Pectin Methylesterase Isoforms in Tomato (Lycopersicon esculentum) Tissues

Effects of Expression of a Pectin Methylesterase Antisense Gene

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We have identified two major groups of pectin methylesterase (PME, EC 3.1.1.11) isoforms in various tissues of tomatoes (Lycopersicon esculentum). These two groups exhibited differential immuno-cross-reactivity with polyclonal antibodies raised against tomato fruit PME or flax callus PME and differences in their accumulation patterns in tissues of wild-type and transgenic tomato plants expressing a PME antisense gene. The group I isoforms with isoelectric points (pIs) of 8.2, 8.4, and 8.5 are specific to fruit tissue, where they are the major forms of PME activity. The group II PME isoforms, with pI values of 9 and above, are observed in both vegetative and fruit tissues. The group I isoforms cross-react with polyclonal antibodies raised to a PME isoform purified from fruit, whereas the group II isoforms cross-react with antibodies to a PME purified from flax callus. Expression of a fruit-specific PME antisense gene impairs accumulation of the group I PME isoforms, with no apparent effect on the accumulation of the group II PME isoforms. The absence of any noticeable effects on growth and development of transgenic plants suggests that the group I PME isoforms are not involved in plant growth and development and may play a role under special circumstances such as cell separation during fruit ripening.

PMEs (EC 3.1.1.11) are cell wall enzymes that de-esterify galactosyluronic acid units of pectic compounds. PME activity has been observed in all parts of higher plants studied. PME has been hypothesized to be involved in fruit softening (Pressey and Avants, 1982; Fischer and Bennett, 1991), abscission and senescence (Sexton and Roberts, 1982; Liners and Van Cutsem, 1992), cell wall maturation and regeneration (Northcote, 1986; Shea et al., 1989), cell growth (Bryan and Newcomb, 1954; Yamaoka and Chiba, 1983; Goldberg, 1984; Ricard and Noat, 1986; Moustacas et al., 1991; Nari et al., 1991), plant responses to infection (Bateman and Millar, 1966; Collmer and Keen, 1986), and salinity stress (Bigot and Van Cutsem, 1978). Different isoforms of PME have been reported in tomato (Lycopersicon esculentum) (Pressey and Avants, 1972; Delincee, 1976; Tucker et al., 1982) and citrus (Evans and McHale, 1978; Versteeg et al., 1978) fruits, mung bean hypocotyls (Goldberg, 1984; Goldberg et al., 1992), and flax callus (Gaffé et al., 1992). The tissue specificity and the role of different PME isoforms in plant growth and development are not known. Also, it is not understood whether the different isoforms are encoded by different genes or represent posttranslational modifications of the same PME protein. Recently, the presence of multiple genes for PME has been demonstrated in tomato and Phaseolus vulgaris (Harriman, 1990; Recourt et al., 1992).

To characterize PME isoforms present in different plant tissues, we have examined immuno-cross-reactivity of various PME isoforms using two polyclonal antibodies raised against a tomato fruit PME and a flax callus PME (Harriman, 1990; A. Mareck, J. Gaffé, and C. Morvan, unpublished results). We have also determined the effects of expression of a fruit PME antisense gene on production of various PME isoforms (Tieman et al., 1992). Our results show that the majority of PME activity present in ripening tomato fruits is due to three fruit-specific isoforms of PME, and production of only these fruit-specific isoforms is inhibited by the expression of a fruit PME antisense gene. Collectively, our results suggest that the PME isoforms present in the tomato plant can be grouped into two types: (a) PME isoforms synthesized during fruit development and (b) PME isoforms present throughout growth and development of the plant as well as in the fruit tissue.

MATERIALS AND METHODS

Plant Material

Wild-type tomato (Lycopersicon esculentum cv Rutgers) and transgenic Rutgers plants were grown under standard greenhouse conditions, as described elsewhere (Tieman et al., 1992). Root, stem, leaf, and fruit pericarp tissues were frozen in liquid nitrogen and stored at −80°C until use.

Protein Extraction and Protein Assay

Tissue samples were ground in liquid nitrogen with a mortar and a pestle. Total proteins were extracted in an equal volume (w/v) of 2 M NaCl by stirring for 2 h at 4°C, centrifuged at 12,000 rpm for 20 min at 4°C in an SS 34 rotor (Sorvall), and concentrated using Aquacide III (Cal-

Abbreviations: pI, isoelectric point; PME, pectin methylesterase.

