Pectin Methylesterase Regulates Methanol and Ethanol Accumulation in Ripening Tomato (*Lycopersicon esculentum*) Fruit*

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We provide genetic evidence that the production of methanol in tomato fruit is regulated by pectin methyl-esterase (PME, EC 3.1.1.11), an enzyme that catalyzes demethoxylation of pectins. The role of PME in methanol production in tomato fruit was examined by relating the tissue methanol content to the PME enzymatic activity in wild-type Rutgers and isogenic PME antisense fruits with lowered PME activity. In the wild-type, fruit development and ripening were accompanied by an increase in the abundance of PME protein and activity and a corresponding ripening-related increase in methanol content. In the PME antisense pericarp, the level of methanol was greatly reduced in unripe fruit, and diminished methanol content persisted throughout the ripening process. The close correlation between PME activity and levels of methanol in fruit tissues from wild-type and a PME antisense mutant indicates that PME is the primary biosynthetic pathway for methanol production in tomato fruit. Interestingly, ethanol levels that were low and unchanged during ripening of wild-type tomatoes increased progressively with the ripening of PME antisense fruit. *In vitro* studies indicate that methanol is a competitive inhibitor of the tomato alcohol dehydrogenase (ADH, EC 1.1.1.1) activity suggesting that ADH-catalyzed production of ethanol may be arrested by methanol accumulation in the wild-type but not in the PME mutant where methanol levels remain low.

Methanol is emitted by actively growing plant tissues (1) and ripening fruit (2). A major source of methanol may be pectin methyl esters (3) that are de-esterified to methanol and pectic substances by a PME-catalyzed reaction (4). Although methanol production is correlated with PME activity in germinating seeds or other plant tissues (1), the role of PME in methanol accumulation in plants has not been firmly established (4, 5).

A developmentally regulated increase in PME gene expression occurs in developing tomato fruit (6) and may be used as a test system to relate PME activity to methanol metabolism. We have created transgenic tomato fruits with severely impaired expression of PME by introducing a fruit-specific PME antisense gene under the control of cauliflower mosaic virus 35S promoter (7). We compared methanol production in the wild-type and the PME antisense tomato fruits to examine whether the arrest of PME gene expression results in diminished production of methanol. Our results show that methanol accumulation in ripening tomato pericarp of either the wild-type or the PME antisense is related to PME activity, suggesting that the PME activity is the primary source of methanol production in tomato fruits. A surprising finding is that the tissue methanol and ethanol content in ripening tomato fruit were inversely related.

**EXPERIMENTAL PROCEDURES**

**Plant Material**—Wild-type tomato (*Lycopersicon esculentum cv Rutger*) and transgenic 37-81 Rutgers, transformed with a PME antisense gene (7), tomato plants were grown at the Purdue Agricultural Experiment Research Farm (8). Fruit from wild-type and PME antisense tomatoes were harvested at the immature green stage (IMG), the mature green (MG), breaker (BR), turning (TU), and red ripe (RR) stages as described earlier (9). The pericarp was frozen in liquid nitrogen and held at −80 °C until being used for the analysis of methanol and ethanol content or purification of ADH.

**PME Activity and Immunoblot Analysis**—Changes in PME activity and protein were determined as described earlier (6, 7).

**ADH Purification**—ADH purification (10) was done with some modifications which consisted of the following: 2 g of frozen, lyophilized pericarp was suspended in 5 ml of buffer consisting of 1 M Tris-HCl, pH 7.4, 10 mM dithiothreitol, 1 mM EDTA, and 1% polyvinylpyrrolidone-insoluble (w/v)(11). The resulting slurry was centrifuged, and the resulting supernatant was supplemented with (NH₄)₂SO₄, precipitate in 50 mM Tris-HCl, pH 7.4, 5 mM dithiothreitol, and dialyzed against the same buffer overnight using dialysis tubing with a molecular weight cutoff of 6,000 to 8,000. The dialyzed solution was loaded onto a 1 × 14 cm column of Cibacron blue (10), and the eluent was concentrated using an Amicon Centricon Prep 10 Ultrafiltration unit. ADH was purified 700-fold based on specific activity and consisted predominantly of ADH-2 isozyme and only traces of ADH-1 isozyme (10). Protein concentration was determined using the Bradford method (Bio-Rad), standardized against bovine serum albumin.

