Fungal cell-wall lysing enzymes have been shown to induce ethylene production in different plant systems. The effect of endogenous plant cell-wall lysing enzymes on ethylene synthesis in fruit has received only limited attention. Therefore, tomato fruit (Lycopersicon esculentum, Mill.) were vacuum-infiltrated with the tomato cell-wall enzymes, polygalacturonase I and II (PG I, PG II) and pectinmethyl esterase (PME). Fruit ethylene levels were observed to increase relative to either salt, buffer, or boiled enzyme controls. This increase in ethylene production occurred in green 'Cherry' tomato fruit as well as in the mutants rin, nor, and Cornell 111. Enzyme-induced ethylene synthesis generally peaked at or before 17 to 20 hr and decreased to lower or basal levels in most immature normal cultivars by 42 hr after treatment. Ethylene was maintained at high levels, however, in some (possibly more mature) green fruit, as well as in all mutant lines. PG II was more effective than PG I in inducing ethylene production and PME seemed to enhance the ethylene-inducing activity of PG II.

Materials and Methods

Plant material. ‘Jumbo’ tomatoes were grown in a greenhouse in Perry, Ga. ‘Cherry’ and ripening mutants rin, nor, and Cornell 111 were grown in field plots at the Russell Research Center, ARS/USDA, Athens, Ga. during Spring and Summer 1986. Stage of ripeness of each fruit was determined either by visual observation or by measurement of ethylene evolution by individual fruit. Freshly harvested fruit of similar size and weight were surface-sterilized with 0.2% NaOCl for 30 sec and then rinsed with distilled water. In most instances, fruit were sealed individually for 1 hr in glass containers fitted with rubber septa. One-milliliter gas samples then were withdrawn from the head volume and analyzed for ethylene on a Perkin Elmer Sigma 300 gas chromatograph equipped with an activated alumina column. Unless indicated otherwise, only those fruit that produced little (< 4 nL·hr⁻¹) or no detectable ethylene were used in experiments with green fruit.

Purification of enzymes. One kilogram of pericarp tissue from ripe ‘Better Boy’ tomatoes was homogenized in 1 liter of cold water. All of the subsequent steps were conducted within refrigerated conditions. The homogenate was adjusted to pH 3.0 by addition of 0.1 N HCl, stirred for 30 min, and then centrifuged at 8000 x g for 20 min. The supernatant solution was discarded and the pellet was suspended in 1.5 liters of 1.0 M NaCl. The suspension was adjusted to pH 6.0 by addition of 0.1 N NaOH, stirred 1 hr, and centrifuged. The supernatant solution was concentrated by ultrafiltration using a PM-10 membrane (Amicon); this represented the crude extract of the enzymes.

The polygalacturonase isoenzymes PG I and PG II were separated by chromatography on a 5 x 50 cm column of Sephadex G-100 in 1.0 M NaCl (18). PG II and PME then were separated on a 5 x 70 cm column of DEAE-Sephadex A-50 in 0.15 M NaCl at pH 6.0. PG I, PG II, and PME were purified further on a 2.5 x 40 cm column of CM–Sephrose using a linear gradient of 0.15 to 1.0 M NaCl at pH 5.5 (18). Purified PG I and PG II contained 650 and 1930 units/ml, respectively. A unit of PG is defined as that amount that releases 1 μmol of reducing groups in 30 min (18). The purified PME contained 7.8 units/ml, with a unit of PME defined as that amount that releases 1 μmol of carboxyl groups in 10 min (18).

Treatment of fruit. Treated and control fruit were penetrated 5 mm in depth with a 25-gauge needle five times in the core.
Fig. 1. Effect of PG II (965 units/ml) in 0.1 M succinate buffer pH 4.5 or in 0.1 M succinate buffer pH 7.0, or 0.1 M succinate buffer pH 4.5 or 7.0 alone on green 'Cherry' tomato ethylene production. All points are means of four replications ± se and treatment solution volumes were 1% of fruit fresh weight.

Fig. 2. Effect of PG II (804 units/ml) + PME (0.6 unit/ml) or boiled PG II + PME in 0.15 M NaCl on green 'Cherry' tomato ethylene production. All points are means of four replications ± se and treatment solution volumes were 1% of fruit fresh weight.

Fig. 3. Effect of PG II (1608 units/ml) + PME (1.3 units/ml) in 0.15 M NaCl, PG II (804 units/ml) + PME (1.3 units/ml) in 0.15 M NaCl, PG II (402 units/ml) + PME (1.3 units/ml) in 0.15 M NaCl, or 0.15 M NaCl alone on green 'Cherry' tomato ethylene production. All points are means of three replications ± se and treatment solutions were 1% of fruit fresh weight.

