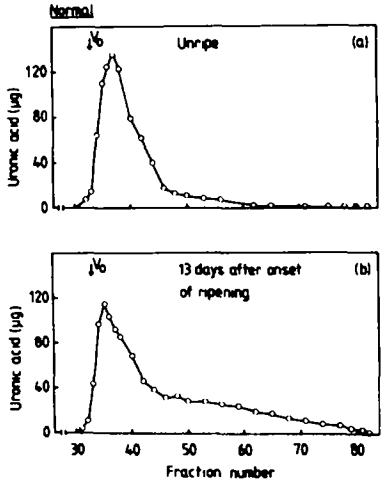

Fig. 3. Sephacryl S-400 profiles of soluble polyuronides from unripe (a) and ripe (b) normal fruit. The amount of acetone-insoluble material used for polyuronide extraction was adjusted so that similar levels of soluble polyuronides were applied to the column in each case.

Fig. 4. Low speed sedimentation equilibrium solute distributions, in 30 mm path length cells, for polyuronides from green fruit (top) and red fruit (bottom) recorded using Rayleigh Interference optics. Rotor speeds were 9341 rpm at 20° with initial loading concentrations of ≈ 0.4 mg/ml.

high M_r forms, but there was also a range of lower M_r material. Accurate estimates of the M_r of these large polyuronides by gel filtration are difficult because the dextran standards commonly used to calibrate these columns are likely to have less extended conformation than the uronide polymers [14]. Therefore, the average M_rs of these polymers from green and ripe normal fruit have also been determined by ultracentrifugation [15]. The technique of low speed sedimentation equilibrium provides a reliable and absolute (i.e. without the need for calibration standards) method for weight average M_r determination of thermodynamically non-ideal polydisperse systems such as the polyuronides under consideration here (see e.g. 16, 17), unlike light scattering techniques which are fraught with problems of clarification of solutions and polydispersity.

Sedimentation equilibrium solute distributions, recorded using Rayleigh Interference Optics in a Beckman Model E Analytical Ultracentrifuge are shown in Fig. 4. Thirty mm path length cells were used at the lowest possible loading concentration (≈ 0.4 mg/ml) to minimize possible effects of thermodynamic non-ideality and/or self-association phenomena. Because of the low speeds employed (to ensure adequate registration of the fringes at the cell base) the concentration at the meniscus remains finite and is obtained by mathematical manipulation of the fringe data. The weight average M_rs for the solute distributions were obtained by extrapolation to the cell base of a particularly useful point average M_r the 'star' average [18], and values of 160 000 ± 1000 and 96 000 ± 4000 were obtained for the green and red fruit polyuronides respectively [15]. Further, the red fruit showed greater effects of polydispersity, consistent with our observations using gel filtration (Fig. 3).

Soluble polyuronides from unripe Nr or rin fruit also exhibit a high apparent M_r on S-400 (unpublished data) but unlike normal fruit there appeared to be relatively little change in their polydispersity during ripening, as determined by chromatography on Sephacryl S-400.

The extent of depolymerisation observed during normal ripening was much less than that reported previously [9]. However, it is possible that *in vivo* some low M_r forms are produced, which are subsequently lost in the preparation of the acetone insoluble material. The greater degree of depolymerization in normal, compared to Nr and rin fruit may be related to their relative levels of PG activity.

EXPERIMENTAL

Plant material. Tomato plants (*Lycopersicon esculentum* Mill var Ailsa Craig) and Nr and rin mutant plants of near-isogenic lines, were grown under glasshouse conditions. Mature green fruit were harvested 35–40 days post anthesis and stored in a humidified atmosphere at 20° until required.

Ethylene determinations. Ethylene evolution of individual fruit was measured by holding the fruit in 600 ml sealed glass jars at 20° for 1 hr and then sampling 1 ml of the enclosed atmosphere. Ethylene was determined by GC [19].

Preparation of soluble polyuronides. Acetone insoluble solids were prepared using a method similar to that described by Huber [9]. Pericarp tissue was homogenized (Polytron, Kinematica GmbH, Luzern, Switzerland) in four vols of Me_2CO at −20°. This homogenate was filtered through Miracloth and the residue washed sequentially with 80% Me_2CO and 100% Me_2CO (each stage 12.5 ml/g tissue). The powder was then either dried over P_2O_5 *in vacuo*, or suspended with constant stirring for 15 min in PhOH–HOAC–H_2O (PAW) (2:1:1 w/v/v) at 4° and at a concentration of 10 ml PAW per g of tissue. This treatment was used to remove endogenous enzyme activity [10, 20]. Following treatment with PAW, Me_2CO was added to a final concentration of 80%, and the Me_2CO-insoluble solids collected

by filtration through Whatman GF/A paper. The powder was then washed with approximately 200 ml of 100% Me_2CO and dried over P_2O_5 *in vacuo*. All material was held dessicated at −20° until required.

Extraction of soluble polyuronide. Acetone insoluble solids were incubated at a concentration of 5 mg/ml in 50 mM NaOAc, 40 mM EDTA, pH 4.5, with constant stirring for 4 hr at room temp. Samples were then filtered through Whatman GF/A and the filtrate containing the soluble polyuronide made up to a known vol.

Analysis of soluble polyuronide. Uronic acid content was measured by the *m*-hydroxydiphenyl method [21] using polygalacturonic acid (Fluka) as a standard. Samples of soluble polyuronide for gel filtration chromatography were prepared by incubating Me_2CO-insoluble solids in 50 mM NaOAc, 40 mM EDTA, pH 4.5, a concentration of 12 mg/ml, unless otherwise stated. After incubation the slurry was centrifuged for 2 min at 12000 *g* and the resultant supernatant filtered through Whatman GFA paper. Samples were made up to a vol of 2 ml and applied to either (a) Sephacryl S-200 (1.6 × 70.5 cm), (b) Sephacryl S-400 (1.6 × 89 cm) or (c) Trisacryl GFO5 (2.1 × 76.5 cm) column. All columns were equilibrated and run in a buffer containing 0.1M NaOAc, 0.02 M EDTA, pH 6.5. Column fractions were analysed for uronic acids as described above.

Extraction and assay of wall bound protein. Total cell wall bound proteins were extracted using the method of ref. [6]. Polygalacturonase activity was determined by following the production of reducing groups [6] and pectinesterase activity by the pH titration method [13]. Protein content was determined by the method of ref. [22] using bovine serum albumin as a standard.

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