Plant Physiol. (1970) 46, 208–211

Senescence

ASSOCIATION OF SYNTHESIS OF ACID PHOSPHATASE WITH BANANA RIPENING[1]

Received for publication February 27, 1970

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ABSTRACT

During ripening of banana (*Musa sapientum* L., var. Gros Michel or Valery) acid phosphatase activity increases 13- to 26-fold in the precipitate and 2- to 4-fold in the supernatant fraction of tissue homogenates. These increases are closely correlated with the onset and peak of the climacteric. The precipitate enzyme may be extracted with Triton X-100, $CaCl_2$ or NaCl; about 80% of it is in a 500*g* precipitate. Studies on effect of tonicity of the grinding medium indicate that the precipitate enzyme is desorbed from membrane or cell wall surfaces, and is not released as a result of lysis of membranes. The development of acid phosphatase during aging of tissue slices is the same as in intact fruit. Short term studies of tissue slices with cycloheximide and actinomycin D indicate that the increase in activity is owed to new enzyme synthesis, which is dependent upon synthesis of RNA. The possible effects of the increase in acid phosphatase on ripening are discussed.

Regardless of whether the amount of total protein increases (4), remains constant (12), or decreases (15) during ripening of climacteric fruits, there is considerable evidence of increased activity of specific enzymes during this period. Increased activity of malic enzyme has been demonstrated in apples (6) and pears (2), and chlorophyllase, in apple and banana (5). Also a 20-fold increase in fructose diphosphate during ripening of banana has been attributed to an activation of phosphofructokinase (1). In this paper we report on a large increase in synthesis of acid phosphatase attending the respiratory climacteric of ripening banana.

MATERIALS AND METHODS

Bananas (*Musa sapientum* L., var. Gros Michel or Valery) were ripened at 19 C naturally or were induced to ripen by soaking whole fruits in a solution of 1000 μl/liter of Ethrel (2-chloroethyl phosphonic acid) containing 0.1% Triton X-100 for 1 hr, or by application of 100 μl/liter of ethylene overnight. The rate of respiration of individual fruits was followed by using a Beckman model 15A infrared CO_2 analyzer. For enzyme assay samples of banana pulp tissue (1 g fresh weight) were ground in a Potter-Elvehjem homogenizer in 10 ml of 0.1 M sodium acetate buffer, pH 5.0, containing 7% Carbowax 4000 (mol wt, ca. 3350) to prevent inactivation of enzymes by leukoanthocyanins (17). The homogenate was centrifuged at 30,000*g* for 30 min at 2 C, and the supernatant fraction was decanted and used as an enzyme source. The precipitate, after washing in grinding medium, was extracted with 10 ml of grinding medium containing 1.0% Triton X-100, was centrifuged at 30,000*g* for 20 min at 2 C, and the supernatant fraction was used as an enzyme source.

Enzyme reaction mixtures, containing 0.3 mg of *p*-nitrophenyl phosphate and enzyme (100 mg fresh weight equivalent) in 0.1 M acetate buffer, pH 5.0, to a final volume of 5 ml, were incubated in a shaker at 40 C. One-milliliter volumes of the reaction mixture were removed at intervals, then added to 3 ml of 0.168 N NaOH and shaken, and the absorbance was determined at 400 nm after 15 min. All determinations of enzyme activity were based on measurements of the zero order rate.

The development of activity of acid phosphatase was studied also during aging of banana sections *in vitro*, by using methods of aging tissue sections similar to those reported by others (7). Green bananas were surface-sterilized with 0.5% sodium hypochlorite for 15 min and cross-sections were cut 3 to 5 mm thick with peel attached, were washed, blotted and aged in Petri dishes either on glass or a 9-cm Whatman No. 1 filter paper wetted with 0.5 ml of 0.4 mM $CaSO_4$. The dishes were stored in a large desiccator, with the bottom and the walls lined with wet filter paper, and ethylene added to 45 μl/liter. Treatment with actinomycin D or cycloheximide was done by soaking the sections in a solution for 1 hr to saturate the free space, after which they were blotted gently and placed in Petri dishes.

