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Products Released from Enzymically Active Cell Wall Stimulate Ethylene Production and Ripening in Preclimacteric Tomato (*Lycopersicon esculentum* Mill.) Fruit[1]

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ABSTRACT

Enzymically active cell wall from ripe tomato (*Lycopersicon esculentum* Mill.) fruit pericarp release uronic acids through the action of wall-bound polygalacturonase. The potential involvement of products of wall hydrolysis in the induction of ethylene synthesis during tomato ripening was investigated by vacuum infiltrating preclimacteric (green) fruit with solutions containing pectin fragments enzymically released from cell wall from ripe fruit. Ripening initiation was accelerated in pectin-infiltrated fruit compared to control (buffer-infiltrated) fruit as measured by initiation of climacteric CO_2 and ethylene production and appearance of red color. The response to infiltration was maximum at a concentration of 25 micrograms pectin per fruit; higher concentrations (up to 125 micrograms per fruit) had no additional effect. When products released from isolated cell wall from ripe pericarp were separated on Bio-Gel P-2 and specific size classes infiltrated into preclimacteric fruit, ripening-promotive activity was found only in the larger (degree of polymerization >8) fragments. Products released from pectin derived from preclimacteric pericarp upon treatment with polygalacturonase from ripe pericarp did not stimulate ripening when infiltrated into preclimacteric fruit.

The solubilization of pectin is a well documented feature of the softening process occurring in tomato and other fruit types during ripening. It is generally accepted that the enzyme polygalacturonase (PG,[2] EC 3.2.1.15), which hydrolyzes cell wall pectic polymers, plays a key role in tomato softening (16). Preclimacteric tomatoes have no PG; the enzyme appears in the pericarp tissue coincident with the appearance of color at the onset of ripening and increases dramatically and continuously during ripening (8, 13, 24, 28). Aside from contributing to texture changes, PG has been implicated as a participant in other features of ripening. Tigchelaar *et al.* (25) proposed, on the basis of studies of the tomato ripening mutants, that PG might control ripening in tomato fruits by releasing wall-bound enzymes involved in control of ethylene synthesis and other ripening-associated events. Although some studies (21, 22, 26) supported the view that PG served an initiating role in ripening, more recent analyses employing immunochemical techniques demonstrated that the onset of ethylene production in ripening tomatoes occurs prior to the appearance of PG transcripts and protein (8, 13, 28, 29). Additionally, Hobson *et al.* (15) have shown that no significant release of protein from tomato cell walls occurs until ripening is far advanced. It seems clear that PG does not play a role in the initiation of ripening in tomato.

However, a number of studies on the biochemistry of host-pathogen interactions have demonstrated that pectin hydrolases and lyases, in addition to hydrolyzing cell wall, can induce a range of host physiological processes via the generation of specific carbohydrate fragments with elicitor activity (2, 11, 12). Although the precise responses are variable among different systems, a commonly observed feature is enhanced ethylene biosynthesis (3).

It has also been demonstrated that specific carbohydrate fragments (oligosaccharins), in addition to mediating defense mechanisms, might also function in regulating certain features of plant development (2). Several researchers have recently reported transient enhancement of ethylene production following treatment of fruit tissue with fungal enzymes (4–6) or cell wall fragments produced by fungal enzymes (20, 27). We considered the possibility that wall fragments generated during autolysis might contribute to the enhanced ethylene biosynthesis characteristic of ripening climacteric fruit (10). We report here our attempts to test this hypothesis. Our results provide evidence that the endogeneous products of in vitro cell wall degradation (18) can stimulate ethylene synthesis and advance the onset of ripening when applied to preclimacteric tomato fruits. However, pectin fragments generated by PG treatment of pectin isolated from preclimacteric tomato had little or no effect on ripening, casting doubt on an exclusive role for PG in the production of biologically active compounds.

