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UPSURGE OF PARTICULATE PEROXIDASE IN RIPENING BANANA FRUIT

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Key Word Index—*Musa cavendishii;* Musaceae; banana; fruit ripening; respiration climacteric; soluble and bound peroxidases; polyacrylamide gel electrophoresis.

Abstract—Peroxidase was isolated from the pulp of ripening banana fruit and assayed with *o*-dianisidine as hydrogen-donor. Cell macerates contained soluble and particle-bound peroxidase. Soluble peroxidase levels did not appreciably differ in pre-climacteric, climacteric and post-climacteric fruit. Particulate peroxidase levels increased 3-fold with the initiation of the respiration climacteric and gradually declined with the onset of senescence. Bound peroxidase was released from cell wall and membrane fractions with washing in 0·8 M $CaCl_2$.

INTRODUCTION

PEROXIDASES (donor:hydrogen peroxide oxidoreductase, E.C. 1.11.1.7) have been implicated in ethylene biogenesis,[1-3] hormone balance,[4,5] membrane integrity[6] and respiration control;[7] and, accordingly, are suggested to be an important factor in the control of ripening and senescence in fruit. Peroxidase activity appears to increase during the ripening of mango,[8] grape,[9] apple[10] and pear fruit.[11] Information on the cellular localization of peroxidase in ripening fruit and senescing plant tissues is lacking. The occurrence of peroxidase bound to cell walls and membranes in developing plant tissues has been reported.[12,13] Recent studies showed the presence of cytoplasmic (soluble), ionically bound and covalently bound fractions of peroxidase in etiolated pea shoots.[14] In that cellular localization of enzymes may delimit their respective function(s), we have examined the distribution of particle-bound and soluble peroxidase in the ripening banana fruit.

RESULTS

Soluble Peroxidase

Levels of soluble peroxidase isolated from the pulp of pre-climacteric, climacteric and post-climacteric fruit were similar under the conditions of extraction described in the Experimental (Fig. 1). Omission of casein dispersion from the extraction medium resulted in

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little recoverable activity (soluble or bound) from green fruit (Fig. 1). We have previously reported that casein dispersion is a potent scavenger of the astringent tannins in green banana fruit.[15] Other compounds reported as useful for preventing enzyme inhibition in other fruits, such as polyvinylpyrolidone[16] and polyethylene glycols[17] were shown to be less effective than casein in preventing inhibition of peroxidase in macerated green banana fruit. Previous studies also showed that casein dispersion was the more efficient tannin scavenger in extracting total protein and mitochondria from green banana fruit.[18] There is, accordingly, convincing evidence that peroxidase activity extracted at low ionic strength is invariant with ripening and senescence of banana fruit.

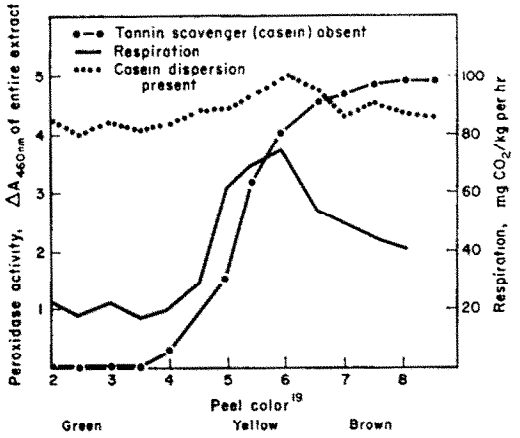

FIG. 1. PEROXIDASE PRESENT IN THE SOLUBLE FRACTION OF CELL MACERATES FROM RIPENING BANANA FRUIT.
The respiration (CO_2 evolution) of intact fruit is shown. Data are the average of triplicate experiments.