**Extraction and Assay of Methanol and Ethanol**—Fifty g of frozen tomato fruit tissue were homogenized for 1 min in 200 ml of ice-cold glass-distilled water containing 1 mM NaN₃. The homogenate was filtered once through Whatman No. 1 filter paper, and the entire filtrate was transferred to a 2-liter round bottom retort-flask and heated. A Thermo-O-Watch L7 SS regulator (Instruments for Research and Industry, Cheltenham, PA) was used to control heat output and to vaporize volatiles from the filtrate at a rate of 50 ml/h. Water vapors and volatile compounds were fluxed through a spiral glass condenser and collected in ice-cold 200-ml round bottom flasks. Distillation was maintained until 100 to 120 ml of the filtrate were recovered. Recovery of ethanol, methanol, and acetaldehyde from test solutions containing 100 mg ml⁻¹ of the volatile was 100% ± 1.5 suggesting that the employed

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* The abbreviations used are: PME, pectin methylesterase; ADH, alcohol dehydrogenase; IMG, immature green; MG, mature green; BR, breaking; TR, turning; RR, red ripe.
protocol led to virtually complete recovery of the compound. The residue
from tissue homogenate filtration was suspended in water and sub-
jected to distillation (as mentioned above) but did not yield methanol
suggesting that the distillation process is not accompanied by heat-
induced release of methanol from the cell wall residue. The distillate
samples were stored at 2 °C and used for gas chromatography analysis
as outlined by Kimmerer and Kozlowski (12). Quantification of resolved
methanol, ethanol, or acetaldehyde was done by comparison against
standard curves made of aqueous solutions containing defined levels of
the compounds.

RESULTS

Relationship between PME Activity and Methanol Accumulation in Tomato Fruit—Development of tomato fruit was ac-
accompanied by a progressive increase in the PME activity (Fig.
1A). The low levels of PME activity at the IMG stage increased
substantially at the MG stage and continued to increase as ripening progressed before declining at the RR stage. In the
PME antisense mutant, the PME activity at the IMG stage was
lower than wild-type and remained unchanged throughout
fruit development and ripening. The steady state PME protein
levels showed a pattern similar to PME activity during the
ripening and development of wild-type fruit (Fig. 1B). In PME
antisense pericarp, the 34-kDa PME isoform was undetectable
whereas the 37-kDa polypeptide, which cross-reacts with poly-
clonal fruit PME antibodies (7), remained unchanged, at all
stages of fruit development and ripening. These results are
consistent with the earlier report showing that the expression
of a fruit-specific PME antisense gene impairs accumulation of
the major PME isoforms (7, 13).

We used the pronounced changes in PME activity during development and ripening of wild-type fruits and the absence of
these changes in the PME antisense fruits to assess the role of
PME in methanol accumulation in tomato fruit. In the wild-
type fruits, a basal level of methanol content in the unripe
pericarp increased progressively through the succeeding ripen-
ing stages in a manner similar to the changes in PME activity
(Fig. 2A). However, methanol remained at a basal level while
PME activity increased between the IMG and MG ripening
stages. Also, PME activity decreased whereas methanol in-
creased between the TU and RR stages of fruit development. In
the PME antisense tomato, the methanol levels in the unripe
pericarp (IMG and MG stages) were 5- to 7-fold lower than in
unripe wild-type pericarp, and fruit ripening was not associ-
ated with an increase in methanol content. Collectively, these
results show a correlation between PME activity and methanol
accumulation in tomato fruit suggesting that methanol produc-
tion in tomato fruit is regulated by PME activity.

Relationship between Methanol and Ethanol Content in Ripen-
ing Tomato Fruit—Analyses of distillates from tomato peric-
arp homogenates revealed that changes in methanol and eth-
anol levels showed opposing trends (Fig. 2). In the wild-type,
low ethanol levels were found at the IMG stage, and ripening-

![Fig. 1. Effect of the introduced PME antisense gene on the levels of PME specific activity (A) and PME protein (B) in developing and ripening tomato fruit. Immature green (IMG), mature green (MG), breaker (Br), turning (Tu), and red ripe (RR) fruits were from field-grown wild-type Rutgers (filled bars) and transgenic 37-81 (open bars) expressing a PME antisense gene tomato plants. PME enzyme activity was determined titrimetrically in the total salt-extractable protein. For PME protein, 10 mg of total salt-extractable protein from field-grown wild-type Rutgers and transgenic 37–81 pericarp was used for gas chromatography analysis as outlined by Kimmerer and Kozlowski (12). Quantification of resolved methanol, ethanol, or acetaldehyde was done by comparison against standard curves made of aqueous solutions containing defined levels of the compounds.](Image)

![Fig. 2. Content of methanol (A) and ethanol (B) in developing wild-type Rutgers tomato and PME mutant (37-81) expressing a PME antisense gene. The results are means of data values ± S.E.](Image)

| Tomato stage | Rutgers | 37–81 |
|--------------|---------|-------|
| Immature Green | 0.042 ± 0.000 | 0.063 ± 0.000 |
| Mature Green | 0.039 ± 0.000 | 0.073 ± 0.005 |
| Breaking | 0.088 ± 0.000 | 0.086 ± 0.002 |
| Turning | 0.167 ± 0.003 | 0.220 ± 0.005 |
| Red Ripe | 0.261 ± 0.006 | 0.416 ± 0.003 |