MATERIALS AND METHODS

Plant Material. Tomato (*Lycopersicon esculentum* Mill.) fruit cv Rutgers and Floradade were obtained from plants grown at the University of Florida Horticultural Unit near Gainesville. Tomato fruit cv. 'Sunny' were obtained from commercial packinghouses in Ruskin and Homestead, Florida, on the day of harvest and immediately transported to Gainesville. All fruit were surface sterilized with 13 mM NaOCl, rinsed with deionized H_2O and surface-dried before use. Uniform samples of mature-green fruit for pectin infiltration were selected based on initial ethylene production rates (9) as follows. 'Floradade' and 'Sunny' fruit were placed in 490 mL glass jars at 23 ± 1°C, held overnight, and the jars sealed for 1 h. This allowed elevated ethylene production resulting from handling the fruit to subside and allowed the fruit to be sealed in the jars with minimal disturbance, improving reproducibility. Gas samples (0.5 mL) were taken through rubber septa inserted in the jar lids, and ethylene concentration in the headspace was determined by gas chroma-

[1] Florida Agricultural Experiment Station Journal Series No. 8887.
[2] Abbreviations: PG, polygalacturonase; DP, degree of polymerization.

tography using an alumina column and photoionization detector. 'Rutgers' fruit used for cell wall preparation were sectioned, seed and placental tissue removed, and pericarp tissue stored at −20°C in sealed polyethylene bags.

Isolation and Preparation of Products Generated from Autolytically Active Cell Wall. Autolytically active cell wall was prepared from ripe 'Rutgers' pericarp as previously described (23). Cell wall (100 mg dry weight) in 50 mL centrifuge tubes was hydrated with 4 mL cold (1°C) Na-acetate buffer (20 mM, pH 4.5) containing 150 mM NaCl. After the wall was fully hydrated (15 min), the samples were incubated for 1 h at 34°C in a shaking water bath. Afterward, the suspensions were filtered through Miracloth and the filtrates passed through glass fiber filter papers (Whatman GF/C) under mild aspiration. The samples were subjected to DEAE-Sephadex chromatography for separation of neutral sugars from uronic acids (18). Pectins were measured using the *m*-hydroxydiphenyl procedure (7) and the pectin-containing fractions diluted with H_2O to the desired concentrations. Other infiltration experiments employed autolytically generated pectins separated into size classes using Bio-Gel P-2 chromatography (18). The final buffer and NaCl concentrations varied depending on the required dilution factor. These values are indicated in the figure legends.

Infiltration Experiments. Individual fruit were infiltrated with 0.5 mL of pectin solutions. Controls included fruit not infiltrated or fruit infiltrated with buffer alone. Fruits were placed in a vaccum chamber and solutions pipetted onto the stem scar area. The pressure in the chamber was reduced to 60 mm Hg and held at that pressure until escape of gas from the stem scar ceased (approximately 1 min). The pressure was then raised slowly to 760 mm Hg during which time the solutions were infiltrated.

Ethylene production was measured as described above at appropriate intervals following infiltration of test solutions. Carbon dioxide production was measured similarly using a gas chromatograph equipped with a Porapak column and thermal conductivity detector. Color development was monitored by measurement of the *a* value on the Hunter Lab scale at the blossom end of the fruit using a reflectance spectrophotometer. The *a* value on the Hunter Lab scale represents relative green or red color with negative values more green and positive values more red.

RESULTS AND DISCUSSION

Effect of Autolysis Products on Ethylene Production. It is known that the time at which ripening is initiated postharvest is greatly dependent on the stage of fruit maturation. Additionally, since ripening is initiated internally and not apparent externally (9), it is possible to unknowingly encounter fruit in which ripening has already been initiated. In order to ensure maximum uniformity in fruit samples employed in these studies, all fruit were first subjected to an initial determination of ethylene production capacity (9). All fruit producing greater than 0.09 nL ethylene g fresh weight^{-1} h^{-1} were discarded. Based on ethylene production capacity, in some experiments fruit were separated into two maturation categories. Infiltration into preclimacteric tomato fruit of 200 μg galacturonic acid equivalents generated from autolytically active cell wall resulted in a burst of ethylene production within 6 h which then declined over 24 h (Fig. 1). Pectin-treated fruit consistently produced slightly higher ethylene levels than buffer-treated fruit during the first 24 h. Differences in magnitude of initial ethylene production between experiments were related to fruit maturity: 6 d pre-breaker fruit (Fig. 1A) produced less than 1 nL ethylene g fresh weight^{-1} h^{-1}, whereas fruit of a more advanced stage of maturation (3-d pre-breaker, Fig. 1B) produced between 3 to 5 nL ethylene g fresh weight^{-1} h^{-1}. Following the initial post infiltration burst of ethylene production, ethylene production in control fruit returned to prein-

