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DIFFERENTIAL EFFECTS OF PECTOLYTIC ENZYMES ON TOMATO POLYURONIDES *IN VIVO* AND *IN VITRO*

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; polyuronide; polygalacturonase; pectinesterase.

Abstract—There are marked differences between polyuronide degradation *in vivo* during tomato ripening and *in vitro* during the autolysis of cell wall preparations. Experiments using purified enzymes and enzymically inactive wall preparations show that the combined action of polygalacturonase (E.C. 3.2.1.15) and pectinesterase (E.C. 3.1.1.11) can mimic this *in vitro* autolysis of cell walls. Assuming these two enzymes are also responsible for polyuronide degradation *in vivo* their combined action must be restricted in some way.

INTRODUCTION

Changes in the composition and solubility of polysaccharides associated with the cell walls of tomatoes appear to play a major role in the softening occurring during ripening [1–4]. Ripening related modifications of the pectic fraction include an increase in soluble polyuronide [1] and a decrease in the M_r of this polyuronide [2,4]. Two enzymes which are likely to be involved in these changes are polygalacturonase (PG) and pectinesterase (PE). The first of these can hydrolyse the α (1–4) link between two adjacent galacturonic acid residues within a galacturonic acid polymer. The PE carries out demethylation at the C6 position of methylgalacturonic acid residues. Since PG can only act on demethylated regions of polygalacturonic acid the action of PE could generate sites on pectic polymers for subsequent PG action. Although this sequence of enzymic events has been postulated to occur during ripening very little experimental evidence has been available. *In vitro* studies [5] have shown that the ability of PG to cause polyuronide solubilization is enhanced in the presence of PE. However, this study used only cell walls from mature green fruit and did not compare *in vitro* activity with that occurring *in vivo*.

Recent evidence [4] suggests that polyuronide degradation *in vivo* is much less extensive than that observed *in vitro*. Neither the cause nor extent of this difference are well understood, but considering the high level of endogenous PE activity and the fact that pectin is not totally deesterified during ripening it is likely that at least PE activity is restricted *in vivo* [6]. In this study the action of PG2 and PE2 on tomato acetone insoluble solids from unripe and ripe fruit is described. The products of this enzyme action are compared with polyuronides solubilised during normal ripening and with those generated by autolysis *in vitro*. The results give some evidence for the relative importance of PG and PE enzymes during ripening.

The isoenzymes used in this work are those which have been shown to be predominant in ripe fruit. Thus PG2 is the major isoenzyme of the two synthesized during ripening [7] and has already been shown to degrade tomato polyuronides *in vitro* [8].

Green fruit contains high levels of both PE isoenzymes, PE1 and PE2. However, during ripening PE1 declines slightly and PE2 accumulates [9]. Previous studies have shown that, although there are minor differences, both of these PE isoenzymes are capable of enhancing polyuronide solubilization by PG *in vitro* [5].

RESULTS AND DISCUSSION

The sugar composition of material solubilized from acetone insoluble solids (AIS) of unripe and ripe tomato fruit in the presence or absence of endogenous pectolytic activity is shown in Fig. 1. Enzymically inactive AIS from green and ripe fruit released $\simeq$ 50 and 100 μg/mg of uronic acid, respectively. These values give an indication of the level of uronic acid solubilized *in vivo* and are in agreement with previous observations showing an increase in soluble uronide during ripening [1,4]. Enzymically active AIS from green fruit release $\simeq$ 50 μg/mg of uronic acid, similar to the enzymically inactive preparation (data not shown). In comparison, and as previously reported [4], the amount of uronic acid released from enzymically active AIS from ripe fruit is greatly increased, compared to the inactive preparation. The nature of this *in vitro* autolysis of ripe AIS was investigated further.

Although the addition of PG2 to enzymically inactive AIS from either green or ripe fruit resulted in an increased solubilization of uronic acid, in neither case was this level as great as that from enzymically active ripe AIS. However, levels of soluble uronic acid comparable to

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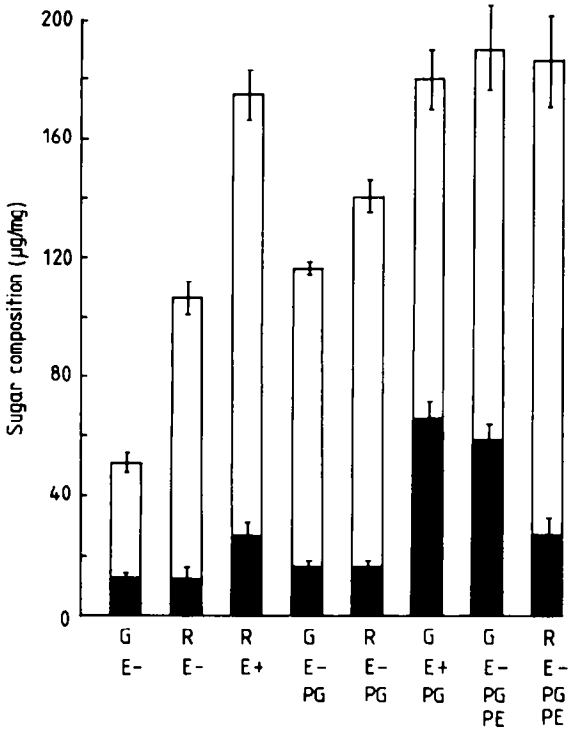