Fig. 4. Effect of PG I (591 units/ml) + PME (0.5 unit/ml) in 0.15 M NaCl, PG II (591 units/ml) + PME (0.5 unit/ml) in 0.15 M NaCl, or 0.15 M NaCl alone on green 'Cherry' tomato ethylene production. All points are means of four replications ± se and treatment solution volumes were 1% of fruit fresh weight.

kPa·min⁻¹ for 5 min, held at 33.25 kPa for 3 min, and then released at the same rate. After infiltration, the stopcock grease was removed with cloth and the fruit were returned to glass containers for periodic ethylene determinations as described above. There were three or four fruit replications for each treatment.

Treatment solutions. Fruit were infiltrated with solutions of tomato PG I, PG II, or PME in 0.15 M NaCl or 0.1 M succinate, boiled enzyme solutions, 0.15 M NaCl, or 0.1 M succinate buffer. In most instances, enzymes were used in combination; PG I +
were conducted with the major isoenzyme from, PG II. The enzymes of PG (PG I and PG II) during ripening (19). The heat-stable protein found in tomato extracts that binds PG II proteolysis and radioimmunoassay studies (23). PG converter is as yet unclear. It is noted, however, that PG I appears early in ripening with the PG II form becoming increasingly predominant as ripening progresses (5). Most experiments in this study were conducted with the major isoenzyme from, PG II.

The effect of PG II on ethylene production in tomato fruit was first determined at pH 4.5, the optimum pH for the enzyme (19), and at pH 7.0, where the enzyme has little activity. Green 'Cherry' tomatoes had low rates of ethylene production when treated with succinate buffers at pH 4.5 and 7.0 (Fig. 1) and with 0.15 M NaCl (data not shown). Fruit treated with PG II in pH 7 buffer produced somewhat more ethylene per hour than the controls. However, fruit infiltrated with PG II in pH 4.5 succinate produced much more ethylene than either the fruit treated with PG at pH 7.0 or control fruit. Of the different sampling times, ethylene evolution was highest at 17 hr and then slowly decreased with time. In preliminary experiments with green 'Cherry' and ‘Jumbo’ tomatoes, induced ethylene generally commenced within 2 to 4 hr, peaked at 17 to 24 hr, and either decreased to lower or basal levels by 36 to 42 hr after treatment or was maintained at high levels (data not shown). In subsequent experiments, sampling times were scheduled to monitor ethylene production at and after the peak to determine whether the treatments would cause fruit to enter the climacteric.

PME is reported to enhance the activity of PG. Demethylation of pectin by PME provides PG with the preferred substrate of pectate (18, 20). In previous studies, it was observed that when green 'Cherry' tomatoes were treated with PG II, PME, or PG II + PME, the PG II + PME treatment tended to induce more ethylene than PG II alone (data not shown). This increase suggests that the exogenous PME enhances the activity of the exogenous PG by demethylation of substrate, thus producing a synergistic effect. PME treatment alone, however, induced no more ethylene than was observed in control fruit, so PME itself does not elicit ethylene synthesis in 'Cherry' tomatoes. In subsequent experiments, PME was added to PG treatments to ensure maximal PG activity.

Green ‘Cherry’ tomatoes treated with PG II + PME produced significantly higher ethylene levels than control fruit treated with boiled enzyme solutions (Fig. 2). This difference indicated that active enzymes are responsible for the increase in ethylene and not salts or other possible solutes in the treatment solutions. Ethylene levels peaked at 20 hr and decreased slightly by 38 hr. Nevertheless, production remained quite high even after 63 hr, as the fruit had entered the climacteric. All enzyme-treated fruit turned red within 1 week, whereas control fruit, which produced only basal levels of ethylene throughout the experiment, were still green. In other experiments, enzyme-treated fruit generally turned red faster than controls, and enzyme-induced ethylene either returned to lower or basal levels by 24 to 48 hr, or entered the climacteric.

‘Cherry’ ethylene production was highly variable over the growing season, which may have been due to a severe drought and excessive temperatures (>38°C) toward the end of the summer. The ‘Cherry’ tomatoes in this experiment were harvested previous to the adverse environmental conditions and had much higher ethylene than fruit harvested late in the season, which were used in other experiments. In any case, the physiological stage of maturity of the green fruit was probably an important factor in the enzyme-induced response. Unfortunately, it is difficult to distinguish mature green from immature green fruit, visually or by ethylene production. Even precise chronological age of the fruits has been found not to be a reliable indicator of time to ripen or ripening behavior (8).

The effect of PG II concentration on ethylene production was determined by treating green 'Cherry' tomatoes with the original PG II solution and with 2- and 4-fold dilutions of the solution. The same amount of PME was added to all treatment solutions to ensure optimal PG activity. Results show that concentrated PG II generally induced more ethylene production than the diluted treatments (Fig. 3). Although the concentrated PG II was not always significantly different from the 2-fold dilution, it did induce significantly higher ethylene synthesis than the 4-fold dilution. All PG II treatments induced significantly higher levels of ethylene than control fruit at 17 and 22 hr but not at 42 hr. The amount of ethylene produced thus was dependent on the concentration of PG II introduced into the fruit.

