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Acid Phosphatase Development during Ripening of Avocado[1]

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ABSTRACT

The activity and subcellular distribution of acid phosphatase were assayed during ethylene-induced ripening of whole fruit or thick slices of avocado (*Persea americana* Mill. var. Fuerte and Hass). The activity increased up to 30-fold during ripening in both the supernatant fraction and the Triton X-100 extract of the precipitate of a 30,000*g* centrifugation of tissue homogenates from whole fruit or slices ripening in moist air. Enzyme activity in the residual precipitate after Triton extraction remained constant. The development of acid phosphatase in thick slices ripened in moist air was similar to that in intact fruit, except that enzyme development and ripening were accelerated about 24 hours in the slices. The increase in enzyme activity that occurs in slices ripening in moist air was inhibited when tissue sections were infiltrated with solutions, by aspiration for 2 minutes or by soaking for 2 hours, anytime 22 hours or more after addition of ethylene. This inhibition was independent of the presence or absence of cycloheximide or sucrose (0.3–0.5M). However, the large decline in enzyme activity in the presence of cycloheximide, as compared with the controls, indicated that synthesis of acid phosphatase was occurring at all stages of ripening.

Ripening of fleshy fruits is an irreversible process of senescence (1, 6, 8, 16–18) that is dependent on synthesis of both RNA and protein (2, 8, 15). Although there is no consistent correlation among different kinds of fruit between changes in the levels of total RNA and protein and the ripening process, there is ample evidence for an increase in activity of many enzymes during ripening (12, 14, 19). It has been suggested that the enhanced rate of incorporation of labeled precursors into total protein during the early phase of the climacteric is involved in synthesis of enzymes which catalyze ripening (2, 9, 15). Although data on incorporation of amino acids in avocado (15) and banana (2) indicate that the capacity for protein synthesis diminishes greatly by the middle of the climacteric rise in respiration, most studies of enzymes show that the increase in enzyme activity continues throughout the entire period of ripening. This report is concerned with the development and subcellular distribution of acid phosphatase throughout the ripening period of 'Fuerte' and 'Hass' avocados, and attempts to determine whether or not increased activity of acid phosphatase during ripening is attributable to enzyme synthesis.

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MATERIALS AND METHODS

Avocados (*Persea americana* Mill. var. Fuerte or Hass) were treated with 300 μl/l of ethylene for 16 hr and then allowed to ripen at room temperature (22 C), in a large desiccator, at high relative humidity, with an opening to allow for gas exchange. Alternatively, 12-mm thick, cross-sectional slices from the midregion of avocados were similarily treated with ethylene. After cutting, the slices were rinsed briefly in running tap water, blotted, and then stored in a humidity chamber until assayed.

Enzyme Extraction. For assay of acid phosphatase, duplicate samples (500 mg fresh weight) of inner mesocarp tissue were taken daily from the midregion of different intact fruits. Most experiments, however, were conducted by aging thick (12 mm) slices and similarly taking daily tissue samples from the slices, after first removing about 2 mm from the outer surfaces. Tissue samples were homogenized in 10 ml of pH 5, 0.1 M acetate buffer containing 7% carbowax 4000, and were centrifuged at 2 C for 15 min at 15,000*g*. Carbowax was added to prevent inactivation of enzyme by tannins (20). The supernatant fraction was used directly as a source of soluble enzyme. The precipitate was washed once in 10 ml of grinding medium by centrifugation as above, and the supernatant fraction was discarded. The precipitate was resuspended with gentle stirring in 10 ml of grinding medium containing 1% Triton X-100, and was centrifuged similarly; the supernatant fraction is referred to as the Triton extract. The residual precipitate was also retained for assay of acid phosphatase in standard reaction mixtures.

Enzyme Assay. Reaction mixtures (5-ml volume) containing 0.1 M acetate buffer, (pH 5), enzyme, and 0.8 mg of *p*-nitrophenylphosphate were incubated at 40 C. One-ml aliquots were removed at zero time and at various intervals, and were added to 3 ml of 0.2 N NaOH to stop the reaction. Absorbance was read at 400 nm. Measurements of enzyme activity were determined during the linear phase. Inorganic phosphate (Table I) was assayed by a modification of the method of Fiske and Subbarow (7).

