

Elicitation of Lignin Biosynthesis and Isoperoxidase Activity by Pectic Fragments in Suspension Cultures of Castor Bean¹

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ABSTRACT

Suspension cultures of castor bean (*Ricinus communis* L.) which have been treated with pectic fragment elicitor rapidly accumulate lignin as measured by derivatization with thioglycolic acid. The responsiveness of cultured cells to elicitor is dependent on the stage of culture growth. In 6-day (maximally responsive) cultures, increases in lignin are first evident 3 hours after addition of pectic fragment elicitor with maximal rates of lignin synthesis between 4 and 10 hours. The abundance of lignin in cultures after 12 hours of elicitor treatment is 10- to 20-fold higher than in untreated control cultures and can thereby account for as much as 2% of the dry cell weight. Only intermediate sizes of pectic oligomer are active as elicitors of lignin. Half-maximal accumulation of lignin occurs at 250 to 300 micrograms per milliliter of an optimal elicitor preparation with an average degree of polymerization of seven. We consider the synthesis of lignin in elicited cultures to be a mechanism of plant disease resistance which is induced by the elicitor. Plant peroxidases have been proposed to catalyze the last enzymatic steps in the biosynthesis of both lignin and hydrogen peroxide. Six extracellular isoenzymes of peroxidase (two anionic, designated A1 and A2, and four cationic, designated C2, C3, C4, and C7) are detectable in healthy suspension cultures of castor bean by native gel electrophoresis. Treatment of cultures with elicitor causes substantial changes in the activity of four of these species (A1, C2, C3, and C7). Elicitor treatment also results in the appearance of three new peroxidase isoenzymes that are not readily detectable in healthy cultures (C1, C5, and C6). Increases in the activities of these isoenzymes are concurrent with or slightly precede the accumulation of lignin in elicited 6-day cultures. By 12 hours after addition of elicitor, C1 becomes the most abundant extracellular isoperoxidase. The differential regulation of expression of peroxidase isoenzymes following elicitor treatment suggests that individual isoenzymes of peroxidase may have specific functional roles in the biosynthesis of disease-lignin.

Resistance of plants to pathogens is frequently a result of the rapid establishment of a localized 'hypersensitive response' by the plant at regions of attempted infection. There are

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multiple biochemical components of the hypersensitive response which act together to halt the spread of an invading organism. These include: (a) production of phytoalexins; (b) secretion of enzymes such as chitinase and β -glucanase which hydrolyze fungal cell walls; and (c) deposition of extracellular molecular barriers such as lignin, callose, and hydroxyproline-rich glycoprotein. Substances which are signals of infection to the plant and can thereby activate these responses are called elicitors. Structural components of fungal origin such as lipids and cell wall fragments as well as fungal enzymes such as pectinase have been shown to be potent elicitors (4, 35).

Pectinases are among the first hydrolytic enzymes produced by microbial plant pathogens during attempted infection (see 35 for references). Homogeneous endopolygalacturonase (an endo-pectinase) from the fungus *Rhizopus stolonifer* has been shown to be a potent elicitor of the biosynthetic capacity to produce casbene, a phytoalexin of castor bean (23). Additional evidence has indicated that the elicitor activity of this endopolygalacturonase is a consequence of the enzyme-catalyzed release of soluble pectic fragments of the plant cell wall which act themselves as primary elicitors (1, 14). Similar conclusions have been reached regarding an endopectate lyase from the bacterium *Erwinia carotovora* which elicits phytoalexin accumulation in soybean (3). Treatment of plant tissue with pectic fragments can thus, at least in part, mimic infection by an invasive organism by simulating the pectin breakdown resulting from microbial endopectinases. We are reporting here a quantitative study of lignin deposition in suspension cultured castor bean cells both prior to and following the treatment of these cultures with PFE.²

Lignin is a three-dimensional phenolic structure resulting from the free-radical polymerization of *p*-coumaryl, coniferyl, and sinapyl alcohols within the plant cell wall. As lignin polymerizes, it forms covalent crosslinks with carbohydrate and protein (22, 36) and renders cell walls highly resistant to mechanical and enzymatic disruption (11, 34). It is most abundant in the vascular and support structures of woody tissue in healthy plants where it can account for a large percentage of the dry weight. Accumulation of lignin or lignin-like material in non-woody plant tissue is commonly associated with attempted fungal infection (34). Deposition of lignin

² Abbreviations: PFE, pectic fragment elicitor; LTGA, lignin thioglycolic acid; PCV, packed cell volume; AIR, alcohol insoluble residue; RT, room temperature.

has been hypothesized to interfere with the enzymatic hydrolysis and mechanical penetration of plant tissue by fungal pathogens and may also impair the movement of water and diffusible molecules between the plant and fungus (29). In some reports, the rapid appearance of lignin or lignin-like polymers has been associated with the expression of disease resistance in race- and cultivar-specific interactions (8, 28). Pectic fragments have previously been identified as elicitors of the accumulation of histochemically detected lignin-like material in cucumber hypocotyls (30). Other elicitors of lignin or lignin-like substances include chitosan (16), extracts from fungal cell walls (24), suspensions of chitin or fungal cell walls (26), and fungal lipids (24).

