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Characterization of Peroxidases in Lignifying Peach Fruit Endocarp

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ABSTRACT

Developing peach (*Prunus persica* L. Batsch 'Redskin') fruit were used to characterize the role of peroxidases in lignification. During development, the endocarp of these drupes becomes lignified while the mesocarp remains parenchymatous. Acidic peroxidase from lignifying endocarp were similar to those of the fleshy mesocarp. The endocarp had a larger amount and number of basic peroxidases than the mesocarp. Cultured peach leaf cells are thought to be lignified because their walls give a positive reaction with phloroglucinol-HCl. These cells also secreted a basic peroxidase. Peroxidases were difficult to extract from endocarp tissue as they lignified. This was also demonstrated by tissue printing on nitrocellulose. Flesh, but not endocarp peroxidase was evident in tissue prints. This suggests that tissue printing may fail to reveal the presence of enzymes which are firmly attached to the cell.

Lignin plays a role in the structural strength of cells and disease resistance. Its occurrence, identification, biogenesis, and degradation have been reviewed (12). Peroxidase is thought to catalyze the polymerization of phenylpropanoid precursors of lignin. However, it has been difficult to tell which peroxidase isozymes are associated with lignification, and which with other biochemical activities. The purpose of the work reported here was to see if the lignifying endocarp of peach would serve to identify which peroxidases are associated with lignification.

Ryugo (14, 15) followed the changes in lignin and phenolic precursors in the developing peach pit and suggested it would be a good system for lignin synthesis studies. Other systems have been used in lignification studies. These include fiber formation in asparagus (9) and cell suspension cultures (3, 4). The cell suspension cultures studies suggested that basic peroxidases were involved in lignification.

Tissue printing has become an important tool in visualizing the location of peroxidase and other enzymes (17). However, some enzymes may be missing in tissue blots because they are not free to move from the tissue to the blotting matrix. We have found that the peroxidases involved in lignin synthesis are an example where tissue blotting failed to reveal the presence of a tissue enzyme.

MATERIALS AND METHODS

Plant Material

Peach fruits (*Prunus persica* L. Batsch 'Redskin') were collected on June 1, 5, 9, and 12, 1989. Most of the flowers were in full bloom on May 1, and the majority of the fruit were mature on August 15. Endocarp tissue gave a faint red lignin reaction with phloroglucinol-HCl at the first harvest date. At the last harvest date, the endocarp showed a bright red lignin reaction, was semi-woody, and difficult to use in enzyme extractions.

Tissue Blotting

Fruit were cut in half and pressed against wet or dry Schleicher and Schuell BA85 nitrocellulose as described earlier (17). No difference was observed using moist or dry nitrocellulose. Location of enzymes on the blots were visualized by the following substrates. Peroxidase was visualized with 10 mM guaiacol −10 mM H_2O_2 and PPO[1] with 0.5% 3,4-dihydroxyphenylalanine. Peroxidase activity in intact tissue was visualized with 0.1% syringaldazine with or without 0.03% H_2O_2 as described earlier (8).

Enzyme Extraction and Assay

Peroxidase from mesocarp and endocarp (40 g) was extracted by homogenizing tissue with a Brinckman Kinetica homogenizer with 80 mL buffer (pH 8), consisting of 0.025 M Tris/glycine, 0.25 M $CaCl_2$, and 1 g insoluble polyvinylpolypyrrolidone. Data shown are the average of three samples. The experiment was repeated on three occasions with similar results. Subsamples (2 g) were homogenized in a mortar and pestle with sand to extract enzymes for electrophoretic separation. As indicated by Church and Galston (4), $CaCl_2$ facilitated the solubilization of peroxidase.