Effect of Cycloheximide on Acid Phosphatase Activity. Thick slices (12 mm) of the entire cross section from the midregion of 'Hass' and 'Fuerte' avocados were induced to ripen with 300 μl/l of ethylene for 16 hr, then were stored at 20 C in moist air. The effect of cycloheximide was assayed at various periods of ripening. Sections about 6 mm thick were cut from the center of the slices, and 10 μg/ml solutions of cycloheximide were administered by aspiration for 2 min at about 475-mm pressure or by partial immersion in the solutions for 2 hr. The sections were then blotted and maintained in moist air for 5.5 hr. Control tissue was similarly treated without cycloheximide, but with 25 μg/ml streptomycin sulfate as a

bactericidal agent. In most assays, sucrose (0.3–0.5 M) was added as an osmoticum. Acid phosphatase was assayed at zero time and after 5.5 hr in control or cycloheximide solutions. This time was selected to provide a period long enough for an increase in enzyme activity that could be measured accurately (Fig. 2).

RESULTS AND DISCUSSION

Comparison of Development of Acid Phosphatase in Intact Fruits and Slices. Four intact fruits and thick slices from one 'Fuerte' avocado were treated with 300 μl/l of ethylene for 16 hr to induce ripening. In tissue slices, the development of both soluble and Triton extractable acid phosphatase begins during the initial 24 hr after addition of ethylene, and the rate becomes greatly accelerated between 48 and 72 hr (Fig. 1). The development of acid phosphatase is more rapid during the first 72 hr in slices aged in moist air than in whole fruit, which agrees with the observation that the ripening period, as measured by softening, is completed approximately 24 hr earlier in slices than in whole fruit. Notwithstanding this difference in the lengths of the ripening period, it is concluded that similarities in the development of acid phosphatase, softening, and normal flavor characteristics of both whole fruit and thick slices indicate that the slice method is applicable for study of avocado ripening, as has been demonstrated for bananas (3, 13). The pattern of development of both soluble and Triton extractable acid phosphatase in avocado is essentially the same as during ripening of banana (3). Substrate specificities of avocado acid phosphatase (Table I) are also quite similar to those of the acid phosphatases that develop during senescence of banana pulp (3) and *Rhoeo* leaf tissues (4), in that for the three tissues the relative affinities for substrates are as follows: ATP, UTP, and ADP > *p*-nitrophenylphosphate, glucose-6-P, and glycero-P > phosphorylcholine and phosphorylethanol amine.

Acid Phosphatase Distribution in Subcellular Fractions from the Midregion of Avocado. Duplicate tissue samples were removed daily during the ripening period from midregional slices of avocado which were induced to ripen by 300 μl/l of ethylene. The tissue homogenates were fractionated by differential centrifugation successively at 1,000, 12,000, and 30,000*g*. After each centrifugation, the activity of acid phosphatase was measured in the supernatant fraction, in a Triton

Table I. *Relative Rates of Hydrolysis of Phosphate Esters by Acid Phosphatase from Ripe Avocado*

Inorganic phosphate was assayed by a modified Fiske-Subbarow method (7); *p*-nitrophenylphosphate was assayed as described under "Materials and Methods." Standard curves were prepared for conversion of absorbance to μmoles hydrolyzed. All substrates were 2 μmoles per reaction mixture.