Investigation of lignin biosynthesis in diseased tissue has been hampered by the lack of suitable assay methods. Currently, the only reliable and quantitative assay for lignin is by derivatization with thioglycolic acid (22, 34, 36). Thioglycolic acid derivatization displaces lignin from its normal covalent attachments to the cell wall and enables it to be extractable from cell walls by alkali. Acidification of the alkaline extract causes the precipitation of LTGA which is not contaminated with either proteins or polyphenols (22). After being resolubilized in alkali, the LTGA can be directly measured by its UV absorbance (36). We have used the thioglycolate procedure to demonstrate that suspension cultures of castor bean which have been treated with PFE show large increases in their rate of lignin biosynthesis within a few hours.

Plant peroxidases catalyze the last enzymatic step in the biosynthesis of lignin, notably the conversion of cinnamyl alcohols into their free-radical forms at the expense of H_2O_2 (11). Plant peroxidases are also believed to be responsible for the generation of H_2O_2 from molecular oxygen and NADH (11). Both of these events are extracellular, and either could serve as a regulatory element for control of lignin biosynthesis. Peroxidases normally exist in plants as multiple isoenzymes. Measurable peroxidase activity can frequently increase severalfold above control levels in infected plants, and in some cases these increases are attributable to increases in the activities of specific isoenzymes. In a fewer number of instances, disease-specific isoperoxidases have been seen to appear in infected tissues (15, 31, 33, 37). However, the functional significance of these isoenzymes is still uncertain.

Extracellular plant peroxidases are commonly classified as either free, ionically bound to the cell wall, or covalently attached to the cell wall. Solutions containing calcium salts efficiently release the ionically bound peroxidase species (32). Extraction of suspension cultures with solutions containing Ca^{2+} has the added advantage that ionically bound peroxidases will be released into the medium and can be separated along with free peroxidases from intact cells. Because the procedure is gentle, nonplasmolyzing, and nondisruptive, contamination with intracellular enzymes is minimized. We have used calcium elution of suspension cultured cells along with native polyacrylamide gel electrophoresis of the resulting cell-free medium to analyze the extracellular isoperoxidase profiles of healthy and elicited cultures. Coordinate with the appearance of new lignin in elicited cultures are a series of changes in the abundance of some of the normally observed isoperoxidase species. In addition, three new peroxidase iso-

enzymes, which are not present at significant levels in healthy cultures, are rapidly expressed following elicitor treatment. These changes suggest that individual isoenzymes of peroxidase may have specific functional roles in the biosynthesis of disease-lignin.

MATERIALS AND METHODS

Cell Cultures

Castor bean (*Ricinus communis* L.) callus culture designated CB-1 was kindly provided by Dr. I. Shechter, Tel-Aviv University. Cell suspension cultures were initiated from fresh callus in liquid B-5 medium (7) containing $0.4 \mu M$ 2,4-D and 2.5 mM $CaCl_2$. Suspension cultures were grown in 50 mL of medium in 250-mL Erlenmeyer flasks at $25^\circ C$ in the dark with shaking at 90 to 100 rpm. These cultures were transferred into fresh medium at 7-d intervals at an initial density of 3.5% (PCV). Callus stock cultures were maintained on the same medium supplemented with $2 \mu M$ benzyladenine and solidified with 0.8% purified agar.

Growth of suspension cultures was measured as PCV. The contents of a single flask were transferred to a 50-mL plastic screw-top tube and centrifuged at $5,000g$ for 5 min in a swinging bucket rotor. Packed cell volume was measured by the volume markings on each tube and is expressed as percentage of culture volume.

