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Changes in Cellulase and Pectinase Activities in Fruit Tissues and Separation Zones of Citrus Treated with Cycloheximide

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ABSTRACT

Cellulase activity increased in separation-zone tissues 1 day after "Valencia" orange (*Citrus sinensis* [L.] Osbeck) was treated with 20 micrograms per milliliter cycloheximide. Exocellulase was detected only in the separation zones of treated fruit, whereas endocellulase was present in zones from both treated and control fruit. Endocellulase activity in separation-zone tissue of treated fruit was nearly three times as great as that in control tissues. Cellulase activity was restricted to separation-zone tissue. Pectinase and an albedo-macerating factor activity were very low and were not influenced by the treatment. The cycloheximide effect in these experiments was apparently caused by ethylene produced by wound tissue.

Ethylene regulates enzyme synthesis by stimulating RNA and protein synthesis (1). Because of this general effect, several enzymes may be involved in cell loosening during abscission. The anatomical changes in the separation zones during abscission suggest that complex enzyme reactions take place (10, 12). Pectinase enzymes in bean (7, 13) and an albedo-macerating factor in citrus (11) apparently increase during separation-zone development. However, others (4, 8) have been able to relate only cellulase activity with the separation process. Ethylene increases the secretion of cellulase (exocellulase) from the cells as well as increases its synthesis (endocellulase) in the cells of some plants (3, 4).

Cellulase activity in the separation zones of citrus leaves and fruit is stimulated by ethylene (1, 2), and the abscission of citrus fruit is related to the level of ethylene in the fruit's internal atmosphere (5, 6, 9). Cycloheximide stimulates ethylene production in citrus fruit, which in turn promotes cellulase activity that causes loosening (6, 9).

This study was done to measure the effect of ethylene on endocellulase and exocellulase and to determine whether or not cellulase is the primary enzyme in and specific to the abscission process in citrus fruit.

MATERIALS AND METHODS

"Valencia" orange (*Citrus sinensis* [L.] Osbeck) trees were sprayed with a H_2O solution of 20 μg/ml cycloheximide containing 0.01% Triton X-100.[1] After 6, 24, 72, and 120 hr, fruit was sampled for ethylene, cellulase, pectinase, and the albedo-macerating factor.

Ethylene (9), pectinase (8), and the albedo-macerating factor (11) were measured by described methods. The albedo discs used in the AMF[2] analysis were cut from mature pummelos (*Citrus grandis* [L.] Osbeck) with a cork borer. Pectinase levels were determined by measuring viscosity change of sodium polypectate after incubation with the enzyme brei for 6 hr at 30 C.

Exocellulase was extracted from 2-mm sections of the separation zones. After vacuum infiltration of the sections with H_2O, they were placed in needle syringes in centrifuge tubes and centrifuged 10 min at 3000 rpm. The centrifugate was made up to 5 ml with pH 7.0 phosphate buffer. One ml of this enzyme extract was incubated 6 hr with 1.5% carboxymethyl cellulose at 40 C. Viscosity was determined in a microviscometer (9). The tissue from the separation zones was then homogenized in a blender with pH 7.0 phosphate buffer. Cellulase activity was determined by described methods (9).

Five days after treatment of fruits with 20 μg/ml cycloheximide, endocellulase activity in 10 g fresh weight of flavedo, albedo, and fruit stems, 5 g of leaf petiole tissue, and 20 leaf separation zones was determined by methods similar to those for cellulase in fruit separation zones.

RESULTS AND DISCUSSION

Exocellulase. Exocellulase was not detected in the separation zones from nontreated fruit, as measured by the viscosity changes of CMC (Fig. 1). The ethylene level in the internal atmosphere averaged 0.1 μg/ml in these fruit. Less than 0.1 μg/ml causes very little loosening of fruit and apparently stimulates very little enzyme activity.