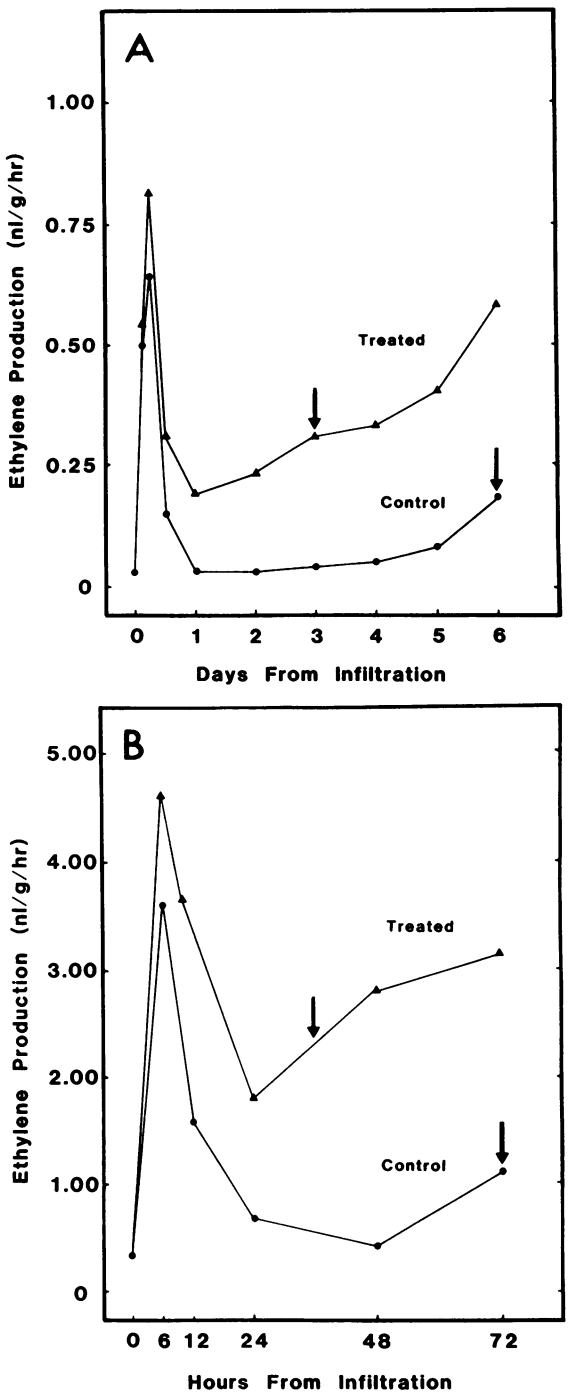

FIG. 1. Effect of tomato cell wall autolysis products on ethylene production of intact preclimacteric tomato fruits. 'Sunny' tomatoes were vacuum infiltrated via the stem scar with 0.5 mL solutions containing approximately 200 μg galacturonic acid equivalents in 24 mM NaCl or 24 mM NaCl alone. Autolytic reaction products were subjected to DEAE-Sephadex chromatography to separate neutral sugars from uronic acids (19). Galacturonic acid equivalents were measured using the *m*-hydroxydiphenyl procedure (7). Average ethylene production prior to infiltration was 0.03 ± 0.01 nL C_2H_4 g^{-1} h^{-1} (A) or 0.19 ± 0.03 nL C_2H_4 g^{-1} h^{-1} (B). Arrows indicate the average time to the breaker stage of development (appearance of red color at the distal [blossom] end of the fruit). ▲ = pectin-treated fruit, ● = control. Four fruit per treatment were monitored for ethylene and color development.

filtration levels until the onset of the ripening-associated increase in ethylene occurred. Ethylene production by fruit infiltrated with pectin did not return to preinfiltration levels before the onset of ripening-associated ethylene production occurred (Fig. 1, A and B).

Figure 2 illustrates the trends of CO_2 and ethylene production by fruits infiltrated with pectin or buffer solutions compared to noninfiltrated controls. It is apparent that vacuum infiltration alone hastened the onset of ripening (denoted as incipient color break) since the initiation of the climacteric and the appearance of red color occurred about 2 d sooner in buffer-infiltrated fruit than in noninfiltrated fruit. The effect of vacuum infiltration on ripening is presumably due to the wound ethylene produced following infiltration. Infiltration of fruit with the autolytically generated pectins advanced the onset of ripening by 5.5 d over buffer-infiltrated controls.