Preliminary pH activity tests ran between pH 3 to 10 indicated that maximum peroxidase activity occurred in the range of pH 4 to 5 using potassium phosphate or acetate buffers (data not shown). Activity was measured in 1 mL final volume reaction mixtures. The 100 μL sample of enyzme was incubated in 1 mM guaiacol, 1 mM H_2O_2 in 0.05 M acetate buffer (pH 5). The change in absorptance at 470 nm was measured every 15 s. Peach tissue from the June 12, 1990

[1] Abbreviations: PPO, polyphenoloxidase; IEF, isoelectric focusing-polyacrylamide gel electrophoresis.

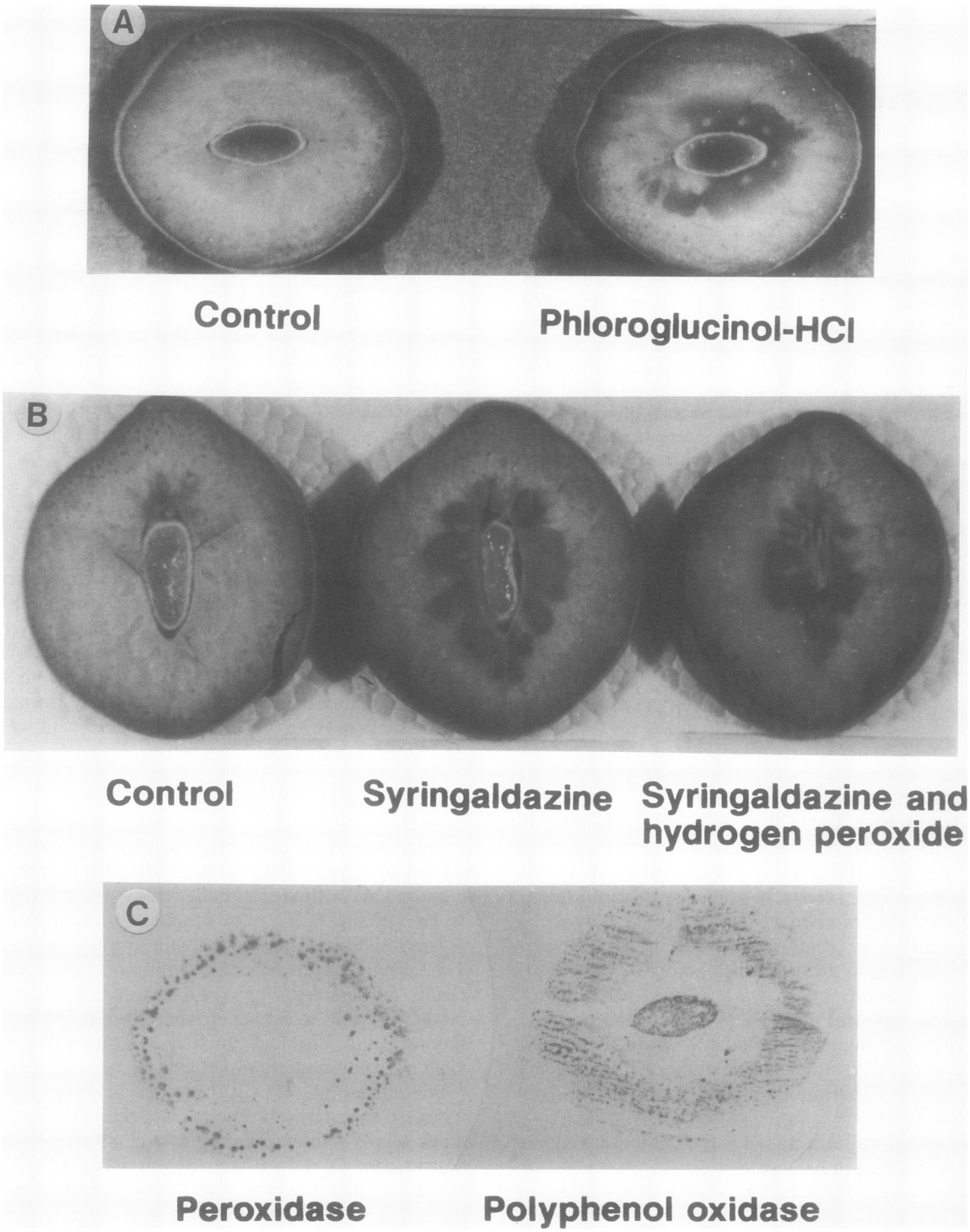

Figure 1. Localization of peroxidase activity and lignin in developing peach fruit. A, Color reaction between endocarp lignin and phloroglucinol and HCl. Control fruit on the left. B, Peroxidase activity in a fruit slice visualized (gray) by treatment with syringaldazine (middle), or syringaldazine plus H_2O_2 (right) compared to control (left). C, Nitrocellulose blots of peach tissue slices treated with guaiacol + H_2O_2 (left) and 1,3-diphenylalanine (right). The blot on the left shows the transfer of peroxidase, and on the right polyphenol oxidase activity. Note that while peroxidase activity was evident in endocarp tissue (1-B) it did not transfer to nitrocellulose (1-C).

Table I. *Calcium (0.25 M $CaCl_2$) Enhanced Release of Peroxidase from Peach Tissue*

Treatment	Peroxidase Activity
	A_{470}/min × mg protein
Endocarp	0.8 B*
Endocarp + $CaCl_2$	2.1 A
Mesocarp	1.1 b
Mesocarp + $CaCl_2$	1.9 a

