Ethylene and the Appearance of an Albedo Macerating Factor in Citrus

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Abstract. Stem and fruit tissues of ‘Valencia’ and navel oranges were treated with ethylene via the decomposition of applied (2-chloroethyl)phosphonic acid (ethephon). Ethephon applications were sufficient to cause uniform loosening of the fruit from the tree. The tissues then were extracted and the extracts assayed by a citrus albedo tissue maceration test for enzyme activity. Maceration activity increased with time after ethylene treatment.

A correlation of ethylene treatment with increased activity of bean and citrus explant tissue enzymes which catalyze the degradation of carboxymethyl cellulose and glucans has been reported (1, 2, 8, 15, 17). It seemed reasonable to examine citrus tissues for other enzymes, such as pectinases, which might show ethylene-enhanced activities. Heid (6) noted in 1941 that pectin decreased in citrus fruit treated with ethylene. However, MacDonnell et al. (12) failed to find pectinase activity in extracts of orange flavedo. Although Pratt and Powers (16) detected a ‘pectin depolymerizing enzyme’ in grapefruit juice, the evidence for a pectinase in citrus is so lacking that it has been postulated that destruction of pectin substances in citrus tissue is due to pectinesterase activity followed by non-enzymatic reactions (9).

As a general approach a tissue maceration assay was employed in searching for ethylene-enhanced activity. Indirect methods then were used in identifying the enzyme(s) involved.

Methods and Materials

Usual methods of pectinase assay involve measurement of viscosity changes or the appearance of galacturonic acid residues with a model substrate, sodium polypectate (4). In preliminary tests we were unable to demonstrate an effect of citrus preparations on the viscosity of polypectate solutions. This is in agreement with the findings of Pollard and Biggs (15). As noted by Karr and Albersheim (10), however, degradation of model substrates does not demonstrate an ability of these enzymes for in vivo polysaccharide degradation; possibly the reverse statement is also true. In order to approach natural conditions as closely as possible, the “pectinase” assay of Morre’ (13) was modified for use with citrus. This assay actually measures tissue maceration rather than pectinases per se. According to Bateman and Millar (5), pectinases are important causal agents in pathogenic tissue maceration, and in many instances appear to be the sole responsible agents.

A crude enzyme extract was prepared from various tissues of navel or ‘Valencia’ (Citrus sinensis L. Osb.) orange. The tissue was frozen after collection and ground to a powder in liquid N. This extraction was frozen after collection and ground to a powder in liquid N. This extract was filtered and the filtrate used as the enzyme source without further treatment. Extracts made at pH 5.0. This extract was filtered and the filtrate used as the enzyme source without further treatment. Extracts made at pH 5.0. This extract was filtered and the filtrate used as the enzyme source without further treatment. Extracts made at pH 5.0. This extract was filtered and the filtrate used as the enzyme source without further treatment. Extracts made at pH 5.0. This extract was filtered and the filtrate used as the enzyme source without further treatment. Extracts made at pH 5.0. This extract was filtered and the filtrate used as the enzyme source without further treatment. Extracts made at pH 5.0. This extract was filtered and the filtrate used as the enzyme source without further treatment. Each experiment was run several times; the data given are from a representative run from each experiment. Because of seasonal variations in growing conditions and physiological state of the citrus tissues, no attempt was made to combine data for tissue collected at different times.

Most of the wt decrease of the cylinders was found to be due to loss of clumps of cells and cell debris. Small quantities of reducing sugars and protein were also washed from the cylinders. There was no wt loss when the tissue was incubated in solutions of fungal cellulase ( Worthington CSE II) and bean cellulase ( donated by Dr. L. N. Lewis), a-amylase (Sigma Lot A-6255)3, or pectinesterase ( Worthington PE81A). Addition of fungal cellulase to the citrus enzyme extract had no effect on maceration. The citrus enzyme extract was also active on cucumber pericarp substrate.

Ethylene was applied to the tissues via the decomposition of ethephon. Orange fruits were collected and taken into the laboratory for treatment. Except as noted later, sufficient ethephon solution (0.5 ml, 7 x 10^-3 M) was injected into the blossom end of the orange to give a final concn in the avg of about 3 x 10^-5 M. In separate experiments, rind and stem pieces were placed in petri dishes on filter paper soaked with the solution. The presence of ethylene in the fruit and petri dishes was confirmed by gas chromatography. No attempt was made to maintain a constant ethylene concn. Ethylene levels in the fruit or container rose slowly to a max between 24 and 48 hr, and then declined slowly ( Palmer and Rogers, unpublished data). The amount of ethylene evolved was sufficient to promote abscission layer formation in the fruit (14). The action of ethephon appears to be confined to generation of ethylene, although Hield et al. (7) reported that local applications of concd solutions of ethephon delayed the loss of chlorophyll in citrus leaves while ethylene hastened the
An increase in maceration activity following ethylene treatment must come either from de novo synthesis of enzyme or increased activity of existing enzyme. A preliminary test of these alternatives was made by treating the enzyme source material with cycloheximide, an inhibitor of protein synthesis.