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1 This research was supported by the Consortium for Plant Biotechnology Research, Inc. This is journal paper No. 14067 of the Purdue University Agricultural Experiment Station.

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biochem) in dialysis tubing (Spectrapor 3). Total protein was
determined by the method of Bradford (1976) using BSA as
a standard.

**IEF**

Horizontal IEF on 1-mm-thick polyacrylamide gels (7% total monomer, 3% cross-linker as percent of total monomer) was performed on an FBE 3000 apparatus (Pharmacia) at 4°C under humidified nitrogen, using a mixture of carrier ampholyte: 3–10 Ampholyne (Pharmacia) and 9–11 ampholyte (LKB) at a final concentration of 2.8% (w/v). Electrode strips were soaked with 25 mM Asp, 25 mM Glu for the anode and 25 mM Lys, 25 mM Arg, 2 M ethylenediamine for the cathode. After a 20-min prefocusing at 10 mA, samples in 0.1 M NaCl were applied on wells at the anodic side of the gel, and focusing was performed for 90 min at 15 W. The pH gradient was estimated by incubating pieces of the gel, 0.5 cm wide, in 1 M NaCl overnight. Because of the short time of focusing, the values of pI are only close approximations.

**PME Activity Assay**

PME enzyme activity was determined titrimetrically as described by Harriman et al. (1991) using 0.4% (w/v) citrus pectin as a substrate. PME activity was located on the gel according to the method of Bertheau et al. (1984). Briefly, after IEF, a part of the gel was soaked for 15 min in 0.1 M sodium phosphate buffer (pH 7.5) and then incubated on an agar (1.5%)-pectin (0.5%) gel in the same buffer for 1 h at 30°C. The de-esterification of the pectic compounds was revealed by incubating the gels for 30 min in 0.1 M maleic acid, followed by 0.02% (w/v) ruthenium red overnight, and destaining in water.

**Immunoblot Analysis**

A part of the same IEF gel was immunoblotted to nitrocellulose and challenged with anti-PME antibodies (diluted 1/3000) followed by alkaline phosphatase-conjugated anti-chicken antibodies, using skim milk as a blocking agent, as described by Harriman et al. (1991). Some blots were subjected to a second challenge using antibodies raised against PME from flax (diluted 1/500) and revealed with alkaline phosphatase-conjugated anti-rabbit antibodies.

**RESULTS**

**PME Activity in Vegetative and Fruit Tissues**

PME activities in various tissues of tomato plants varied significantly (Fig. 1). The lowest levels of PME specific activity were recorded in leaf and stem tissues and 10-d-old pericarp. PME activity levels in 20-d-old fruit and roots were about 5- and 20-fold higher than in leaf tissue, respectively. The PME specific activity continued to increase during tomato fruit development until the mature green stage of fruit development before declining slightly during fruit ripening. An increase in PME specific activity of about 50- to 60-fold was observed as fruit matured and ripened. Expression of a fruit-specific PME antisense RNA gene did not affect PME activity in transgenic leaf, stem, and root tissues, whereas an 80 to 90% reduction of PME activity was observed in 30-d and older transgenic fruits. Twenty-day-old transgenic fruit showed about 60% reduction in PME activity, as compared to the wild-type fruit, whereas no inhibition of PME activity was measured in the 10-d-old transgenic fruit. With reduction in PME activity by the antisense RNA gene, the specific activity of PME in 30-d and older transgenic fruits was of the same order of magnitude as in the wild-type root tissue.

**PME Isoforms in Vegetative and Fruit Tissues**

Five isoforms of PME were observed in immature tomato fruit after IEF and staining for PME activity. All isoforms had basic pIs, with the majority of PME activity associated with the isoform having a pI of approximately 8.5 (Figs. 2A and 3A). Other PME isoforms had pIs of 8.2, 8.4, about 9.0, and above 9.0 (Fig. 3A). All vegetative tissues tested showed only two PME isoforms with pIs of about 9.0 and above 9.0. Most PME activity in the vegetative tissues was associated with PME isoforms having a pI 9.0 and above. The PME isoforms with pIs 8.2, 8.4, and 8.5 observed in fruit were not detectable in vegetative tissues.