RESULTS AND DISCUSSION

Distribution of Acid Phosphatase. A comparison of the distribution and activity of acid phosphatase in green and ripe bananas is shown in Table I. During ripening, there occurred a 59% increase in the activity of acid phosphatase in the 30,000*g* supernatant fraction, which was not affected by the addition of 1.0% Triton X-100. The precipitate fraction was washed with 10 ml of the grinding medium and then centrifuged, and the supernatant fraction was discarded. A subsequent extraction of the precipitate with grinding medium yielded no measurable enzyme activity in presence of substrate, thus indicating that all residual supernatant enzyme was removed. In contrast, a Triton X-100 extraction of the washed precipitates from both green and ripe bananas showed considerable enzyme activity (Table I), with 7 times more acid phosphatase activity in ripe than in green bananas. Most (ca 70%) of the Triton-extractable enzyme activity was demonstrated when the precipitate was incubated in the enzyme assay medium. For convenience, however, all assays of the precipitate-bound enzyme were made with Triton X-100 extracts.

[1] This investigation supported by Grant GB-8316 from the National Science Foundation.

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Approximately 80% of the precipitate-bound enzyme is recovered in a Triton X-100 extraction of a 500*g* precipitate; the remaining 20% is found in a 12,000*g* precipitate of the 500*g* supernatant fraction (Table II). Thus, most of the precipitate-bound enzyme is associated with a heavy fraction that contains cell wall debris, starch grains, large organelles such as nuclei and plastids, as well as the portion of the plasma membrane, endoplasmic reticulum, and smaller particles that get trapped in the cell wall debris. The most active lysosomal particles, in contrast, are obtained from a light mitochondrial fraction (9). Ultrastructural studies of cells from carrot cultures show acid phosphatase localized in the cell wall region (3). Others find that in fresh carrot root sections, acid phosphatase is uniformly distributed in the plasma membrane and in what appear to be plasmodesmata (S. Poolsawat and S. M. Caplin, personal communication).

Since the precipitate from a hypotonic (ca. 0.12 M) grinding medium subsequently yields a large amount of acid phosphatase activity when extracted with Triton X-100, it appears that the precipitate enzyme was not from cell particles enclosed by a membrane. This view was supported by the fact that there is no difference in distribution of enzyme between the precipitate and supernatant fractions, whether ripe banana tissue is ground in a medium containing 0.1 M acetate buffer plus 0.3 M sucrose, or in 0.1 M or 0.025 M acetate buffer, all with Carbowax 4000 (0.02 M). Were the enzyme in a membrane-enclosed organelle, a greater amount of enzyme activity would be expected in the supernatant fraction derived from a hypotonic grinding medium. Also, the enzyme may be extracted from the precipitate fraction with NaCl or $CaCl_2$. In fact, with 0.2 M $CaCl_2$ the amount of enzyme extracted from the precipitate is essentially the same as with 1% Triton X-100 (Table III). These results may be construed to indicate that the acid phosphatase extracted from the precipitate with a solution of Triton X-100 or salt is adsorbed to membrane or cell wall surfaces by weak bonding, rather than being released from a membrane-enclosed particle.

Acid Phosphatase Activity and the Respiratory Climacteric. The relationship between the increase in acid phosphatase and the respiratory climacteric was determined both for Gros Michel bananas ripening normally and for Valery bananas induced to ripen by application of Ethrel, which breaks down to yield ethylene (16). Figure 1 shows acid phosphatase activity and the Q_{CO_2} for Gros Michel bananas. Figure 2 illustrates the results of similar assays of Valery bananas, which were induced to ripen by soaking whole fruits in a solution of Ethrel (4.16 ml/liter) containing 0.1% Triton X-100 to facilitate penetration. The results of these and other experiments demonstrate that the initial level of acid phosphatase in the supernatant fraction of green banana tissue is greater than that of the precipitate fraction. Both the rate of increase in, and ultimate level of enzyme activity during the climacteric, however, are substantially greater in the precipitate fraction than in the supernatant fraction. In the experiment shown in Figure 2 there is a 26-fold increase in acid phosphatase in the precipitate and a 3.5-fold increase in the supernatant fraction.