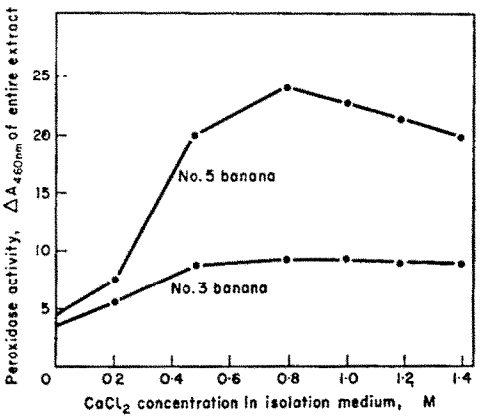

FIG. 2. EXTRACTION OF BOUND PEROXIDASE FROM BANANA PULP PARTICULATE FRACTION BY VARIOUS CONCENTRATIONS OF $CaCl_2$.
Values are means of three experiments.

Particulate Peroxidase

The particulate fraction of banana tissue which sedimented at 60 000 *g* in 30 min was observed to contain considerable peroxidase activity when it was incubated with *o*-dianisidine assay medium. The bulk of this particulate activity was associated with the slowest sedimenting portion of the centrifugal pellets and was not readily released when pellets were washed in 0·05 M sodium maleate, pH 6·0 (Table 1). Extraction of the washed particulate fraction with a medium containing 0·05 M sodium maleate, pH 6·0 and 0·8 M $CaCl_2$ resulted in nearly complete solubilization of the bound peroxidase (Table 1). The $CaCl_2$-washed and sedimented particulate material exhibited negligible peroxidase activity when histochemically assayed. Reextraction of this fraction with medium containing $CaCl_2$ resulted in little additional release of peroxidase (Table 1).

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Influence of Salt Concentration on Release of Particulate Peroxidase

Solubilization of peroxidase from the cellular particulates was optimal when 0·8 M $CaCl_2$ was present in the extraction medium (Fig. 2). Release of enzyme was gradual with an increase in salt concentration to 0·2 M and increased more rapidly in ripe fruit when the salt concentration was raised to 0·8 M. Increasing concentrations of $CaCl_2$ from 0·8 to 1·4 M resulted in a gradual decline in activity recovered in the supernatant.

TABLE 1. EXTRACTION OF BOUND PEROXIDASE WITH $CaCl_2$

Fraction	Relative peroxidase activity
Soluble*	100
Buffer wash†	8
Buffer-$CaCl_2$ wash‡	359
Buffer-$CaCl_2$ wash-2	45

* 20 g No. 5 banana powdered in liquid N_2; extracted with 50 ml medium containing 0·05 M sodium maleate, casein dispersion (1 g), Polyclar-At (0·5 g), pH 6·0.

† Pellet suspended in 50 ml medium containing 0·05 M sodium maleate, pH 6·0 and centrifuged (60 000 *g* for 30 min).

‡ Washed pellet suspended in 50 ml medium containing 0·05 M sodium maleate and 0·8 M $CaCl_2$, pH 6·0 and centrifuged (60 000 *g*, 30 min).

Equimolar levels of $MgCl_2$ and NaCl were less effective than $CaCl_2$ in eliciting release of bound peroxidase at salt concentrations less than 1 M (Fig. 3). Similar release of enzyme was observed with all three salts at a concentration of 1·6 M.

TABLE 2. DISTRIBUTION OF PARTICULATE PEROXIDASE

Fraction	Activity released (%)*
5000 *g*, 5 min	12·5
20 000 *g*, 15 min	33·3
60 000 *g*, 30 min	54·2

* Per cent of total activity associated with cellular particulates. Release of enzyme and assay as described in text. Data are for one experiment and are representative of four similar experiments.