**Changes in PME Isoforms during Fruit Development**

Figure 2A shows changes in the patterns of PME isoforms during tomato fruit development and ripening. Ten-day-old pericarp showed a PME isoform distribution similar to that observed in root, leaf, and stem tissues, with the major activity associated with isoforms of pI 9.0 and above. Three additional isoforms of PME with pIs 8.2, 8.4, and 8.5 appeared in 20-d-old pericarp tissue. In 30-d-old pericarp significant PME activity was associated with the isoforms having pIs 8.4 and 8.5. However, at the mature green stage of fruit development, the PME activity associated with a pI 8.4 was
not detectable, and the majority of PME activity was present in the isoform having a pi 8.5. No further changes in PME isoform patterns were observed in ripening pericarp. The levels of the PME isoforms with pi 9.0 and above remained similar throughout development and ripening of fruit (Fig. 3A). We have observed that PME isoforms with pi 9.0 and above tend to aggregate and fall out of solution during freezing and thawing of tissue extracts (M. Tiznado and A.K. Handa, unpublished results). This may explain the relatively low abundance of these isozymes in 30-d, mature green, and breaker stage samples shown in Figure 2. Fresh protein extracts were analyzed to obtain the PME isozyme patterns shown in Figure 3.

**Immuno-Cross-Reactivity of PME Isoforms**

In an earlier investigation, polyclonal antibodies raised against PME purified from mature green tomato pericarp showed cross-reaction with PME activity present in 20-d and older pericarp but did not cross-react with PME activity present in 10- or 15-d-old pericarp or in vegetative tissues (Harriman et al., 1991). These polyclonal antibodies were tested for their cross-reaction to various PME isoforms present in both vegetative and fruit tissues (Fig. 2B). These antibodies recognized PME isoforms with pis 8.2, 8.4, and 8.5 present in 20-d or older pericarp. No recognition occurred with PME isoforms with pi 9.0 and above at any stage of fruit development or ripening. These antibodies also failed to cross-react with any PME isoform present in the vegetative tissues. The polyclonal antibodies raised against flax callus PME did not cross-react with pi 8.2, 8.4, and 8.5 fruit PME isoforms (data not shown) but did cross-react with PME isoforms with pi 9.0 and above in both vegetative and fruit tissues (Fig. 2C).

**PME Isoform Patterns in Transgenic Tomato Tissues Expressing a PME Antisense RNA Gene**

The three PME isoforms with pis 8.2, 8.4, and 8.5 were not detectable either by enzyme staining or by immunoblotting at any stage of transgenic fruit development (Fig. 3). All PME activity present in the transgenic pericarp was associated with isoforms having pi 9.0 or above (Fig. 3) and did not cross-react with fruit-specific PME antibodies. Similar results

![Figure 3](https://plantphysiol.org)
were obtained with transgenic 20- and 30-d pericarp and vegetative tissues (data not shown). Based on activity staining of the IEF gel, no apparent reduction in the isoforms with pl 9.0 and above was observed in transgenic pericarp or vegetative tissues as compared to wild-type pericarp or vegetative tissues.

**DISCUSSION**

The five isoforms of PME that we have observed in various tissues of tomato plants can be classified in two groups. The first group was constituted of isoforms with pl's of approximately 8.4 (8.2, 8.4, and 8.5) and represented the major PME isoforms in fruit tissue. The second group, isoforms with pl 9.0 and above, was the major form in 10-d-old fruits and the vegetative parts of the plant but was the minor form in 30-d and older pericarp. The first group of PME isoforms seemed specific to fruit tissue, whereas the second group was observed in all plant tissues studied, including pericarp. The presence of multiple isoforms of PME in various plant tissues has been reported by several laboratories (Pressey and Avants, 1972; Delincee, 1976; Evans and McHale, 1978; Versteeg et al., 1978; Tucker et al., 1982; Goldberg, 1984). However, patterns of PME isozymes in different tissues of a particular plant species have not yet been investigated. It would be interesting to determine whether other plant species have PME isozyme patterns similar to tomato.

