

Avocado Pectinmethylesterase Activity in Relation to Temperature, Ethylene, and Ripening¹

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ADDITIONAL INDEX WORDS. *Persea americana*, respiration

ABSTRACT. Investigation of optimum conditions for extraction, titrimetric assay and activity of avocado (*Persea americana* Mill cvs. Fuerte, Hass) pectinmethylesterase (PME) showed maximum extraction was obtained from lyophilized mesocarp using 0.4 M NaCl. Best assay conditions required a substrate of 0.5% pectin in 0.1 M NaCl. The enzyme was released from the tissue by salt but not by nonionic detergents. The Arrhenius plot between 3 and 32°C was a straight line which indicates no involvement with membrane lipid. Incubation with air or ethylene did not affect the enzyme. PME activity declined rapidly as ripening was initiated and reached a minimum shortly before the peak in respiration and ethylene production.]

The avocado fruit ripens only after harvest and shows a rapid transition from a hard to a soft flesh consistency. This change is intimately related to activity of cell wall degrading enzymes. PME is responsible for the deesterification of pectin required before polygalacturonase starts the depolymerization of pectins associated with fruit softening (7, 11). The presence of PME in avocado fruits and its rapid decline in activity after harvest are well documented (1, 5, 15, 17).

There has been, however, considerable difference in the methods of extraction and assay used by various workers as well as concern that the conditions necessary for optimum activity may change during ripening (16). A report (5) on the influence of ethylene on PME activity in tomato has added further complexity. PME was reported to lose activity upon incubation with ethylene at 25°C and this inactivation was reversed at 6°, while the enzyme was stable at 25° but not at 6°. Tomato PME exposed to ethylene showed marked stability in another report (12). The purpose of this investigation was to study conditions of extraction and assay of PME at several stages of ripening as well as the relation between temperature and activity of the enzyme in the presence and absence of ethylene.

¹Received for publication June 25, 1979.

The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper must therefore be hereby marked advertisement solely to indicate this fact.

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Materials and Methods

SAMPLING METHOD. Sections 2 cm-wide were removed on the day of harvest from the equatorial part of 'Fuerte' and 'Hass' fruit in order to compare the extraction of PME from fresh and lyophilized avocado mesocarp. A small wedge of each section was used immediately for the determination of PME. The remaining tissue was rapidly sliced, frozen in liquid N₂, lyophilized and ground to a fine powder with a porcelain mortar and pestle. This powder was stored in a desiccator at room temperature, or at -20°C for several months over which time there was no loss of activity.

Postharvest variation of PME in relation to respiration and ethylene production was measured on samples taken from single fruit around the equatorial diameter of the fruit at appropriate intervals with a 6 mm diameter cork borer. Holes were immediately closed with warm lanolin.

MEASUREMENT OF RESPIRATION AND ETHYLENE PRODUCTION. Avocado fruit were placed in separate jars at 20°C and flushed continuously with humid 100 ml/min, ethylene-free air. The outflow from each jar was passed automatically, at appropriate intervals, through a Beckman 215 A Infrared Gas Analyzer for the determination of CO₂ evolution and then a 1 ml sample was injected automatically into a Varian 1400 Gas Chromatograph with a flame ionization detector for the determination of ethylene production.

EXTRACTION AND ASSAY OF PME. Fresh hard tissue was homogenized with 10 ml of 2°C extracting solution in a Polytron (Brinkmann Instruments). Soft or lyophilized tissue was macerated in a ground-glass mortar and pestle. The mixture was centrifuged after a 15-min desorption period at 6,000 x g for 10 min. PME was assayed by a modification of the procedure of Rouse and Atkins (14). Five ml of the supernatant below the lipid layer were added to 50 ml of an 0.5% (w/v) solution of pectin NF (Purified polygalacturonic acid, methyl ester, Sunkist Growers, Inc.) in 0.1 M NaCl. The mixture was brought rapidly to pH 7.57 with NaOH and the release of carboxyl groups by the action of PME on the substrate was followed for 10 min with an automatic titrator (Metrohm Herisau) using 0.095 N NaOH at 24°C or the temperature under study. A water jacketed cuvette connected to a constant temperature bath maintained at ± 0.02°C was used for temperature studies. Activity of PME in the mixture was checked periodically at 24° over the entire period and found to be constant. Boiled enzyme showed no activity. PME units were expressed as µeq ester hydrolyzed/min (g fresh wt).