**Results and Discussion**

*Effect of ethylene on maceration activity.* Changes in maceration activity following ethylene treatment (from decomposition of ethephon) of citrus tissues are shown in Fig. 1 and Table 1. Maceration activity is given in pectinase units. For the data in the Figs., 2 cm of stem adjacent to the fruit were used (fruiting stem). The button tissue (point of attachment of stem to fruit) is made up of a small amount of stem tissue, calyx remnants, and rind (albedo and flavedo). Amounts of maceration activity found in non-fruiting stem bark, stem wood, and in rind albedo and flavedo are given in Table 1, along with data for an interaction of temp and ethylene on fruit-button and fruit-stem tissue. After ethephon injection, the fruits were held at 25°C or 38°C until assayed.

Maceration activity response to 3 concn of injected ethephon is indicated for 2 assay times in Fig. 2. Each 10-fold increase in the concn of injected ethephon within that concn range leads to a corresponding increase in ethylene evolution (Palmer and Rogers, unpublished data).

From the data of Figs. 1 and 2, it appears that for the fruit button the increase in maceration activity is correlated with time after ethephon treatment and with the concn of ethephon injected. The results are similar to those for ethylene-mediated increases in cellulase activity in citrus leaf explant abscission zones reported by Ratner et al. (17). The small maceration response in fruiting stem tissue appears to be analogous to the cellulase response in non-abscission zone tissue (1, 8, 17). Maceration activity in the non-fruiting stem sections (Table 1) is confined mostly to the bark, and in this tissue activity declined in the presence of ethylene. Rind tissues showed low levels of maceration activity and little response to ethylene. The citrus rind apparently contains an inhibitor of maceration activity. Addition of rind extract to fungal pectinase caused a 60% decrease in measurable activity. Stem tissue had no inhibitory effect.

Data on the effect of temp on the ethylene-mediated increase in maceration activity are given in Table 1. The substantial rise is probably a multiple response to temp increases. Decomposition of ethephon proceeds more rapidly as the temp rises (Rogers, unpublished data), and presumably ethylene diffusion and protein synthesis also increase.

*Effect of cycloheximide on maceration activity.* 'Valencia' fruits were treated by injection with ethephon at the blossom end at zero time and with cycloheximide (0.5 ml, 4 x 10⁻³ M) just below the button at zero time and at intervals thereafter. Maceration activity was assayed at 48 hr (Fig. 3). Cycloheximide applied before 10 hr completely inhibited the rise in maceration activity, but thereafter applications decreased in effectiveness. A similar curve for citrus leaf explant cellulase was found by Ratner et al. (17).
As indicated by Abeles (1), the almost complete inhibition which resulted from early applications of cycloheximide suggests de novo synthesis of the macerating factor, but a rigorous proof requires further work. This proof was supplied for bean cellulase by Lewis and Varner (11).

![Graph](attachment:graph.png)

**Fig. 3.** Effect of cycloheximide on the rise in maceration activity following ethephon injection. Sampling as for Fig. 1. All samples were assayed at 48 hr after ethephon applied. Cycloheximide was injected at times given on the graph. Tissues for data at 48 hr received no cycloheximide.

**Characteristics of the macerating factor.** We appear to be dealing with a soluble enzyme system. The first extraction in the cold appeared to be almost complete; further extractions produced no more than 10% (w/w) of the first activity. Addition of the residue back to the extract did not increase the measurable activity. Lewis and Varner (11) reported recovery of 2 cellulases in bean leaf explants, a soluble fraction extractable in dilute buffer, and a particulate fraction extractable in dilute buffer plus 1 M NaCl. We tried the high salt extraction and found a marked interference with the assay used. Pollard and Biggs used a similar concn of sodium chloride, at pH 8.2, in extracting citrus abscission zone tissue and found essentially no cellulase activity. They employed sodium polypectate as a model substrate (15).

Addition of Ca or Mg salts to the incubation medium had little effect on activity, while addition of EDTA increased activity. Activity was eliminated completely when the substrate equilibrium solution produced substantial increases in activity. It appears that the Ca status of the substrate is of primary importance in the assay used.

It is impossible at this time to identify the enzyme(s) involved in maceration. The fact that added fungal cellulase did not alter maceration activity, and was ineffectual alone is in accord with the findings of plant pathologists that cellulases are not important to maceration (5). Although the maceration factor did not alter the viscosity of polypectate solutions, maceration activity on the tissue was inhibited by Ca ions. This inhibition is a characteristic of certain pectinases. Of the possible pectinases (exo- and endo-polygalacturonases and exo- and endo-transeliminases, according to Bateman and Millar), the exo-pectinases may be tentatively ruled out on the basis that no exo-enzymes have been found to be involved in maceration (5). Pectinases with transeliminase activity produce an unsaturated galacturonate moiety which absorbs maximally at 235 mμ (pectin substrate) or 230 mμ (pectic acid substrate), and which reacts with thiobarbituric acid to yield a product which absorbs maximally at 548 mμ. The incubation medium under our conditions did show a peak at 220 mμ. Reaction with thiobarbituric acid did not yield a chromogen. Albersheim reported that laype action on pectins in the presence of 2, 4-D produced a reaction product which inhibited degradation of the pectin (3). We could find no 2, 4-D-mediated inhibitory reaction for our maceration factor.

On a tentative basis it seems that an endo-polygalacturonase comprises a part of the maceration factor. Morre’ (13) also concluded from his tests that the bean pectinase which he measured was an endo-polygalacturonase. The presence of other enzymes which catalyze polysaccharide hydrolysis can not be excluded.

**Literature Cited**