Immuno-cross-reactivity of the two groups of PME isoforms indicated important differences between the proteins of the two groups. Antibodies raised against tomato fruit PME (Harriman et al., 1991) recognized only the first group of PME isoforms, with no cross-reaction with the second group of PME isoforms present in either fruit or vegetative tissues of the plant. Furthermore, polyclonal antibodies raised against flax callus PME did not recognize the PME isoforms of the first group, whereas a reaction was observed with PME isoforms of the second group.

Differences between the two groups of PME isoforms were further suggested by the inhibition of synthesis of only one group of PME isoforms in transgenic plants expressing an antisense gene for a fruit PME clone that was isolated with fruit PME antibodies used for the immunoblotting in this study. In these transgenic plants almost complete inhibition of accumulation of the group I isoforms was obtained with no apparent effects on accumulation of the group II isoforms in either fruit or vegetative tissues. The introduced PME antisense RNA gene was expressed in both the fruit and vegetative tissues and accumulated to a relatively large abundance (Tieman et al., 1992; Hall et al., 1993). In a recent work Recourt et al. (1992) showed a 65 to 70% identity between the PME genes isolated from pea pod and the PME genes obtained from fruit cDNA libraries, whereas two fruit PME cDNA clones and three PME genomic clones isolated using a fruit PME cDNA shared more than 90% identity (Ray et al., 1988; Harriman, 1990). The homology recorded between fruit PME genes and pod PME genes could be related to the conservation of active sites segments, including the positions of Cys's in tomato, fungal, and bacterial PME genes (Markovic and Jornvall, 1992; Recourt et al., 1992).

Our results show that accumulation of different isoforms of the group I enzymes were differentially regulated during fruit development (Fig. 2, A and B). All three isoforms of the group I PMEs showed accumulation in 20-d-old fruit, and the isoforms with pl's 8.4 and 8.5 were present at similar levels. In 30-d-old fruit, the level of the isoform with pl 8.5 was higher compared with the isoform with pl 8.4. In mature green fruit, the pl 8.4 isoform was undetected and the pl 8.5 isoform represented most of the PME activity present in fruit. No further change in the isoform patterns was observed during fruit ripening. The appearance and disappearance of the pl 8.4 isoform coincided with the phase when fruit was undergoing cell enlargement. During the ontogeny of tomato fruit, cell division ceases between 10 and 15 DAF, and fruit reaches its maximal size sometime before entering the mature green stage (Bunger-Kibler and Bangert, 1982). It would be tempting to suggest that the pl 8.4 isoform is involved in fruit cell enlargement. However, we did not see any change in the size of transgenic fruit, which lack the group I PME isoforms. In field trials, the average fruit weights of the wild-type Rutgers and transgenic Rutgers (3781A) were 104 ± 2 and 100 ± 3 g, respectively. If PME is indeed involved in cell enlargement (Ricard and Noat, 1986), then it is possible that the group II isoforms may substitute for the group I isoforms in fruit cell enlargement.

The significance of two types of PME isoforms is unknown. Major roles for PME suggested in plants include (a) localized reduction in pH due to demethoxylation of pectins resulting in cell extension and growth (Lamport, 1986; Ricard and Noat, 1986), (b) de-esterification of pectins, making them more susceptible to degradation by polygalacturonase or other pectinases and enhancing cell separation during fruit ripening, senescence, and abscission (Fischer and Bennett, 1991; Liners and Van Cutsem, 1992), and (c) generation of free COO⁻, which facilitates formation of new cross-linkages in cell walls (Fry, 1986). Experiments with transgenic plants suggest that the group I PME isoforms, specific for fruit tissue, are not necessary for either fruit development and ripening or plant growth and development (Tieman et al., 1992; Hall et al., 1993). It is possible that the two groups of PMEs, and even isoforms within one group of PMEs, have different modes of de-esterification of pectins (Markovic and Kohn, 1984). The role of PME in plant growth cannot be explained by studying a specific isoform from fruit tissue. It will be necessary to isolate and clone the second group of genes found in the vegetative tissues and study the effect of their inhibition or overexpression in transformed plants.

**ACKNOWLEDGMENTS**

The authors thank Dr. A. Mareck (Universite de Rouen, France) for the gift of antibodies raised against PME from flax callus, Dr. Nicholas C. Carpita for the review of manuscripts, and Mrs. D. Serra for tomato cell transformation.

Received October 25, 1993; accepted January 17, 1994.

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**LITERATURE CITED**


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