Table I. *Distribution and Activity of Acid Phosphatase in Green and Ripe Bananas*

Duplicate 1-g fresh weight samples of tissue from green and ripe bananas were ground in 10 ml of 0.1 M acetate buffer, pH 5.0, containing 7% Carbowax 4000. The homogenate was centrifuged at 30,000*g* for 30 min at 1 C. Acid phosphatase was assayed in the supernatant fraction, in presence and absence of 1% Triton X-100. The precipitate, after being washed once with grinding medium, was extracted with 10 ml of grinding medium, with and without 1% Triton X-100.

Enzyme Source	*A*, at 400 nm per 15 min per 20 mg fresh wt[1]	
	Green banana	Ripe banana
SN[2]	0.220	0.350
SN + Triton	0.215	0.380
Ppt extracted with grinding medium	0.000	0.000
Ppt extracted with grinding medium + Triton	0.127	0.855

[1] For Tables I to IV, $A \times 0.20 = \mu$moles of *p*-nitrophenyl phosphate hydrolyzed per 15 min per 20 mg fresh weight.

[2] SN: Supernatant fraction.

Table II. *Distribution of Acid Phosphatase in Ripe Bananas*

Procedures are as described in Table I, except for the centrifugation procedures.

	A at 400 nm per 15 min per 20 mg fresh wt
500*g* SN[1]	0.315
500*g* ppt + Triton	0.398
12,000*g* SN	0.220
500–12,000*g* ppt + Triton	0.086
30,000*g* SN	0.205
12,000–30,000*g* ppt + Triton	0.000

[1] SN: Supernatant fraction.

Table III. *Effects of NaCl and $CaCl_2$ on Extraction of Acid Phosphatase from the Precipitate of a Tissue Homogenate*

Samples (1-g fresh weight) of banana tissue were ground in 10 ml of 0.1 M acetate buffer, pH 5.0, containing 7% Carbowax 4000 and were centrifuged at 12,000*g* for 15 min. The supernatant fractions were discarded. The precipitates were washed and then extracted with 10 ml of grinding medium, and also with grinding medium plus the addition of NaCl, $CaCl_2$, or Triton X-100, and the supernatant fraction was used as the enzyme source.

Extraction of Precipitate	*A* at 400 nm per 15 min per 20 mg fresh wt
Grinding medium	0.000
+ 0.2 M NaCl	0.125
+ 0.2 M $CaCl_2$	0.145
+ 1% Triton X-100	0.165

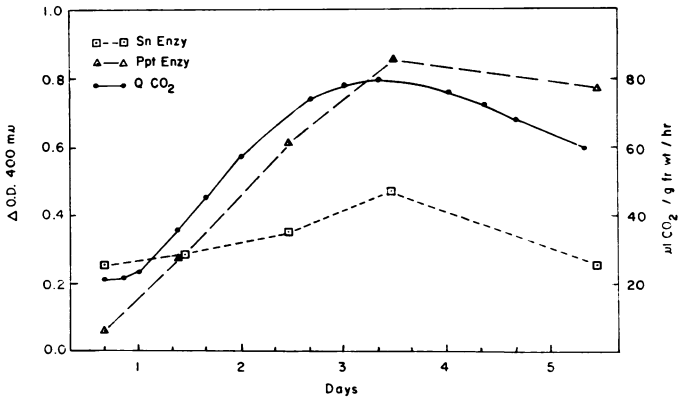

FIG. 1. Relationship between the respiratory climacteric and the development of acid phosphatase activity in the supernatant (Sn) and precipitate (Ppt) enzyme (Enzy) fractions from Gros Michel bananas.

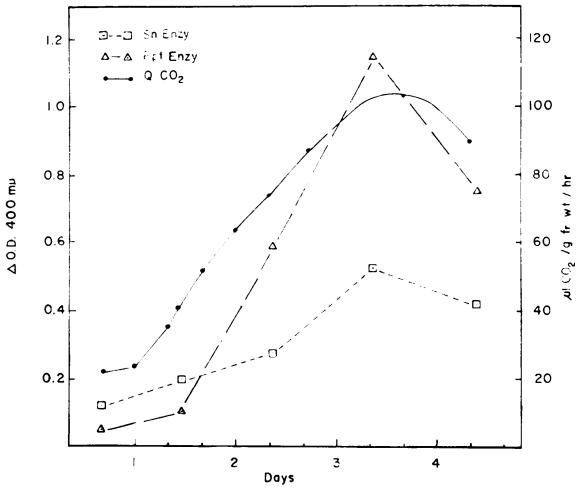

FIG. 2. Same as Figure 1, but for Valery bananas induced to ripen by application of Ethrel.