The ethylene-inducing activities of the isoenzymes PG I and PG II then were compared. PG I and PG II were adjusted to equal activities and applied to green 'Cherry' tomatoes (Fig. 4). Equal amounts of PME were added to both PG treatments. PG II + PME induced significantly more ethylene synthesis than did PG I + PME, and both induced higher ethylene levels than control fruit (Fig. 4). Overall ethylene levels were low in these fruit, however, even after color development (data not shown). Nevertheless, PG II may be more effective in vivo than PG I due to a smaller molecular size and hence reduced steric effects.

Table 1. Effect of PG II (804 units/ml) + PME (0.6 unit/ml) in 0.15M NaCl vs. 0.15M NaCl alone on ethylene production by green tomato ripening mutants.

<table>
<thead>
<tr>
<th>Fruit type</th>
<th>Treatment</th>
<th>Hours after treatment</th>
<th>1</th>
<th>24</th>
<th>44</th>
<th>68</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cornell-111</td>
<td>PG II + PME</td>
<td>80 ± 10</td>
<td>1115 ± 318</td>
<td>1360 ± 214</td>
<td>1412 ± 426</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>77 ± 10</td>
<td>112 ± 41</td>
<td>153 ± 70</td>
<td>265 ± 91</td>
<td></td>
</tr>
<tr>
<td>rin</td>
<td>PG II + PME</td>
<td>63 ± 10</td>
<td>732 ± 123</td>
<td>412 ± 161</td>
<td>405 ± 180</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>62 ± 10</td>
<td>34 ± 10</td>
<td>18 ± 0</td>
<td>12 ± 4</td>
<td></td>
</tr>
<tr>
<td>nor</td>
<td>PG II + PME</td>
<td>72 ± 17</td>
<td>678 ± 153</td>
<td>590 ± 134</td>
<td>538 ± 166</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>118 ± 90</td>
<td>42 ± 8</td>
<td>47 ± 21</td>
<td>58 ± 14</td>
<td></td>
</tr>
</tbody>
</table>

Means of four replications ± se.
The latter enzyme is essentially PG II plus PG converter and has a molecular weight more than twice that of PG II (17). Red, pink, and green ‘Jumbo’ tomatoes were treated with the combination of PG II + PME and showed a similar pattern of ethylene production to the green ‘Cherry’ tomatoes (data not shown). Evidently the enzyme-induced ethylene response occurs in ripe as well as green fruit.

PG II + PME treatments also increased ethylene in the tomato ripening mutants Cornell 111, rin, and nor (Table 1). All fruit were producing relatively low levels of ethylene at time of treatment and were showing no signs of chlorophyll breakdown. After treatment with enzyme solutions, all three mutant lines showed a dramatic increase in ethylene production over control fruit, which was maintained for over 3 days. All enzyme-treated Cornell 111 fruit showed signs of chlorophyll breakdown after 5 days, but so did two out of four control fruits. Two of the enzyme-treated rin fruit underwent chlorophyll breakdown and even showed signs of lycopene development at the blossom end. The other two enzyme-treated fruit and all controls remained green. None of the nor fruit showed any color change until weeks later, when both enzyme-treated and control fruit started to turn yellow at about the same time.

In conclusion, the native pectin-lyzing enzymes such as PG I and PG II induce ethylene when infiltrated into preclimacteric green tomato fruits. The role of PME is less clear, but this pectin-modifying enzyme also appears to influence tomato ethylene production. The stage of maturity in green tomatoes, in terms of responsiveness to ethylene, may determine whether the enzyme-induced ethylene leads into the climacteric and ripening or returns to lower or basal levels. Unfortunately, the stage of maturity of green fruit is difficult to access without dissecting the fruit. Whether this enzyme-induced ethylene is a direct effect of enzymatic pectin demethylation and hydrolysis or an indirect effect of possible hydrolysis products is unknown. There have been some preliminary studies on inducement of ethylene in tomato by galactose, galacturonic acid (10, 13), and pectin fragments (6). Cell-wall fragments, produced by fungal enzymes, have been implicated as inducers of ethylene in citrus fruit (2) and pear suspension cells (22). Tighchelaar et al. (21) suggested that PG may initiate fruit ripening by releasing wall-bound enzymes involved in ethylene production or other processes. Although there is good evidence showing that ethylene stimulates synthesis of PG in ripening tomatoes (9), the ripening scheme may not be that simple, as it is now evident that PG induces synthesis of ethylene. It is possible that low and as yet undetectable levels of PG in green fruit promote increased ethylene production so that internal levels of this hormone reach some threshold. Mature fruit that are sensitive to ethylene might then respond with increased synthesis of PG, lycopene, and autacatalytic ethylene. The increased levels of PG could conceivably induce more ethylene, resulting in the very high levels of prolonged ethylene production observed during the climacteric.

**Literature Cited**