Effect of Pectin Concentration on Ripening. One-half mL solutions containing a range of concentrations of autolytically generated products were infiltrated into preclimacteric tomato fruit and the effect on the onset of ripening measured by observing the number of days to the first appearance of color at the distal end of the fruit (breaker stage). Control fruit reached the breaker stage after 10.5 d (Fig. 3). The response to pectin infiltration occurred at all concentrations tested but maximized at a concentration of 50 $\mu g\ mL^{-1}$, at which concentration fruit required only 2.5 d to reach the breaker stage. Fruit treated with other concentrations of pectin began to ripen 5 to 7 d after infiltration.

Since fruit to fruit variability in maturity was minimized by the use of ethylene production rates for fruit selection, the variability in response to the infiltration treatments was probably primarily due to poor distribution of the infiltrated materials. Infiltration of various anionic dyes into tomato fruit indicated that the test solutions were not uniformly distributed (data not shown), but rather migrated only a short distance from the stem scar along vascular branches to the columella and especially to the outer pericarp in a somewhat random pattern. Based on these observations, it is probable that the infiltrated pectins are acting in localized areas of the fruit to stimulate the onset of ethylene production and ripening. Although the region of infiltration was apparently restricted to the proximal one-half of the fruit, color development proceeded in a normal fashion from distal to proximal ends.

The optimum quantity of galacturonic acid equivalents required to stimulate ripening in these experiments (25 $\mu g\ fruit^{-1}$, or approximately 0.2 $\mu g\ g\ fresh\ weight^{-1}$) is considerably lower than the amount of galactose (400 $\mu g\ g\ fresh\ weight^{-1}$) required to stimulate ethylene production in mature-green tomatoes (19). However, Kim *et al.* (17) pointed out that endogenous galactose levels in ripe tomato fruit may exceed 200 $\mu g\ g\ fresh\ weight^{-1}$. Interestingly, Huber and Lee (18) reported that tomato placental tissue galactose (covalently associated with pectin) was metabolized (presumably to monomer) in the period during which placental tissue ethylene production is initiated (9). Thus, the precise nature of cell wall metabolites capable of inducing ethylene biosynthesis may be structurally and compositionally quite variable.

Effect of Fragment Size on Ripening-Promotive Activity of Pectins. Autolytic reactions result in the generation of polymeric, oligomeric and monomeric uronic acids (18). In an effort to identify the component(s) active in accelerating ripening initiation, autolytic reaction products following anion-exchange chromatography were separated into size classes using Bio-Gel P-2. Infiltration experiments were performed using three size categories including a mixture of monomer, dimer, and trimer (DP 1–3), a mixture of higher oligouronides (DP 4–6), and products excluded from P-2 (DP > 8). Concentrations were adjusted to be proportionally equivalent to the contribution of each size class in the unseparated mixtures. This corresponded to 25, 12.5, and 12.5 μg galacturonic acid equivalents mL^{-1} for the DP 1 to 3, DP 4 to 6, and larger size classes, respectively. When these preparations were infiltrated into preripe tomato fruit there was no difference in ethylene production or color development between the NaCl-infiltrated control fruit and the fruit treated with pectin fragments of the two lower size classes (Fig. 4). Treatment of the fruit with the products excluded from P-2 stimulated the onset of ripening in terms of ethylene production and color development. The time to reach the breaker stage was 2 d for fruit treated with the large fragments compared to 4 to 5 d for control fruit and fruit treated with the smaller size classes. The