The results of these two experiments demonstrate a very close correlation between the onset and peak of both the increase in acid phosphatase activity and CO_2 evolution, and in the relative magnitude and rate of increase of acid phosphatase and CO_2 evolution, in that the larger increase in respiration rate (Fig. 2) is associated with the larger increase in enzyme activity. Also, the more rapid rate of postclimacteric decline in acid phosphatase is associated with the more rapid rate of decline in CO_2 evolution.

The increase in acid phosphatase in the supernatant or precipitate fractions is not due to a decline in the amount of some inhibitor during aging, inasmuch as dilution of the enzyme solutions from green bananas, with substrate concentration kept constant, did not cause an increase in activity per unit of enzyme.

Development of Acid Phosphatase in Sections Aged *in Vitro*. The development of acid phosphatase occurs *in vitro* in tissue sections, in a manner that is quantitatively and qualitatively like in intact fruits. Changes in skin color, softening, and the rate of respiration paralleled these changes in whole fruit, as recently reported (7).

Effect of Actinomycin D and Cycloheximide. The similarity of development of acid phosphatase in whole fruit and in sections aged *in vitro* indicated that tissue sections would be suitable for investigation of whether the increase in enzyme activity is owed to new synthesis, or activation, of enzyme. Sections of green banana, 5 mm thick, were aged for 24 hr in a humidity chamber containing 45 μl/liter of ethylene, and were then assayed for the effect of metabolic inhibitors on development of acid phosphatase activity between 24 and 48 hr, the period during which the increase in enzyme activity is rapid. The aged sections were washed and then soaked for 1 hr in water or 100 μg/ml of actinomycin D or cycloheximide; they were then blotted and aged in a humidity chamber for 23 hr. In the Triton X-100 extract of a 16,000*g* precipitate fraction, the increase in acid phosphatase was inhibited 48 and 67% by actinomycin D and cycloheximide, respectively. The development of enzyme activity in the supernatant fraction was inhibited 85% by actinomycin D. Cycloheximide caused a decline in the level of enzyme to 30% less than the 24-hr controls.

In the experiment illustrated in Table IV, 3-mm-thick sections were removed from Valery bananas during the period of the climacteric rise, and the effect of the inhibitors during a 5-hr period was determined. The 43% increase in acid phosphatase in the precipitate fraction (Triton extraction) was inhibited 71% by actinomycin D, and 100% by cycloheximide; both actinomycin D and cycloheximide completely inhibited the 58% increase in enzyme activity in the supernatant fraction.

The experimental results support the view that the increased activity of acid phosphatase during banana ripening is owed to new enzyme synthesis, rather than enzyme activation. Very little acid phosphatase may be extracted from the precipitate fraction of preclimacteric bananas with Triton X-100, and this extraction procedure yields increasing amounts of enzyme during the climacteric rise (Figs. 1 and 2). It is unlikely that during this period the enzyme exists preformed, but is in an inactive state, and its activation or extractibility increases during, and decreases after, the respiratory rise. Consistent with the interpretation that increased enzyme activity is attributable to *de novo* enzyme synthesis is the suppression of the increase in acid phosphatase by cycloheximide during a 5-hr period. The inhibition of synthesis of acid phosphatase by actinomycin D in 5 hr indicates a dependency on RNA synthesis, which may include synthesis of mRNA specific for acid phosphatase. Since during the climacteric there is no increase in total protein (12), the increase in acid phosphatase appears as specific synthesis of one of the enzymes involved in senescence (10).