Table I

Comparison of ADH activity between wild-type Rutgers and transgenic 37–81

Pericarp tissue from different developmental stages was frozen and then pulverized by mortar and pestle. The tissue was then homogenized in 1 M Tris-Cl, pH 7.4, 10 mM dithiothreitol, and 1 mM EDTA, and ADH activity was measured as described in Fig. 3. These results are the average ± S.E. of one experiment with four replications.
related increases in methanol were accompanied by further reduction in ethanol content. In the PME antisense tomato pericarp, the arrest in methanol accumulation was associated with an increase in ethanol, especially in the RR fruit. Assuming that tomato pericarp contains 95% H₂O (w/w), our results indicate that levels of methanol increased from 2.5 mM to 6.5 mM during development and ripening of the wild-type tomato tissue while methanol levels in the PME antisense pericarp ranged between 0.3 and 0.5 mM (Fig. 2A) and remained unchanged during fruit development and ripening. Ethanol levels in the wild-type ranged from 0.48 mM at the MG stage down to 0.075 mM at the RR stage while ethanol levels in the PME antisense tissue ranged from 0.715 mM in the MG stage to 3.98 mM in the RR stage (Fig. 2B). ADH-catalyzed reduction of acetaldehyde to ethanol is the only known pathway for ethanol biosynthesis (14) and thus may be a putative target for methanol-regulated ethanol production. Table I supports the data in Fig. 2 that methanol is an inhibitor in vivo since the transgenic fruit had an overall higher ADH activity than did the wild-type fruit indicating that methanol does possibly regulate ADH. Also, there are indications (Table I) that the ADH activity in the PME antisense pericarp was overall higher than in the fruit tissue of wild-type fruit suggesting that ADH activity in the mutant was not a limitation to the enzyme-catalyzed conversion of acetaldehyde to ethanol. In a Lineweaver-Burk plot (Fig. 3) the apparent $K_m$ for ethanol was determined to be 0.38 mM whereas the apparent $K_m$ for methanol was 5.1 mM. Methanol inhibition seems to be competitive much like pyrazole, a known inhibitor of ADH (10). Methanol was found to have no effect on tomato ADH activity in the absence of ethanol (data not shown) and has been found not to be a substrate for ADH in vitro in tomato or other plants (10). An apparent $K_m$ value of 1.74 mM for acetaldehyde for tomato ADH (10) suggest that methanol levels in normally ripening fruit may not be sufficiently high to completely arrest acetaldehyde conversion to ethanol production (Fig. 2B). In the PME antisense pericarp, the methanol levels, approximately an order of magnitude lower than the wild-type, may have been sufficiently low as to be apparently not competitive with ADH-catalyzed reduction of acetaldehyde to ethanol thus resulting in ethanol accumulation.

**DISCUSSION**

Our results provide evidence that methanol production in tomato fruit is primarily regulated by PME. PME is a ubiquitous enzyme present in higher plants (15), and PME activity may partially account for methanol accumulation and emission in plant tissues (1, 2, 16). The present results showing that methanol levels are related to PME activity further support the hypothesis that demethoxylation of pectins is, at least in part, a metabolic origin of methanol. The lag in methanol accumulation (Fig. 2A) compared with the increase in PME activity between IMG and MG stages in wild-type fruit may reflect the inaccessibility of cell wall pectin methyl esters to PME action at early ripening stages. Production of several cell wall hydrolases, including polygalacturonase, lead to a change in the cell wall structure (17) and may influence the availability of pectic substrate for PME activity and in consequence, accumulation of methanol during ripening of wild-type fruits. The low level of methanol production in PME antisense fruit pericarp may have resulted from the low PME activity which probably arises from the group II PME isoforms, whose synthesis is not impaired by the introduced PME antisense gene (13). Since methanol production does not increase during ripening of the PME antisense fruit, these results suggest that the activity of group II PME isoforms may contribute marginally to methanol accumulation.

The results also suggest that methanol may regulate ethanol metabolism in ripening fruits. The decline in methanol may have created metabolic conditions for accumulation of ethanol in the PME antisense tissue. An increase in ADH activity during tomato fruit ripening (Table I (11)) suggests that the capacity of fruit tissue for alcoholic fermentation is not a limitation to the production of ethanol although ethanol accumulation may be restricted by trace amounts of acetaldehyde in ripening tomato (results not shown) However, in the PME antisense tissue the decline in methanol may have created metabolic conditions for accumulation of ethanol.

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