* Number followed by the same letter were not different (P = 0.05) according to Duncan's multiple range test. Different case letters indicate separate comparisons.

sampling date were used in chromatography experiments in an attempt to separate peroxidase isozymes. Reaction mixtures from chromatographic studies consisted of 100 μL from each fraction plus 100 μL of 10 mM each guaiacol and H_2O_2 in acetate buffer. The fractions were incubated for 10 min and then measured at 492 nm in a microtiter plate reader (SLT Lab Instruments-EAR 400 AT, Salzburg, Austria).

Chromatography

Peach peroxidases were separated on a Pharmacia (Piscataway, NJ) Sephadex MonoQ column with a 0.02 M Tris (pH 7.4) buffer. The NaCl gradient was 0.0 to 0.5 M over a 14 min run and 1 mL fractions were collected for enzyme activity assays. The column was flushed with 1 M NaCl between assays.

Electrophoresis

Isoelectric focusing electrophoresis on pH 3 to 9 gels was accomplished by means of the Pharmacia PhastGel system following directions supplied by the manufacturer. Peroxidase isozymes were visualized with the guaiacol stain described above. The peach 'Sunhigh' leaf callus tissue culture peroxidase was a gift of Dr. M. Wisniewski of this laboratory. The

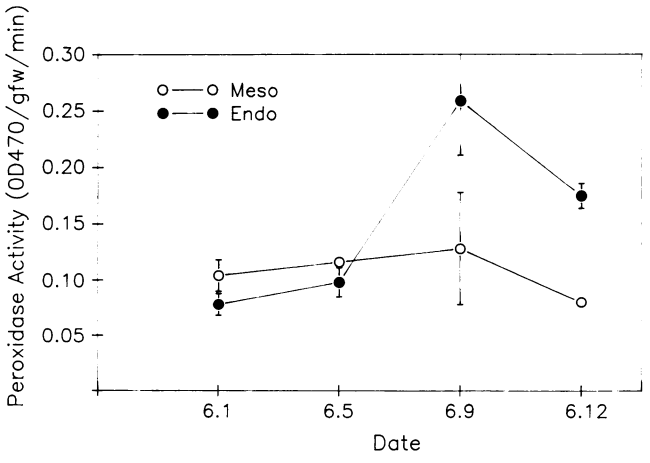

Figure 2. Changes in the levels of total peroxidase during early pit hardening in peach fruit. Open circles represent endocarp and closed circles mesocarp tissue. The bars or circle diameters represent the standard error of the mean of three samples.