Changes in Particulate Bound Peroxidase with Ripening

Pulp obtained from preclimacteric, climacteric and postclimacteric fruit was freed of soluble peroxidase. The sedimented cell debris was washed twice with 0·05 M sodium maleate as described in the Experimental. The tissue homogenates (free of soluble peroxidase) were suspended in isolation medium containing 0·8 M $CaCl_2$ with a Potter–Elvehjem tissue

homogenizer and centrifuged at 60 000 *g* for 30 min. Peroxidase activity observed in the supernatant fractions was taken as a measure of particulate peroxidase. Bound peroxidase estimated by this procedure was observed to increase by approximately 3-fold at the onset of the respiration climacteric (Fig. 4). When ripening was complete and the peel color progressed from yellow to brown, the level of bound peroxidase gradually declined. The increase in bound enzyme was in contrast to the observed constant level of soluble peroxidase (Figs. 1 and 4).

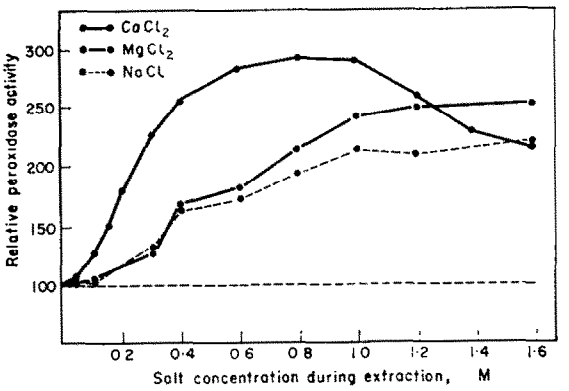

FIG. 3. EXTRACTION OF BOUND PEROXIDASE FROM THE PARTICULATE FRACTION OF NO. 5 BANANA BY VARIOUS CONCENTRATIONS OF $CaCl_2$, $MgCl_2$ AND NaCl.
Values are means of two experiments.

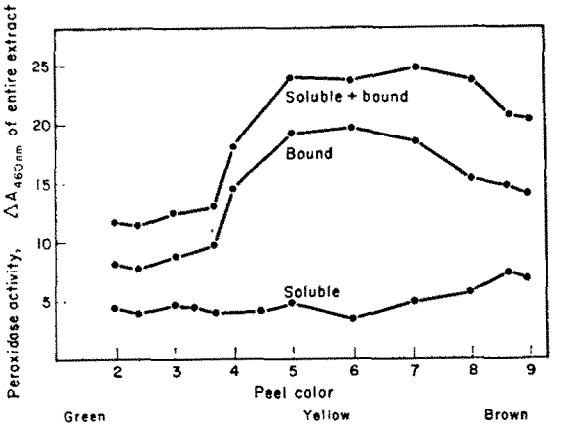

FIG. 4. ACTIVITY OF PARTICULATE AND SOLUBLE PEROXIDASE AS A FUNCTION OF FRUIT RIPENING.
Values are means of three experiments.

Distribution of Bound Peroxidase in the Cell Particulates

Cell homogenates free of soluble peroxidase were suspended in isolation medium containing 0·05 M sodium maleate, pH 6·0 and fractionated by differential centrifugation at 5000 *g* (5 min), 20 000 *g* (15 min) and 60 000 *g* (30 min). The three sedimented fractions were suspended in isolation medium containing 0·8 M $CaCl_2$ and centrifuged at 60 000 *g* (30 min). Peroxidase activity recovered in the respective supernatant fractions was taken as a measure of bound peroxidase in the three fractions of cell particulates (Table 2). Although activity was distributed throughout all particulate fractions, the enzyme was notably high in the lighter fraction which contained mitochondria and other subcellular membranes.[18]

Peroxidase Inhibitor

It has been submitted that an observed rise in peroxidase activity with ripening of mango fruit was a reflection of the disappearance of a peroxidase inhibitor.[8] The inhibitor, present in green fruit, was labile to incubation at 50° and was manifested by an anomolous (non-linear) enzyme concentration-activity relationship. We have observed no such indication of a peroxidase inhibitor in banana fruit. Incubation of extracts at 50° for 30 min prior to assay did not influence activity, and the relationship between enzyme concentration and peroxidase activity was linear. However, we have observed that IAA oxidase activity associated with the

cytoplasmic peroxidase exhibited anomolous enzyme kinetics (Fig. 5). Such anomolous kinetics were not observed when horseradish peroxidase was similarly assayed. IAA oxidase activity may be a reflection of specific peroxidase isozymes.