Fig. 1. Sugar composition (uronic acid □, neutral sugar ■) of material released from unripe (G) and ripe (R) tomato acetone insoluble solids in the presence (E+) and absence (E−) of endogenous enzyme activity and in the presence of purified polygalacturonase (PG) and pectinesterase (PE).

those released from enzymically active ripe AIS are produced when PE2 is added along with PG2 to either green or ripe enzymically inactive AIS. The addition of PG2 alone to enzymically active green AIS, which has been shown to contain endogenous PE activity [4], resulted in a similar high level of uronic acid solubilization. These results indicate that polyuronide solubilization during autolysis in ripe AIS could be the result of endogenous PG and PE activity.

Although uronic acid is the major solubilized sugar, appreciable amounts of neutral sugar are also released (Fig. 1). Enzymically active AIS from ripe fruit released significantly more neutral sugar than the comparable inactive preparation. In this case the addition of PG2 and PE2 to enzymically inactive AIS from fruit resulted in a similar level of soluble neutral sugar to that found in an active ripe AIS. However, when PG2 and PE2 were added to enzymically inactive green AIS far more neutral sugar was released. It should be noted, that the AIS used in these experiments contains polysaccharides from both the middle lamella and primary cell wall. Analysis of the neutral sugar released by combined PG2 and PE2 action on green AIS has shown it to be rich in galactose (68%) and arabinose (17%) (Seymour, G. B., unpublished results). This could imply solubilization of primary wall associated pectin which is thought to be relatively rich in arabinogalactans [10], as well as middle lamella pectin. However, during ripening it is generally considered to be the middle lamella which is the primary site of pectolytic activity, and so it may be PG sites specifically on middle lamella pectin which are important during ripening. It should be noted that the addition of PG2 to enzymically inactive AIS from ripe fruit resulted in a slightly greater uronide solubilization than from green AIS. Perhaps this represents a ripening related increase in sites for PG action which are important for pectin solubilization.

The degree of polymerisation of the soluble uronides

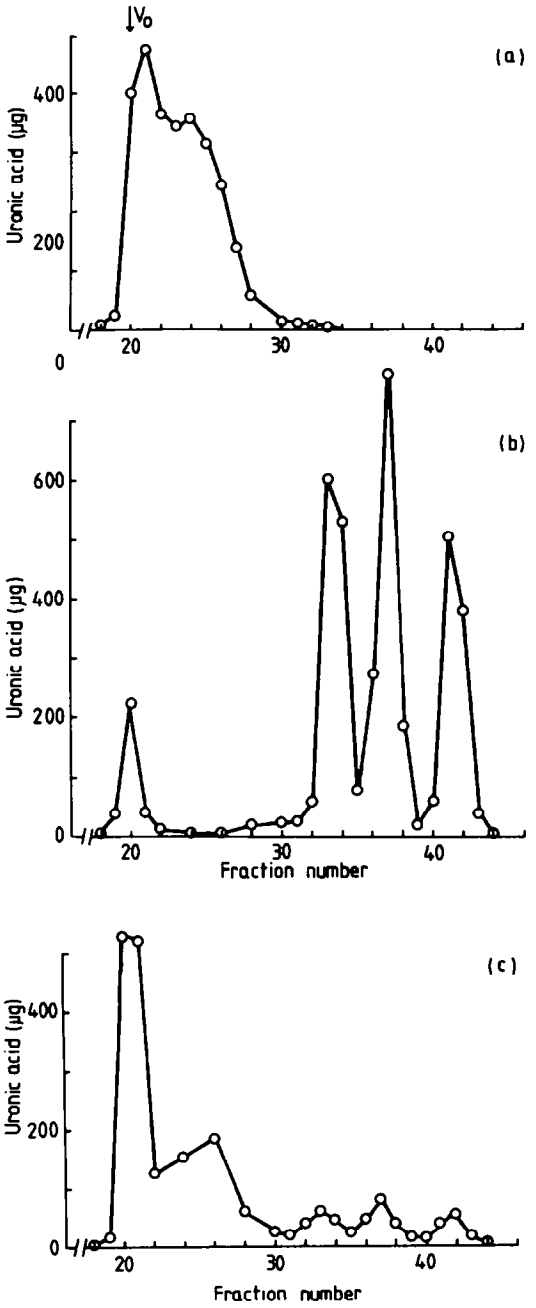

Fig. 2. Trisacryl GF05 profiles of acetate–EDTA soluble polyuronides from ripe tomato acetone insoluble solids (AIS) treated to remove endogenous enzyme activity and in the absence of any exogenous pectolytic activity (a), from AIS having endogenous pectolytic activity (b), and from AIS treated to remove endogenous enzyme activity, but with exogenous polygalacturonase (c).