Elicitor Preparation

PFE was prepared from a solution of 2% polygalacturonic acid (Sigma grade II) adjusted to pH 5.4 with NaOH. To prepare elicitor fractions with different average polymer lengths, the substrate solution was enzymatically hydrolyzed for various time intervals between 0 and 41 h at $30^\circ C$ with 0.031 units/mL of pure endopolygalacturonase (23). To stop hydrolysis, solutions were brought to pH 6.4 with concentrated NaOH and then autoclaved. In addition, a limit digest was prepared with 2.5-fold more enzyme and a pH of 4.9 (optimum) for hydrolysis. Average polymer length was determined for all samples by measurement of reducing termini (23) with D-galacturonic acid monomer as standard.

Large scale preparations of PFE were prepared as above using the conditions for partial hydrolysis and a digestion time of 23.5 h. The resulting material had an average polymer length of 7 and was stored as a lyophilized dry powder.

Elicitation of Cells

Cell suspension cultures were elicited by the aseptic addition of elicitor in aqueous solution. Solutions of elicitor used for treatment of cells contained 50 mg/mL PFE and had been filter sterilized. Immediately following the addition of elicitor, cultures were returned to standard growth conditions (90–100 rpm, $25^\circ C$, dark). These conditions were maintained throughout the interval of elicitor treatment (usually 12 h).

Lignin Measurement

Lignin was assayed quantitatively by derivatization with thioglycolic acid (36) from alcohol-insoluble residues of sus-

pension cultured cell material. The contents of a single 50-mL culture were first vacuum-filtered over 9 cm Whatman GF/A to remove excess medium. The resulting cell material (approx. 8 g from a 6 d culture) was homogenized (Polytron) in 160 mL of absolute MeOH at top speed for 1 min. The homogenate was then vacuum-filtered over 5.5-cm Whatman GF/A and rinsed with MeOH, and the residue was transferred to a glass beaker for drying (24 h at 60°C). The resulting AIR was used for lignin determination.

To 50 mg of AIR in glass screw-cap tubes was added 5 mL of 2 N HCl and 0.5 mL of thioglycolic acid. The sealed tubes were placed in a boiling water bath and shaken initially to hydrate the AIR. After 4 h at 100°C, the tubes were cooled and the contents transferred to polypropylene centrifuge tubes. Following centrifugation at 30,000g for 10 min at RT, the supernatant was discarded and the pellet washed once with 5 mL of H₂O. The resulting pellet was resuspended in 5 mL of 0.5 N NaOH, sealed with parafilm, and agitated gently at 25°C for 18 h to extract the lignin thioglycolate. The samples were centrifuged (30,000g, 10 min) and the supernatant solutions transferred to glass conical centrifuge tubes. One mL of concentrated HCl was added to each tube and the LTGA allowed to precipitate at 4°C for 4 h. Following centrifugation in a clinical centrifuge at top speed for 10 min, the orange-brown pellets were dissolved in 10 mL of 0.5 N NaOH, and the *A* at 280 nm was measured.

Histochemical Staining

Intact cells were stained for the presence of suberin fatty acids with the histochemical reagents Sudan black B and Nile blue (13).

Enzyme Extraction

To obtain samples containing both free and ionically bound extracellular enzymes, dilute CaCl₂ (50 mM) was first used to displace the ionically bound enzyme fraction from samples of cell suspension. Aliquots of cell suspension (2 × 0.7 mL; 1.4 mL total volume) were removed from cultures to a polypropylene microfuge tube with a 1-mL Pipetman fitted with a disposable tip from which 2 cm had been cut from the tapered end with a razor blade. One molar CaCl₂ (70 μL) was added, and the tube was capped and agitated occasionally for 10 min at RT. The sample was centrifuged at 5,000g for 5 min at 4°C to pellet the cells, and 0.7 mL of supernatant solution was then transferred to a new tube. This fraction was further centrifuged at 16,000g. An aliquot (0.6 mL) of the final supernatant was removed for electrophoretic analysis. In some cases (after 7 d growth), cultures were too dense to allow the representative sampling of cell suspensions by the above technique. In these circumstances, samples of cell suspension were removed by pouring small amounts (5–10 mL) into a new vessel. One molar CaCl₂ (0.05 volume) was added and the extracellular enzyme fraction was similarly isolated.