INCUBATION OF PME WITH AIR OR C₂H₄. Lyophilized powder equivalent of 2 g of fresh tissue obtained from fruit on the day of harvest was extracted with 10 ml of 2°C 10% NaCl (w/v) using a mortar and pestle. The mixture was centrifuged after a 15-min desorption period at 6,000g for 10 min. Three ml of the supernatant were placed in a small erlenmeyer flask at constant temperature and flushed continuously with 100 ppm of C₂H₄ in CO₂-free humid air. A second 3 ml sample from the supernatant, was placed in another erlenmeyer flask at constant temperature and flushed continuously with CO₂-free humid air. Samples were removed at appropriate intervals for determination of PME activity at 25°C. The assay mixture consisted of 0.5 ml of enzyme solution and 10 ml of a 0.5% pectin solution (w/v) in 0.1 M NaCl.

Results and Discussion

COMPARISON OF FRESH AND LYOPHILIZED TISSUE. Activity of PME extracted from freeze-dried unripe avocado mesocarp was 30 to 50% greater than from fresh hard tissue

Table 1. Pectinmethylesterase extraction from fresh and lyophilized preclimacteric 'Fuerte' and 'Hass' avocado mesocarp.^z

Cultivar	PME activity ^y		Increase in lyophilized fruit (%)
	Fresh fruit	Lyophilized fruit	
Fuerte	4.1	5.3	29
Hass	6.8	9.9	46

^zExtracted with 0.4 M NaCl solution; assayed with 0.5% pectin solution (w/v) in 0.1 M NaCl.

^yUnits are $\mu\text{eq ester/min}$ (g fresh wt). Means of three fruit assayed in duplicate.

EFFECT OF NA CL ON EXTRACTION AND ASSAY SOLUTIONS.

Maximum yield of PME was achieved using 0.4 M NaCl (Table 2). Salt was also beneficial in the assay medium (Table 3). Either 0.05 or 0.1 M NaCl in the pectin solution increased activity by 10% while higher levels were inhibitory. It should be noted for data shown in Table 3 that addition of 5 ml of the extract in 0.4 M NaCl to 50 ml of pectin solution made the final assay concentration of NaCl 0.036 M where no salt was present in the pectin and 0.127 M when pectin solution contained 0.1 M NaCl.

Table 3. Effect of NaCl concentration in the pectin solution used in the assay of PME from lyophilized preclimacteric 'Fuerte' avocado mesocarp.^z

NaCl concn	PME activity ^x	SE ^y
0	5.04	0.09
0.05 M	5.44	0.07
0.1 M	5.42	0.06
0.2 M	4.04	0.03
0.4 M	2.69	0.02

^zExtracted with 0.4 M NaCl solution; assayed with 0.5% pectin solution (w/v).

^yStandard errors of triplicate determinations on each of two tissue samples.

^xUnits are $\mu\text{eq ester/min}$ (g fresh wt).

(Table 1). This is primarily because cells of freeze dried tissue are more easily ruptured than those of hard fresh tissue. The values found here for avocado PME are at least 2x greater than those reported before (5, 17) and are believed to result from optimum extracting and assaying conditions.

Table 2. Effect of NaCl concentration in the extracting solution on the activity of PME from lyophilized preclimacteric 'Fuerte' avocado mesocarp.^z

NaCl concn (M)	PME activity ^x	SE ^y
0	2.14	0.03
0.1	3.65	0.02
0.2	4.49	0.03
0.4	5.03	0.05
0.8	4.73	0.04
1.6	3.02	0.03
3.2	1.87	0.04

^zAssayed with 0.5% pectin solution (w/v) in 0.1 M NaCl.

^yStandard errors of triplicate determinations on each of two tissue samples.

^xUnits are $\mu\text{eq ester/min}$ (g fresh wt).

EFFECT OF PECTIN CONCENTRATION IN THE ASSAY SUBSTRATE.

PME activity increased gradually as concentration of the substrate increased up to 0.25% and then remained constant (Fig. 1). A pectin concentration of 0.5% was selected as the one that would insure maximum PME activity.

EFFECT OF VARIOUS ADDITIONS TO THE EXTRACTING SOLUTION.

The nonionic detergent Triton X-100 is effective in releasing some bound enzymes (6). Neither 0.1 nor 1.0% Triton were

effective in desorbing the enzyme and Triton added to NaCl was no better than NaCl alone in extracting PME. Triton did not influence either the extraction or the activity of avocado PME.