Substrate	Hydrolysis	
	Soluble enzyme	Triton extract of precipitate
	μmoles/15 min·g fresh wt	
Glucose-6-P	10.0	8.0
ATP	51.3	51.3
UTP	50.3	59.2
ADP	51.3	26.3
β-Glycerol-P	15.0	8.3
p-Nitrophenyl-P	19.5	9.5
Phosphorylcholine	1.3	7.5

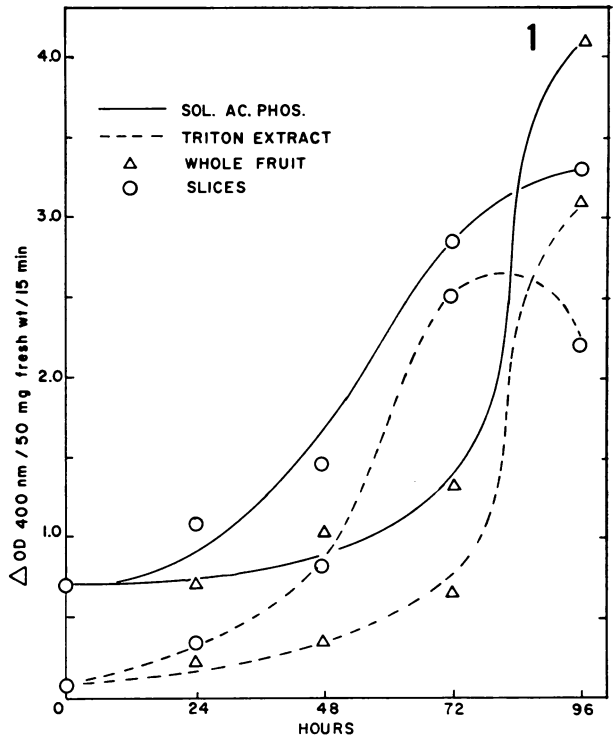

FIG. 1. Comparative development of acid phosphatase during ethylene-induced ripening of whole fruits and thick slices of 'Fuerte' avocado. Duplicate tissue samples were removed at 24-hr intervals from whole fruit and slices, and acid phosphatase was extracted and assayed as described under "Materials and Methods."

Table II. *Distribution of Acid Phosphatase in Subcellular Fractions of Tissue Slices from the Midregion of Avocado during Ethylene-induced Ripening*

The data are means and SE values for duplicate samples of tissue.

Subcellular Fractions	Specific Activity of Phosphatase		
	Zero time	24 hr	48 hr
	ΔA 400/50 mg fresh wt·15 min		
Supernatant fraction 30,000*g*	1.06 ± 0.19	2.20 ± 0.01	2.60
Triton extract of precipitate			
1,000*g*	0.24	0.55	0.90
12,000*g*	0.08	0.17	0.28
30,000*g*	0.05	0.06	0.01
Total	0.37 ± 0.01	0.78 ± 0.07	1.19 ± 0.07
Residual precipitate			
1,000*g*	1.04	1.22	0.89
12,000*g*	0.08	0.09	0.20
30,000*g*	0.03	0.01	0.12
Total	1.15 ± 0.06	1.32 ± 0.07	1.21 ± 0.02
Grand total	2.58 ± 0.20	4.30 ± 0.07	5.00 ± 0.05

X-100 extract of the precipitate, and in the residual precipitate after extraction with Triton. The results are shown in Table II. There was a 2.5-fold increase in the soluble enzyme and a 3.2-fold increase in the Triton extract of the precipitates, although the activity of the residual precipitate remained essentially constant. From analysis of the data in Table II, it can

Table III. *Effect of Cycloheximide on Development of Acid Phosphatase in Hass and Fuerte Avocado Slices*

Thick slices were ripened with ethylene in moist air at 20 C. At times indicated, 6-mm thick sections were cut from the center of the slices and control or 10 μg/ml cycloheximide solutions with and without sucrose as an osmoticum were administered by aspiration for 2 min or partial immersion in the solutions for 2 hr. Control solutions contained 25 μg/ml streptomycin sulfate as a bactericide. The data are means and SE values for duplicate or triplicate samples of tissue.