In 6 hr the ethylene in treated fruit was 0.25 μg/ml, and the exocellulase activity increased markedly. The viscosity of the CMC was lowered 22% by the exocellulase in 6-hr samples and 30% in the 24-hr samples. The exocellulase secretion rate then decreased, since the 120-hr sample preparation lowered the viscosity by only 50% (Fig. 1). The important point is the rapidity with which ethylene affects the secretion of cellulase. It seems that in this case ethylene is influencing the permeability of the plasma membrane. With these samples it was not possible to withdraw ethylene after a period of time to see if secretion of cellulase would decrease, as others have done with various explants (3).

Ethylene increased up to nearly 1.0 μg/ml in the internal atmosphere of the fruit by 5 days. Under usual conditions, the attachment force would have been reduced from 6.8 to 9.2 to 2.7 to 4.2 kg.

[1] Mention of a trademark name or a proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

[2] Abbreviations: AMF: albedo-macerating factor; CMC: carboxymethyl cellulose.

Endocellulase. The separation-zone tissue of nontreated fruit contained a small amount of endocellulase, as measured by viscosity changes of CMC (Fig. 2). The endocellulase in the separation-zone tissue of nontreated fruit reduced the viscosity of the CMC from 14 to 22%. Ethylene in these fruit was less than 0.1 μg/ml (Fig. 2). Attachment of citrus fruit in many cases decreases gradually as the fruit matures. This may result from the small amounts of endocellulase found in the separation-zone cells. Also, a number of seasonal change studies indicate that cellulase is a causal factor for the gradual loosening of citrus fruit. Only total cellulase was measured in these samples (unpublished data). Judging from results on nontreated fruit, exocellulase would be too low to measure, but some cellulase must be secreted through the membrane to the cell wall for degradation to take place.

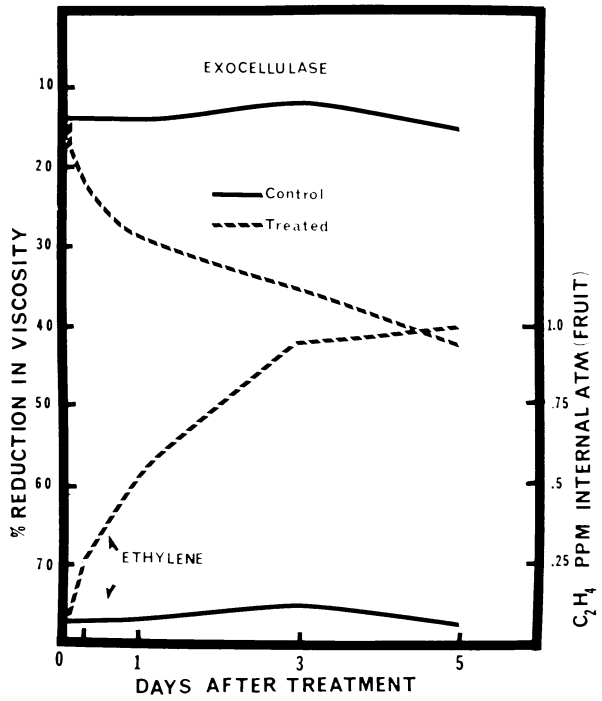

FIG. 1. Exocellulase activity of separation-zone tissue and ethylene content of "Valencia" orange fruit sprayed with 20 μg/ml cycloheximide.