Electrophoretic Analysis

The peroxidase isoenzyme profiles from healthy and lignifying suspension cultures were examined by native polyacryl-

amide gel electrophoresis. Slab gels 1 mm thick with a 9 cm resolving gel (7.5%) and a 2.5 cm stacking gel (3.75%) were prepared with a 29:1 ratio of acrylamide to bisacrylamide. Cathodic enzymes were resolved at 150 V in the buffer system of Reisfeld *et al.* (27) and anodic enzymes were resolved at 200 V in the buffer system of Davis (2). These gel systems resolve at pH 3.8 and pH 8.3, respectively. Any protein whose pI falls within this range should be resolved by both gel systems, although this does not happen for any of the isoperoxidase species reported in this paper. Samples for electrophoresis (25 μL) contained enzyme extract (18 μL) and 4× sample buffer (6 μL) resulting in final concentrations of 10% glycerol, 1× stacking gel buffer, and 0.002% tracking dye (methyl green for cathodic gels or bromphenol blue for anodic gels). Samples for cathodic gels also contained 8 μg Cyt *c* per lane as a tracking protein which migrates just behind the dye front and is visualized by stains for peroxidase activity. When the dye front had reached 0.5 cm from the bottom of the gel, electrophoresis was stopped, and the gel slabs were immersed in 50 mM Na acetate (pH 5), containing 0.02% 3-amino-9-ethylcarbazole (a synthetic peroxidase substrate) and 5 mM H₂O₂. After 60 min at RT, the gels were rinsed in H₂O and then fixed in 25% MeOH/10% acetic acid. The gel slabs with zones of peroxidase visualized as dark red bands were dried and photographed.

RESULTS

Oligomer Size Dependence of PFE Activity

Oligogalacturonide preparations with various average degrees of polymerization were obtained by limited enzymatic hydrolysis of polygalacturonic acid as described in "Materials and Methods." Six-day cultures were treated for 12 h with 0.5 mg/mL culture of the various oligogalacturonide preparations and then assayed for lignin content of the AIR. Galacturonic acid oligomers of intermediate size clearly elicit the accumulation of lignin in suspension cultured castor bean with 7 being the most effective average degree of polymerization (Fig. 1). Oligomer fractions with size averages of less than 6 or more than 20 were essentially inactive as elicitors of lignin biosynthesis. This indicates that some intermediate degree of enzymatic hydrolysis is necessary to convert endogenous pectic substances into elicitors.

A large-scale PFE preparation was obtained by enzymatic hydrolysis of polygalacturonic acid to an average polymer length of 7. This preparation was used for all other reported experiments.

Identity of Lignin

The thioglycolate assay is specific for lignin as well as for substances such as suberin in which lignin-type polymers are present (9). We used the dyes Sudan black B and Nile blue (13) to stain for the lipid-polyester component of suberin which would be present in samples containing suberin. No differences were observed in the intensity of staining of elicited cells compared to unelicited cells by these dyes, suggesting that lignin, and not suberin, is rapidly biosynthesized in elicited cultures.

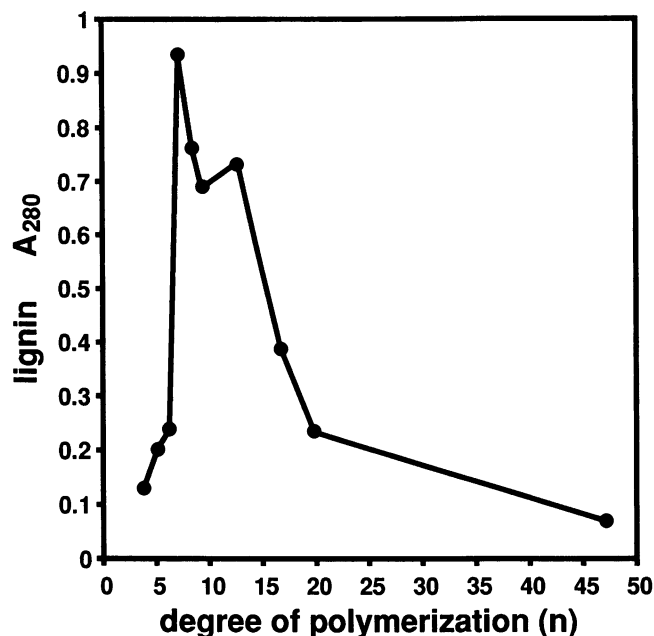


Figure 1. Influence of PFE oligomer size on elicitor activity. Six-day suspension cultures were treated with 0.5 mg/mL culture of oligo-D-galacturonate preparations of various average degrees of polymerization. After 12 h, the cells were harvested and the lignin from 50 mg of AIR was measured. Results are expressed as 280 nm absorbance of LTGA in 0.5 N NaOH (10 mL). Cultures were 32% PCV at the time of addition of elicitor.

A large-scale preparation of LTGA from cultures elicited for 24 h was performed by scaling up the procedure described in "Materials and Methods." Before finally being dissolved in alkali, the LTGA pellet was washed once more with water and then lyophilized to dryness. The UV spectrum of a solution containing 100 $\mu\text{g/mL}$ of this LTGA preparation in 0.5 N NaOH from 6-d elicited cells is presented in Figure 2. It is very similar to previously reported spectra for LTGA (36). The absorbance of this solution at 280 nm was 0.834.