Experiment No.	Date	Avocado Variety	Time Lapse after Adding Ethylene	Sucrose Concn and Infiltration Method	Treatment		
					Zerotime	5.5 hr (controls)	5.5 hr (cycloheximide)
					A_{400}/50 mg fresh wt tissue·15 min		
I	June 26	'Hass'	22 hr	None; 2 min aspiration	0.34 ± 0.20	0.26 ± 0.10	0.21 ± 0.05
II	July 30	'Hass'	60 hr	0.4 M; 2 min aspiration	2.70 ± 0.10	2.30 ± 0.03	1.80 ± 0.12
III	August 22	'Hass'	40 hr	0.5 M; soaking for 2 hr	0.93 ± 0.04	0.65 ± 0.01	0.55 ± 0.00
IV	December 5	'Fuerte'	24 hr	0.3 M; soaking for 2 hr	0.40 ± 0.02	0.42 ± 0.00	0.28 ± 0.00
V	December 10	'Fuerte'	72 hr	0.3 M; 2 min aspiration	1.57 ± 0.00	0.80 ± 0.07	0.65 ± 0.05

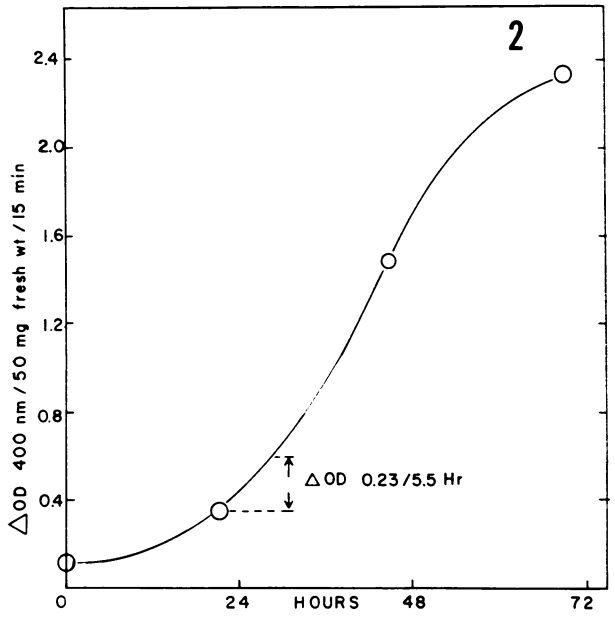

FIG. 2. The development of acid phosphatase during ethylene-induced ripening of thick slices of 'Hass' avocado. Triplicate samples of tissue were removed at zero time and daily intervals for assay of activity of acid phosphatase in a Triton X-100 extract of a 15,000*g* precipitate fraction of tissue homogenates. At 22 hr, samples of tissues were also prepared for study of the effect of cycloheximide on the activity of acid phosphatase during a 5.5-hr period, the results of which are shown under experiment I, in Table III.

be demonstrated that the percentage of the total activity in each of the fractions of the Triton extract and residual precipitate is remarkably constant during ripening. About 75% of the bound enzyme occurs in the heavy (1,000*g* precipitate) fraction, which is very heterogenous and includes cell wall debris, nuclei, plastids, and any membrane material trapped in or attached to the wall debris. It is concluded that the increases in acid phosphatase activity in the soluble fraction and Triton extracts are real, and are not attributable to a redistribution of enzyme among different subcellular fractions as a result of the ripening process or extraction procedures.

Effect of Cycloheximide on Acid Phosphatase Activity. Several experiments were conducted on the effects of cycloheximide on the development of acid phosphatase activity in both the soluble fraction and the Triton X-100 extract of the 15,000*g* precipitate fraction of tissue homogenates. Because of the similarity of response, only the results of the Triton extract are reported. In the first experiment, cycloheximide was administered by aspiration in absence of an osmoticum in the medium. As shown in Table III (experiment I) there was a decline in enzyme activity of 23 and 38% in the control and cycloheximide-treated tissues, respectively, during the 5.5 hr period. In contrast, the thick slices from which the 6-mm thick sections were removed showed a substantial increase (ΔA = 0.23/5.5 hr) in enzyme activity (Fig. 2). Thus, it appeared that the lack of an increase in activity during the assays for the effect of cycloheximide was attributable to the effect of solution hypotonicity, or the aspiration procedure, or both.