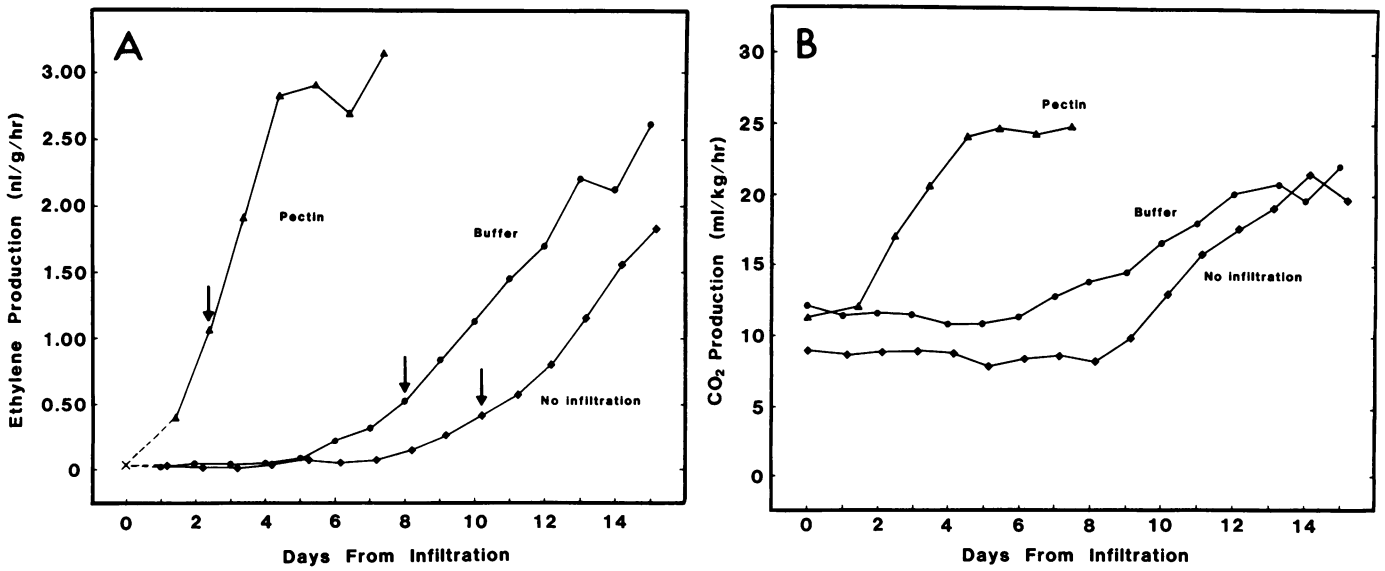

FIG. 2. Effect of autolysis products and infiltration on ethylene production and respiration of intact preclimacteric tomato fruit. 'Floradade' tomatoes were vacuum infiltrated with 253 μg galacturonic acid equivalents in 4.8 mM NaAc buffer (pH 4.5) containing 24 mM NaCl, infiltrated with the same solution minus autolysis products, or subjected to vacuum treatment without solution infiltration. Other details as in Figure 1 legend. Arrows indicate the average time to the breaker stage of development. ▲ = pectin-treated fruit, ● = buffer-treated fruit, ♦ = noninfiltrated fruit. Five fruit per treatment were monitored for CO_2, ethylene, and color development.

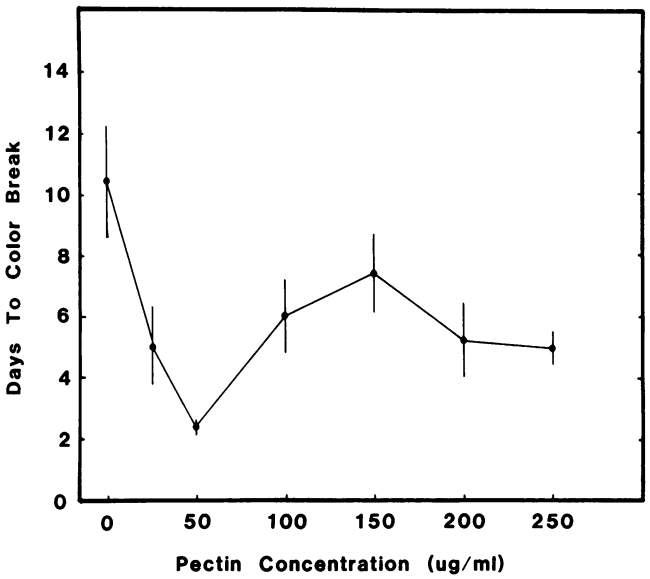

FIG. 3. Effect of pectin concentration on ripening. Preclimacteric 'Floradade' tomatoes were vacuum infiltrated with solutions containing 0, 25, 50, 100, 150, 200, or 250 μg galacturonic acid equivalents mL^{-1} in 4.8 mM NaAc (pH 4.5) containing 24 mM NaCl. Other details as in Figure 1 legend. The number of days to the breaker stage of development was recorded for five fruit per treatment. Bars indicate standard deviation.