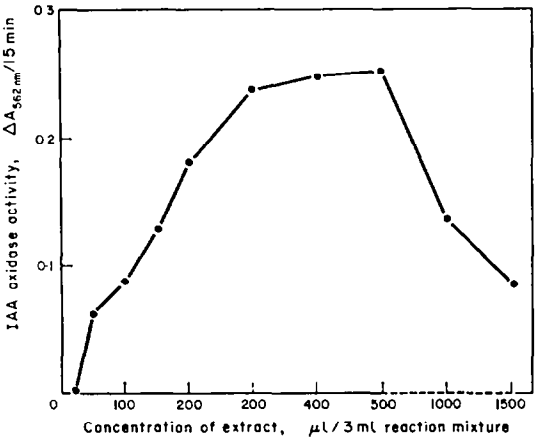

FIG. 5. INDOLE-3-ACETIC ACID OXIDASE ACTIVITY AS A FUNCTION OF THE VOLUME OF CRUDE EXTRACT PRESENT DURING ASSAY.
Sample assayed was the soluble peroxidase extracted from a No. 4 banana. Negligible IAA oxidase activity was observed in the particulate fraction. Data are for one experiment and are representative of two similar experiments.

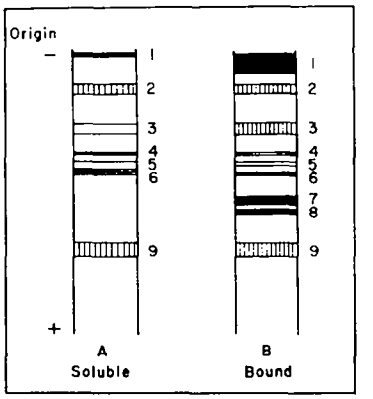

FIG. 6. ISOENZYMES OF, (a) SOLUBLE, AND (b) BOUND PEROXIDASE RESOLVED BY POLYACRYLAMIDE GEL ELECTROPHORESIS.

Isozyme Profiles

Inconsistent peroxidase isozyme profiles were observed when extracts were applied directly to polyacrylamide gels. Freshly prepared isolates were passed through a column containing Sephadex G25 (coarse) and dialyzed against 100 volumes of distilled water prior to electrophoresis. Reproducible isozyme patterns were observed with benzidine–H_2O_2 (Fig. 6). Zones designated as 1, 7 and 8 were the most active species in the bound peroxidase fraction whilst zone 1 was the predominant isozyme in the soluble peroxidase. Major zones 7 and 8 were not observed in the soluble peroxidase fraction suggesting these were the unique and predominant species of the bound peroxidase. Incubation of gels in *o*-dianisidine–H_2O_2 medium revealed an identical distribution of activity except that staining was less intense and zones appeared less distinct. No peroxidase entered the gels when the electric field was reversed.

DISCUSSION

Peroxidase associated with cell particulates was observed to increase with the ripening of banana fruit. The inherent difficulties in judging whether an enzyme is localized on cell surfaces *in situ* make it impossible to interpret definitively the data reported in this communication. The extreme conditions of high ionic strength necessary to release the bound enzyme is evidence that peroxidase association with cell particulates is not an artifact of preparation. Preliminary data in our laboratory showed that 'bound peroxidase' was not released by changing the pH of the extraction medium to either 4·0, 5·0, 7·0 or 8·0. We had previously

shown that the release of 'soluble peroxidase' from banana pulp was independent of hydrogen ion concentration in the pH range of 4·0–7·0.[15] The apparent difference in isozyme profiles is also evidence that the bound peroxidase represents a component of cellular peroxidase distinct from the soluble enzyme. Investigations in progress in our laboratory have revealed that the 'bound' and 'soluble' peroxidase fractions differ in substrate specificity and pH optima. Our preliminary histochemical and biochemical data lead us to suggest that the particulate-associated peroxidase is localized extracellularly in the middle lamella. These results will be reported in a later communication.