In four subsequent experiments with 'Hass' and 'Fuerte' avocados, 0.3 to 0.5 M sucrose was included in the medium which was administered to the sections either by aspiration for 2 min or by partial immersion of the sections for 2 hr. From these results (Table III) it is clear that none of the treatments enable the increase in acid phosphatase activity that occurs in tissue slices maintained in moist air. The fact that in all experiments there was either a loss of acid phosphatase activity or a failure of the normal increase in activity during the 5.5-hr period may be construed to indicate that all the infiltration treatments caused severe impairment of the tissue's capacity for enzyme synthesis.

In all five experiments (Table III) there was a substantially greater loss of acid phosphatase activity in the presence of cycloheximide than in the controls, thus indicating that synthesis of the enzyme is going on at all stages of ripening. Inasmuch as cycloheximide is a potent inhibitor of protein synthesis in plants (3–5, 11), the greater loss of enzyme activity in the presence of cycloheximide is probably attributable to inhibition of synthesis, resulting in the predominance of acid phosphatase degradation. It is apparent that the enzyme(s) involved in degradation have a low enough rate of turnover that their level is not significantly affected by cycloheximide over the 5.5-hr period. Half-times for enzyme degradation in presence of inhibitors of protein synthesis have been determined over periods of 6 hr or less for tryptophan pyrrolase in rat liver (10), invertase in sugar cane (11), and ribonuclease in bean endocarp and turnip root tissues (Sacher, unpublished results). Since the kinetics of enzyme degradation are first order and yield linear semilogarithmic plots, an analysis, based on the mean of plots of data from five experiments (Table III) on acid phosphatase activity at zero time and 5.5 hr later in the

presence of cycloheximide, should provide a good estimation of the half-time for degradation of acid phosphatase in avocado tissue during the ripening period. The mean half-time was calculated to be 8.3 ± 1 hr for the five experiments. If experiment V ($T_{0.5}$ = 4.7 hr) was excluded, the mean for experiments I through IV was 7.8 ± 0.5 hr. The latter value is probably characteristic of most of the ripening period.

From other studies, however, it is clear that the sensitivity of avocado fruit tissue to infiltration of liquid develops only after ripening is initiated, either naturally or induced by addition of ethylene. It was demonstrated that vacuum infiltration of isotonic medium into thick slices of preclimacteric avocados has no adverse effect on the development of acid phosphatase, softening, or other characteristics of ripening during the subsequent 72- to 96-hr ripening period (C. Grant and J. A. Sacher, unpublished results). The data in the present report, in contrast, refer to adverse effects on enzyme synthesis that occur as a result of infiltration of liquid into avocado tissue 22 or more hr after ripening was induced by ethylene.

Addition of cycloheximide to preclimacteric avocado slices would probably prevent ripening, as has been shown for pear (8), by inhibiting synthesis of one or more enzymes with a high rate of turnover; this could be rate limiting to ripening, but would not shed light on the role of protein synthesis in the development of acid phosphatase activity.

Pulp tissue from other fruits may not be adversely affected by infiltration of liquids. Vacuum infiltration of isotonic mannitol solutions into pear fruits during the preclimacteric period or during the middle of the climacteric rise in respiration did not impede ripening or protein synthesis, whereas H_2O-infiltrated fruits did not ripen (9). In contrast, 3-mm thick slices from banana during the middle of the climacteric rise showed a normal rate of increase in acid phosphatase, when soaked for 1 hr in H_2O and then maintained on damp paper for 5 hr; this increase was inhibited completely in slices similarly treated with dilute solutions of cycloheximide or actinomycin D (3). From these few studies it is evident that the biosynthesis metabolism of fruits may vary greatly in their response to infiltration of liquids, a method that is used with both intact fruits and slices to infiltrate materials (isotopes, inhibitors, or potential ripening regulators) in order to study synthesis of protein or RNA, or to ascertain their effects on some other aspect of ripening.

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