Polyvinylpyrrolidone (PVP) is often used in extracts of plant material to prevent inhibition of enzymes by phenols. PVP (Polyclar AT, GAP Corporation) suppressed rather than stimulated PME either alone or with acetate buffer. Addition of 0.04 M acetate buffer to the NaCl extraction medium gave a 20% reduction of activity compared to salt alone. Addition of a sulfhydryl reagent, dithioerythritol (DTE), likewise brought about no stimulation of PME activity. PME is not inhibited by phenols released upon cell rupture and that activity is not dependent on an exposed —SH group.

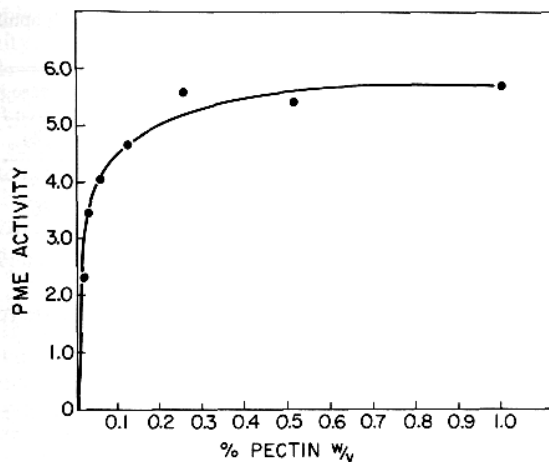


Fig. 1. Effect of pectin concentration in the assay substrate, on the activity of PME from lyophilized preclimacteric 'Fuerte' avocado mesocarp. (Extracted with 0.4 M NaCl solution; assayed with pectin solution in 0.1 M NaCl. Activity in $\mu\text{eq ester/min (g fresh wt)}$).

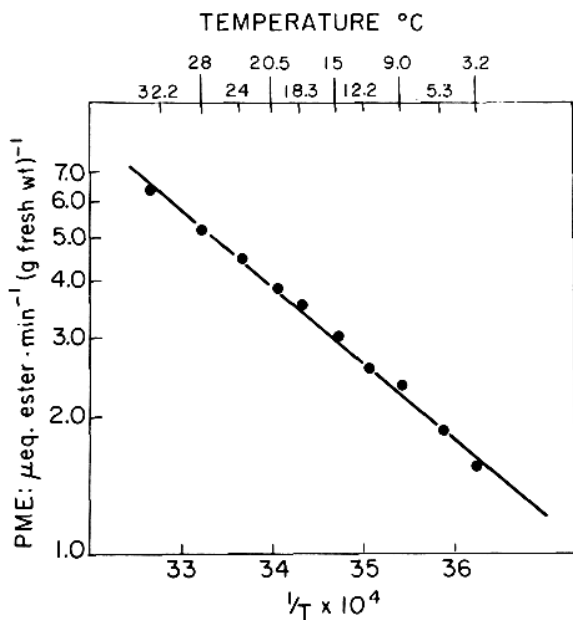


Fig. 2. Effect of temperature of assay on activity of PME from lyophilized preclimacteric 'Fuerte' avocado mesocarp. (Extracted with 0.4 M NaCl solution, assayed with 0.5% pectin solution (w/v) in 0.1 M NaCl.)

DESORPTION TIME AND SOLUBILITY OF PME. Less than 45% of the enzyme was released in extracts made up of water alone or with dilute buffer or detergent added. The crude NaCl extract was assayed directly as well as supernatant solutions from pellets sedimented at various forces from 3,000 x g for 10 min to 63,000 x g for 1 hr. There was no significant difference in the PME activity of any of the supernatant solutions. Thus, the enzyme appears to be completely soluble in salt solution. Ninety percent of the enzyme was released in 5 min and the remaining 10% in an additional 10 min in 0.4 M NaCl. Only the activity of the occluded supernatant solution retained in the first pellet was found when the centrifugal pellet derived from the first extraction was reextracted with 0.4 M NaCl.

EFFECT OF TEMPERATURE AND ETHYLENE. The report by Gertman and Fuchs (5) of the unusual effects of temperature and ethylene on the activity of PME suggested the enzyme might be associated with a lipid component, in which case a break in the

Arrhenius plot would be expected (13). The plot of log of activity against $1/T$ between 3 and 32°C is a straight line (Fig. 2). Bubbling 100 $\mu\text{l/liter}$ of ethylene through the extract did not change the plot. This is typical of a soluble, lipid-free enzyme and gives no evidence for direct involvement of ethylene with the enzyme.