Irrespective of the *in vivo* localization of peroxidase, the data presented here indicate that total peroxidase activity increases concomitantly with the respiration climacteric. Further research will examine the role of this enzyme in ethylene biogenesis, auxin catabolism and membrane dissolution.

EXPERIMENTAL

Plant material. 'Valery' banana (*Musa cavendishii*), grown in Honduras, were generously donated by the United Fruit Company, *ca.* 7 days postharvest. Fruits were preclimacteric and had a solid green peel color (No. 2).[19] Bananas were gassed with 100 ppm ethylene for 4 hr and ripened at 20°, 80–90% relative humidity in a ventilated incubator. Pulp from the center 0·66 of banana length was sampled for isolation of peroxidase.

Respiration. Respiration of intact bananas was monitered by trapping carbon dioxide in 0·1 N NaOH and estimating bicarbonate ion by a double end point titration with phenolphthalein and methyl orange.[20]

Isolation of soluble peroxidase. A 20-g sample of banana pulp was sliced into 1 mm sections and immersed in 50 ml of cold (4°) extraction medium containing sodium maleate, pH 6·0 (0·5 mmol), casein dispersion[18] (1·0 g) and insolubilized polyvinylpyrolidone 'Polyclar-At', GAF Corporation (0·5 g). The banana tissue was vacuum infiltrated for 15 min at 4° with a water aspirator. Infiltrated tissue and extraction medium were macerated for 1 min with a Servall Omni-mix blender at four 15-sec intervals. The resulting homogenate was immediately centrifuged at 60 000 *g* for 30 min and the clear supernatant fraction was decanted and assayed for peroxidase within 30 min after recovery. The sedimented fraction was retained for isolation of bound peroxidase. All operations were performed at 4°.

Isolation of bound peroxidase. The sedimented fraction of cellular particulates was resuspended in 50 ml of cold (4°) isolation medium (as above) containing 0·8 M $CaCl_2$, centrifuged (as above) and was decanted and assayed for peroxidase within 30 min after recovery. In certain experiments, the concentration of $CaCl_2$ was varied or replaced by NaCl or $MgCl_2$. The pH of extracts was always maintained at 6·0.

Distribution of peroxidase in the particulate fraction. The sedimented (60 000 *g*, 30 min) portion of cell homogenates, prepared in the absence of added salts, was resuspended in extraction medium and fractionated by differential centrifugation at 5000 *g* (5 min), 20 000 *g* (15 min) and 60 000 *g* (30 min). The respective cell particulate fractions were resuspended in extraction medium containing added $CaCl_2$ (40 mmol) and centrifuged at 60 000 *g* for 30 min. The respective supernatant fractions were collected and assayed within 30 min after recovery.

Peroxidase assay. Aliquots (10–200 μl) of extracts were assayed for peroxidase at pH 6·0 in 0·3 M sodium phosphate with *o*-dianisidine as hydrogen-donor and 0·003% H_2O_2 as oxidant.[21] Oxidation of *o*-dianisidine was measured at 460 nm with a DBG-recording spectrophotometer (Beckman Inst.). The absorbancy change per min was generally expressed as activity of the total volume of respective extracts.

IAA oxidase assay. Soluble and particulate peroxidase fractions were assayed for IAA oxidase activity by coupling indole-3-acetic acid oxidation to 4-dimethylamino-cinnamaldehyde and spectrophotometric assay of the red product.[22]

Polyacrylamide gel electrophoresis. Polyacrylamide gels containing tris (hydroxychloroaminomethane) and glycine were prepared according to established procedure.[23] Electrophoresis was at a constant current of 3 mA/tube for 40 min. Gels were stained for peroxidase by incubation in *o*-dianisidine assay medium or in benzidine-H_2O_2.[23]

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