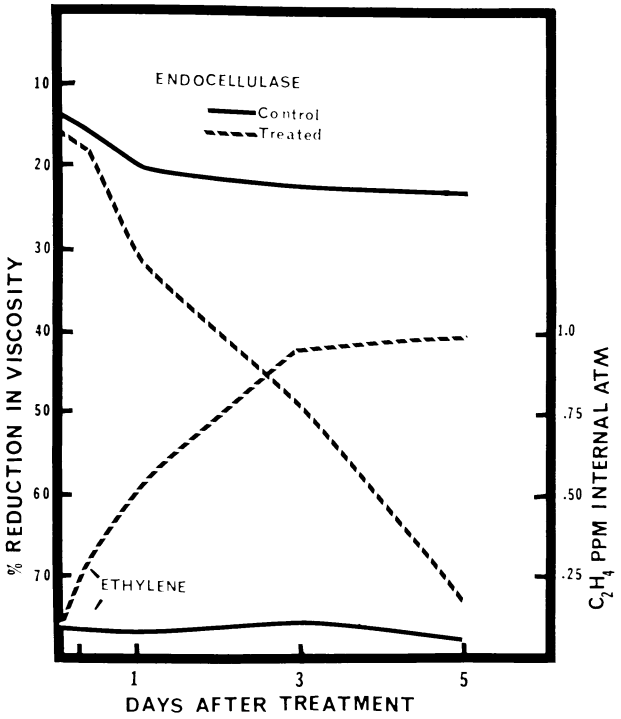

FIG. 2. Endocellulase activity of separation-zone tissue and ethylene content of "Valencia" orange fruit sprayed with 20 μg/ml cycloheximide.

Table I. *Increase in Cellulase Activity in Fruit, Fruit Stems, and Leaf Tissues*

Results are 5 days after a 20-μg/ml cycloheximide spray.

Tissue[1]	Reduction in Viscosity	
	Control	Sprayed
1 g fresh wt	%	
Separation zone (leaf)	4	42
Leaf petiole	<1	2
Separation zone (fruit)	21	74
Flavedo	<1	2
Albedo	2	3
Fruit stem	<1	<1

[1] Discs cut with cork borer as 2-mm sections from separation zones and fruit stems.

Tissue from separation zones of treated fruit contained large amounts of endocellulase. More endocellulase had been synthesized in each successive sample, as measured by viscosity changes of CMC (Fig. 2). The change in endocellulase activity the first 6 hr was less than that in later samples. I think this indicates a time lag for synthesis of cellulase. The endocellulase in the 120-hr samples lowered the viscosity of the CMC 70%. Earlier studies (8, 9) showed increased cellulase activity in separation zones of fruit treated with ethylene-generating chemicals.

Pectinase and Albedo-macerating Factor. No pectinase activity was detected during the sampling period (data not shown). These data agree with earlier data for citrus (8) and cotton and beans (4).

AMF activity was small and not affected by ethylene. The fresh weight of albedo discs decreased 5% when treated with the enzyme from 6-hr samples, but the trend did not continue. The enzyme from the 120-hr samples caused only a 9% reduction in fresh weight of the discs. I doubt that this activity is high enough to cause cell loosening. The lag period for the increase in AMF is too long to implicate ethylene as a factor in its stimulation, especially since cellulase is influenced so much and so quickly by ethylene.

Cellulase Activity in Other Citrus Tissues. If cellulase is specifically involved in dissolution of the cells in the separation zone and stimulated by ethylene, it should not be found in other citrus tissues. This appeared to be the case. Only the tissues from the separation zones of leaves and fruit had cellulase activity (Table I). Ethylene stimulated cellulase formation only in the separation-zone cells. The flavedo and albedo of the rind, petioles, and fruit stem tissue did not contain cellulase, as measured by viscosity of CMC. The 2 to 3% reduction in viscosity after 6-hr incubation is too small to indicate cellulase activity.

General Comments. The AMF in this report was very low and was not influenced by ethylene. The reason for results that contrast with those of others (11) is not known. Possibly the

pummelo albedo in the present tests was too old. However, commercial pectinase did cause a weight reduction of the albedo discs.

The results reported here confirm the conclusions of Abeles *et al.* (3, 4) that ethylene affects cellulase in separation zones in two ways. First, an increase in exocellulase, and second, an increase in endocellulase was measured. Thus, the permeability of the membranes must have changed so that cellulase present in the cell was secreted to the cell walls. This probably happened first, and later new cellulase was synthesized because of the effect of ethylene on protein synthesis (1). Therefore, the results of this study indicate that cellulase is the primary enzyme in and specific to the fruit abscission process in this citrus variety.

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