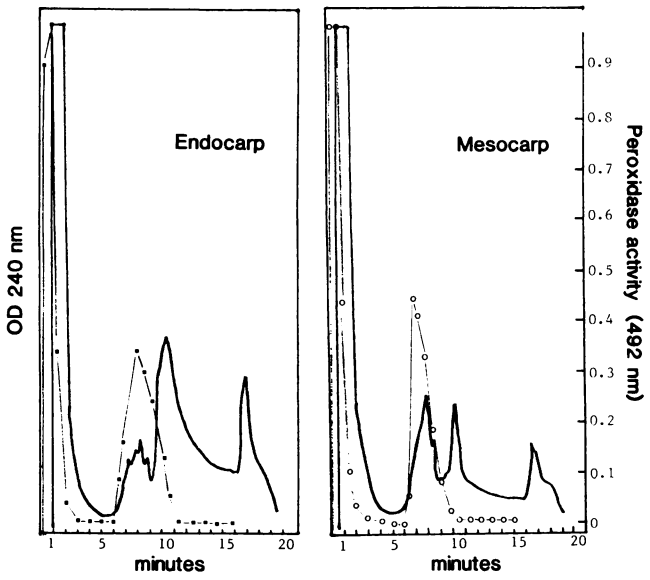

Figure 3. Distribution of peroxidase activity separated on a Sephadex MonoQ column run at pH 7.4 is shown. The UV absorbing material in the fractions indicated is shown by the solid curve without symbols. The peroxidase activities of the endocarp fractions is represented by solid squares (left) and mesocarp fractions by open circles (right).

callus tissue cell walls tested positive for lignin with phloroglucinol-HCl and were resistant to hydrolysis by cellulysin, macerase, hemicellulase, and Driselase (data not shown).

RESULTS

Figure 1A shows that the endocarp of immature peach tested lignin-positive with the phloroglucinol-HCl reagent. Figure 1B shows that the endocarp also possessed peroxidase activity. The observation that endocarp tissue oxidized syringaldazine without added H_2O_2 suggested that endocarp cells have an endogenous supply of oxidant.

Figure 1C demonstrates that endocarp peroxidase did not transfer to nitrocellulose, whereas mesocarp peroxidase did. Mesocarp enzymes appeared to be localized in discrete areas associated with vascular bundles. Blotting experiments were repeated with PPO which revealed that PPO was transferred from both mesocarp and endocarp tissue. However, more activity was transferred from the mesocarp than endocarp tissue.

Table I presents data showing that 0.25 M $CaCl_2$ increased the amount of peroxidase isolated from peach tissue. $CaCl_2$ added to extraction buffer caused a 2.6-fold increase in peroxidase isolated from the endocarp and a 1.7-fold increase in peroxidase isolated from the mesocarp.

Figure 2 shows that total peroxidase activity in the endocarp increased during the early period of pit hardening. Peroxidase activity was similar in mesocarp and endocarp samples harvested on 6.1 and 6.5. In contrast, peroxidase activity in the 6.9 and 6.12 endocarp samples increased two-fold compared to mesocarp samples. The decline in activity of 6.12 samples reflects the difficulty of extracting peroxidase from the endocarp as it becomes lignified.

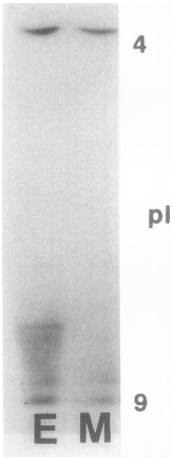

Figure 4. Isoelectric focusing gel of peach peroxidases. E, Endocarp peroxidases; M, mesocarp peroxidase. The harvest date of the fruit was June 9, 1989.

Samples of mesocarp and endocarp peroxidase from the previous experiment were separated with a Pharmacia MonoQ column and the peaks of activity run on IEF gels. Figure 3 shows that there are no major differences between peroxidase isozymes isolated from endocarp and mesocarp tissue. Two major peaks were observed. The first peak corresponded to the basic peroxidases, and the second to the acidic peroxidases shown in Figure 4.