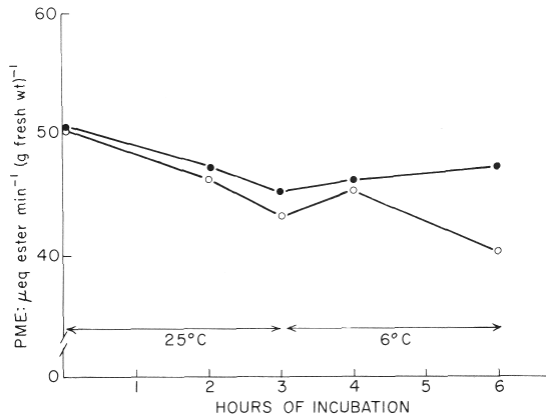


Fig. 3. Effect of incubation temperature in the presence of air (○—○) or C_2H_4 (●—●) on the activity of PME from lyophilized preclimacteric 'Fuerte' avocado mesocarp.

POSTHARVEST VARIATION OF PME. A single fruit was sampled each day for PME while respiration and ethylene production were followed continuously on the same fruit. PME was highest at picking time, remained at 70% of the initial value throughout the preclimacteric period, and decreased precipitously just as the respiratory climacteric was initiated to about 20% of the initial activity at least one day before the climacteric peak of respiration (Fig. 4). Ethylene production rate on day 4 when PME level had already dropped substantially was 1 $\mu\text{l/liter}$ which was equivalent to 0.08 $\mu\text{l/liter}$ in the gas phase and 0.70 $\mu\text{l/liter}$ in the internal atmosphere of the fruit (8). The drop in PME cannot be initiated by this very low level of ethylene production. A decrease in activity of tomato PME was shown to be due to the accumulation of the reaction product, polygalacturonic acid (2, 9). We found, however, that making the assay medium as much as 0.06% in citrus sodium poly-pectate was without effect on avocado PME activity.

Conclusions

The enzyme was incubated under CO_2 -free air or 100 $\mu\text{l/liter}$ of ethylene in CO_2 -free air for 3 hr at 25°C and for 3 hr at 6° followed by assay at 25° as described by Gertman and Fuchs (5). There was a slight decline in activity of both the air and ethylene treatments at 25° (Fig. 3). There was no significant change in activity for either treatment at 6° . Similar results were obtained using lyophilized tomato PME (Sigma Chemical Co. No. P 6763). The reversible effects on PME activity resulting from changes in incubation temperatures reported by Gertman and Fuchs (5) could not be confirmed.

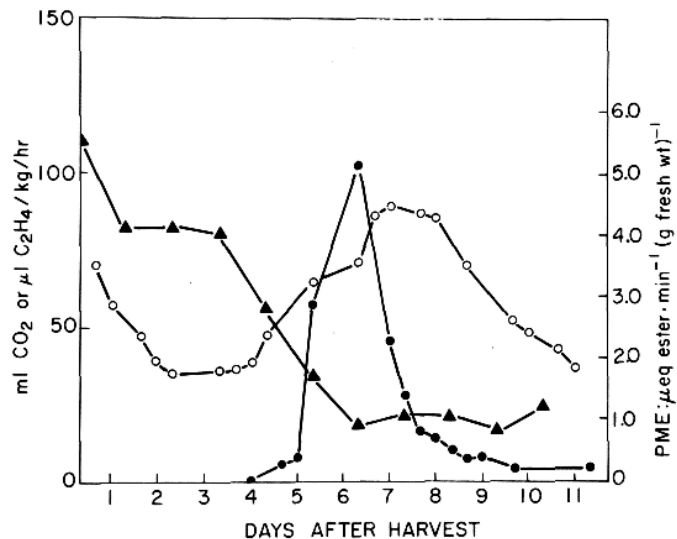


Fig. 4. Postharvest variation in PME (\blacktriangle — \blacktriangle), CO_2 (○—○) and C_2H_4 (●—●) in a single 'Fuerte' avocado fruit.

Our observations show that the postharvest level of PME from avocado fruit is highest at harvest and declines as ripening progresses. Neither the extracted avocado enzyme nor tomato PME are sensitive to ethylene and the enzyme responds to temperature just as a typical soluble enzyme. Pectin in most fruits, including avocado, is highly methylated at full maturity (3, 4, 10). Partial deesterification of pectin appears to be necessary to provide attachment sites for polygalacturonase (7), thus the need for PME activity may decline early in the ripening process.

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