It is a reasonable assumption that an enzyme that is synthesized

Table IV. *Effect of Actinomycin D and Cycloheximide on Development of Acid Phosphatase in Banana Sections*

Sections (3 mm thick) were cut from Valery bananas during the climacteric rise; then they were washed and soaked in water or a solution of 100 μg/ml of actinomycin D or cycloheximide for 1 hr, were blotted damp, and were incubated for 5 hr on a 9-cm filter paper wetted with 0.5 ml of H_2O or of the inhibitor used. Acid phosphatase was assayed at 0 and 5 hr in both the precipitate and supernatant fractions of a 30,000*g* centrifugation of tissue homogenates.

Treatment	Increase in *A* at 400 nm per 15 min per 20 mg fresh wt	
	Supernatant fraction	Precipitate + Triton X-100
Zero time	0.240	0.240
5 hr H_2O	0.380 (158)[1]	0.345 (143)
5 hr Actinomycin D	0.232 (100)	0.270 (112)
5 hr Cycloheximide	0.255 (100)	0.255 (100)

[1] Numbers in parentheses express enzyme level in percent of the zero time control.

Table V. *Relative Rates of Hydrolysis of Phosphomonoesters by Soluble and Precipitate-bound Acid Phosphatase from Banana*

Enzyme prepared as in Table I except without Carbowax. Inorganic phosphate was assayed by a modified Fiske-SubbaRow method.

Substrate	μMoles Hydrolyzed per 15 min per g fresh wt	
	Soluble enzyme	Ppt enzyme
Glucose-1-P	2.5	Not assayed
Glucose-6-P	6.0	Not assayed
ATP	14.0	25.0
UTP	12.5	30.0
ADP	Not assayed	18.5
AMP	6.3	7.5
Glycerol-P	5.0	7.5
p-Nitrophenyl phosphate	6.5	18.5
Phosphorylcholine	Not assayed	1.3
Phosphorylethanolamine	Not assayed	1.3
Phosphorylserine	Not assayed	1.3

rapidly and in large amounts during a 3-day period of fruit ripening (Fig. 2) is involved in changes occurring during ripening. Both the supernatant and precipitate enzymes hydrolyze a number of phosphomonoesters (Table V), but whether they are different forms of the same enzyme is not known. Ripening of bananas is associated with the appearance of at least two new isozymes of soluble acid phosphatase (R. E. Young, personal communication). Although cause-effect relationships are difficult to establish with certainty, acid phosphatase is an enzyme that could affect senescence in numerous ways. For example, breakdown of ATP to ADP could contribute to some release of the rate of respiration from acceptor (ADP) control by accelerating a cycling of ATP to ADP beyond that dictated by actual metabolic needs. Such an occurrence would not exclude the possibility that the increased respiration could also be affected by changes in membrane permeability which attend ripening (11, 12). From assays of the amounts of ATP and ADP during ripening it was concluded that the respiratory climacteric of avocado could not be explained by acceptor control (18).

There are some possible consequences of accelerated activity of both soluble and precipitate-bound acid phosphatase(s) which could adversely affect membrane properties indirectly, such as hydrolysis of ribose nucleotides, which would impair synthesis of RNA and protein needed for membrane maintenance. More direct effects could be due to hydrolysis of one or more among membrane ATP and phosphomonoesters, such as phosphorylcholine, phosphorylserine, and phosphorylethanolamine. The latter are hydrolyzed by the precipitate enzyme (Table V) and by human prostatic acid phosphatase (13).

Our studies of the comparative physiology of senescence indicate that the development of acid phosphatase(s) may be a common feature of senescence of fruit and leaf tissues (see also 14). For example, synthesis of acid phosphatase in a heavy precipitate fraction from aging *Rhoeo* leaf sections increases about 3-fold in 24 hr, and is enhanced by abscisic acid and suppressed by auxin or kinetin, in presence or absence of abscisic acid (P. De Leo, Elsa Hodge, and J. A. Sacher, to be published). Also, current work (J. Sacher) shows clearly that ripening of avocado is attended by development of acid phosphatase(s) that is qualitatively and quantitatively like that in banana. The correlations which emerge are that an increase in the heavy precipitate enzyme is common to all three tissues (banana, avocado, and *Rhoeo*), either attending or preceding loss of membrane integrity, whereas an increase in soluble acid phosphatase occurs only in the two climacteric tissues.

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