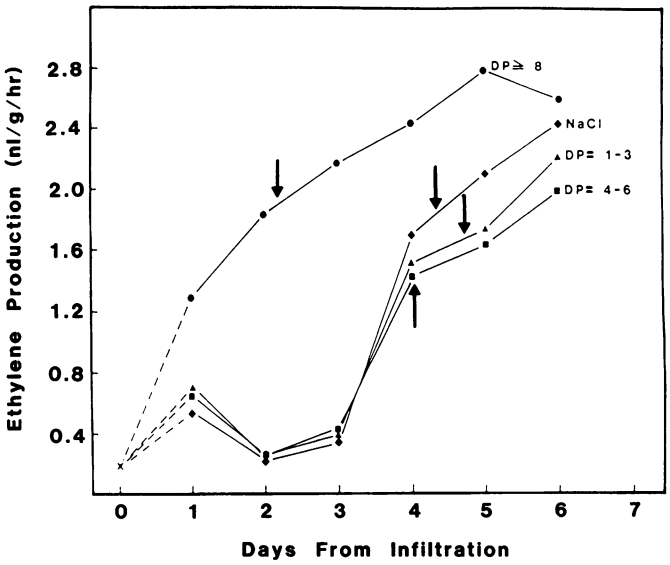

FIG. 4. Effect of pectin fragment size on ethylene production of preclimacteric 'Sunny' tomato fruits. Autolytic reaction products were separated into size classes using Bio-Gel P-2. Fractions were pooled to give solutions with pectin fragments representing three different size classes and the concentrations adjusted to be proportionally equivalent to the contribution of each size class in the unseparated mixture. This corresponded to 25, 12.5, and 12.5 μg galacturonic acid equivalents mL^{-1} for the DP 1 to 3, DP 4 to 6, and DP > 8 size classes. Other details as in Figure 1 legend. The control fruit were vacuum infiltrated with 24 mM NaCl. Arrows indicate the average time to the breaker stage of development. ● = DP ≥ 8, ■ = DP 4 to 6, ▲ = DP 1 to 3, ♦ = control. Five fruit per treatment were monitored for ethylene and color development.

amount of pectin in the active fraction applied to the tomato fruits was only about 6 μg $fruit^{-1}$ (approximately 0.05 μg g fresh $weight^{-1}$).

Compositional analysis of the pectin fragments from autolysis reaction mixtures from ripe tomato fruit indicted that the larger, Bio-Gel P-2 void pectins contain a high proportion of neutral sugars compared to the smaller fragments (18), indicating that these pectin fragments may be highly branched. Although branching was shown to be necessary for elicitor activity of glucan fragments from cell walls of *Phytophthora megasperma* f. sp. *glycinea* (11), in most reports of pectin-derived elicitors the active fragments have been shown to be linear, unbranched oligouronides with a degree of polymerization of 12 to 13 (11). Furthermore, Hahn *et al.* (14) demonstrated that the activity of an endogenous oligouronide elicitor released from soybean cell wall by acid hydrolysis was destroyed upon incubation with an endopolygalacturonase (14). The presence of high neutral sugar polymers in the P-2-excluded fraction does not preclude the possibility that less highly substituted polymers are responsible for the elicitor activity. Alternatively, the presence of other glycanases and glycosidases in tomato and other fruits (16) raises the possibility that infiltrated polymers were metabolized, resulting in the release of neutral polymers with elicitor activity. Finally, protein is also released during autolytic reactions (JW Rushing, DJ Huber, unpublished data), some of which may have been carried through the ion-exchange and P-2 purification steps.

An attempt was made to determine if treatment of pectins from cell wall from preclimacteric tomato pericarp with PG from ripe fruit pericarp could result in the production of pectic compounds with ripening promotive activity. There was little difference between the ripening of fruit infiltrated with pectin incubated with active PG or with boiled PG, but wound ethylene production between 6 and 24 h after infiltration was higher in the fruit from the pectin/active PG treatment (data not shown). These results do not support the idea that pectin fragments generated from preclimacteric tomato pectin by PG act to stimulate ripening. However, we can speculate that other enzymes present in ripening fruit but not in preclimacteric fruit may be necessary for production of the active compounds present in ripe fruit, perhaps by modification of PG-generated polymers, or by production of novel cell wall fragments.

Recent work has indicated that the magnitude of ethylene production during ripening in different tomato genotypes is correlated with the relative activity of PG in the fruit (1). It may be that maintenance of the elevated ethylene production rates characteristic of climacteric fruits (absent from nonclimacteric fruits) during ripening is part of a stress response related to the effect of pectic polymers or other compounds released during the process of fruit softening. It is apparent from this and other work that cell wall metabolism and specifically products of pectin degradation during fruit softening may be involved in the stimulation and coordination of ethylene production and the progression of ripening in fruit tissues.

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