IEF gels shown in Figure 4 indicated that the acid peroxidases are similar in endocarp and mesocarp tissue. However, there was a greater diversity in basic peroxidase isozymes isolated from endocarp tissue.

Although the development of leaf cell wall peroxidases are not directly comparable to peach fruit tissue, we observed that a basic peroxidase (pI 9.0) was the only isozyme produced by cultured leaf cells. The cell walls of the peach leaf cells grown in culture were lignin positive according to a phloroglucinol-HCl test (data not shown).

DISCUSSION

As shown in Figure 1A, peach endocarp cells form lignified stone cells while the mesocarp, with the exception of vascular tissue, remained parenchymatous. The purpose of work reported here was to compare the peroxidase isozymes produced by lignifying and nonlignifying tissues.

The formation of red oxidation products of syringaldazine shown in Figure 1B demonstrate that peroxidase was present in endocarp tissue. These products were formed in the presence or absence of added H_2O_2. This agrees with the report of others (2, 18) who have shown that peroxidases are dual function enzymes. In the presence of Mn^{2+} and reduced pyridine nucleotides, peroxidases are capable of generating, as well as utilizing, H_2O_2. The results presented here suggest endocarp lignification peroxidases are dual function enzymes.

The presence of peroxidase and PPO in peach has been described earlier (13). PPO is a membrane protein which is activated by disruption and attack by proteolytic enzymes (16). As shown in Figure 1C, PPO was present in both endocarp and mesocarp tissues. However, more PPO was transferred to the nitrocellulose from the mesocarp. Blotting studies with peroxidase indicated that the mesocarp enzyme transferred readily to nitrocellulose but that the endocarp enzyme did not. The absence of endocarp peroxidase in nitrocellulose blots also demonstrates its strong affinity for cell walls or other components of the cell. Our results indicate that tissue blotting may fail to reveal the presence of tightly bound proteins. This suggests that lignification peroxidases may become enmeshed in the wall or membranes involved in its synthesis. Results shown in Table I also suggest that peroxidases are more firmly bound to endocarp tissue than mesocarp tissue.

Peroxidases are involved in the last step in lignin formation. Results shown in Figure 2 indicated a two-fold increase in total peroxidase activity during lignification. In addition to this increase in activity, data presented in Figure 4 demonstrated that there was an increase in number of basic isozymes in lignifying tissues. Dalet and Cornu (5) reported that they found no differences in the amount or distribution of peroxidase isozymes in cherry clones with different degrees of lignification.

The electrophoretic pattern of acidic (pI 4) isozymes from endocarp and mesocarp tissue were similar. This suggests that acidic peroxidases are not associated with lignification. Other workers (3, 4) have suggested that basic peroxidases are associated with lignification of cells in culture. We have confirmed their observations with cultures of peach leaf cells. The walls of these cells are phloroglucinol-HCl positive and resistant to hydrolysis by a number of cell wall degrading enzymes. These cultures also release a basic (pI 9) peroxidase in culture medium.

The positive charge of basic proteins may facilitate their binding to negatively charged cell walls. Using unspecific histochemical techniques, peroxidases have been detected in vacuoles, tonoplast plasmalemma as well as inside cell walls (7). Immunocytochemical localization has been used to pinpoint the location of peroxidase in cells. Suberization-associated acidic peroxidase was associated with the inner side of potato cell walls (6). In peanut, acidic peroxidases were localized on plasmalemma while the basic peroxidase was located in cell walls (10). However, in other reports acidic peroxidases were associated with cell walls of corn (11). We observed that the basic peroxidase of cucumber cotyledons was associated with the plasmalemma (1).

In conclusion, although an increase in the amount and diversity of basic peroxidases was observed during lignification, other enzymes involved in producing phenylpropanoid precursors may play a more important role in controlling lignin formation.

ACKNOWLEDGMENTS

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