was investigated using gel filtration on Trisacryl GF05 (Fig. 2). Enzymically inactive AIS from both green and ripe fruit yielded soluble polyuronides which eluted at or around the void volume of the column (Fig. 2a). The elution profile of the soluble polyuronides obtained from enzymically active AIS from ripe fruit is shown in Fig. 2b. In this case mainly low M_r oligomers were present with degrees of polymerisation between about six and one. The presence of monomer was confirmed by paper chromatography (unpublished results). The addition of PG2 alone to enzymically inactive AIS from both green or ripe fruit resulted mainly in large polymers but with some small oligomers (e.g. see Fig. 2c). When PE2 was added along with the PG2, column profiles similar to that shown in Fig. 2b were obtained from both green and ripe AIS. Again these results show that autolysis of ripe AIS can be minimized by the combined action of PG2 and PE2.

It is clear that *in vivo* solubilization and depolymerization of polyuronide does not occur to the same extent as *in vitro*. The nature of this restricted pectolytic activity *in vivo* is not clear. It seems that PG2 alone can act on enzymically inactive AIS to release uronic acid polymers comparable in size and amount to those observed during normal ripening, whilst the result of PG2 and PE2 combined is markedly different. This may suggest that the extent of PE action *in vivo* is the main restriction on polyuronide solubilization. However more experiments are required to elucidate the precise mechanism for this.

It is interesting to note that the degradation of the soluble polyuronide either during autolysis or by the combined action of PG2 and PE2, is not totally random. The products consist of two discrete sized fractions and monomer galacturonic acid. In view of the recent discovery of cell wall derived elicitors it would be interesting to determine if these oligomers occur *in vivo*. The results using enzymically inactive AIS without added enzymes, and a previous study of ripening related soluble polyuronides [4] both failed to identify these small oligomeric uronides. However, the possibility exists that these are lost during preparation of the AIS. Oligomers with the same elution profiles are produced in small amounts by the action of PG2 alone on enzymically inactive AIS. Assuming PG action *in vivo* is similar to that *in vitro* the possibility exists that these wall fragments do occur *in situ*. Their possible function as elicitors, however, remains to be elucidated.

EXPERIMENTAL

Plant material. Tomato plants (*Lycopersicon esculentum* Mill var Ailsa Craig) were grown under glasshouse conditions. Mature green fruits were harvested 35 to 40 days post anthesis or left on the plant until they reached the red ripe stage. The harvested fruit was then used immediately.

Purification of pectolytic enzymes. Polygalacturonase isoenzyme 2 and pectinesterase isoenzyme 2 were purified from ripe Ailsa Craig tomato fruit as described previously [7, 9].

Acetone insoluble solids. Actone insoluble material was prepared from unripe and red ripe fruit as described previously [4]. Where appropriate, endogenous pectolytic activity in the acetone powders was removed by stirring the powders in phenol–HOAc–H_2O (2:1:1 w/v/v) [4]. Polyuronides were extracted from the acetone insoluble material by incubation in 50 mM Na acetate, 40 mM EDTA, pH 4.5 at $\simeq$ 12 mg/ml in the presence or absence of polygalacturonase or polygalacturonase and pectinesterase for 4 hr at room temp. with constant stirring. The amount of each enzyme activity used was equivalent to that present in a 1M NaCl extract of enzymically active acetone powder of the same weight from ripe fruit, for PG2 this was 6.5 nmol/min/mg and for PE2 0.63 μ equiv./min/mg [4].

Analysis of soluble polyuronide. The polyuronide extractable from the acetone insoluble preparations of tomato fruit was analysed for uronic acid content by the *m*-hydroxydiphenyl method [11]. Total neutral sugars, after correction for uronic acids, were estimated by the method of ref. [12], using galactose as a standard. The molecular size distribution of the polyuronides was examined by gel filtration chromatography on Trisacryl GF05 (2.1 × 76.5 cm). The column was equilibrated and run in a buffer containing 0.1 M NaOAc and 0.02 M EDTA pH 6.5. The column was calibrated with maltooligosaccharides (Sigma).

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