Influence of Culture Age on Lignin Biosynthesis and Peroxidase Isoenzyme Activities

Suspension cultures ranging in age from 3 to 9 d were treated with PFE (0.5 mg/mL culture) and cells were harvested 12 h later. Control cultures lacking additions were harvested simultaneously with the elicited cultures. Lignin contents from 50 mg of AIR were measured and are plotted in Figure 3 along with the standard growth curve of healthy (unelicited) cultures as measured by PCV.

Six-day cultures representing the "late log-phase" of culture growth (30–40% PCV at time of addition of elicitor) accumulate the most lignin in response to PFE. Cells assayed significantly before or after this time accumulate markedly less lignin in response to elicitor. Lignin contents from control cultures remained low during all stages of growth.

In a similar experiment, PFE was added to cultures of increasing age and aliquots of cell suspension were removed at 0 and 12 h after addition. The ionically bound proteins were released into the medium by treatment with CaCl_2 , and

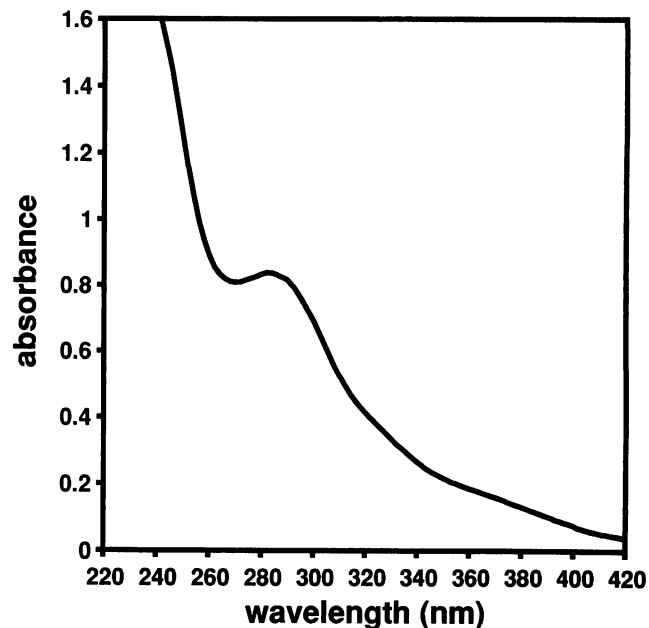


Figure 2. UV spectrum of 100 $\mu\text{g/mL}$ LTGA in 0.5 N NaOH. $A_{280 \text{ nm}}$: 0.834.

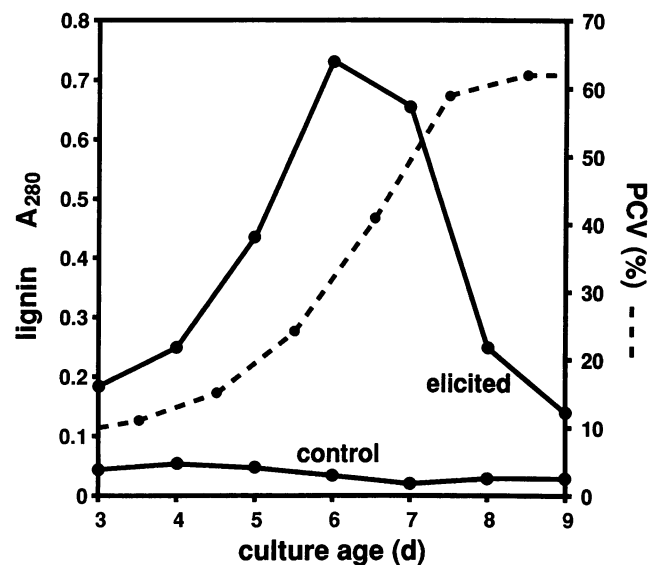


Figure 3. Influence of culture age and elicitor treatment on lignin biosynthesis. Cultures of the indicated age were treated with PFE for 12 h, and the lignin was assayed from 50 mg of AIR. Control cultures lacking treatment during the 12 h interval were harvested simultaneously and also assayed for lignin. The growth curve measured as PCV is superimposed.

the isoperoxidases in the resulting pool of extracellular proteins were analyzed by native gel electrophoresis. Two extracellular anionic isoperoxidase species, designated A1 and A2, are present in castor bean suspension cultures (Fig. 4a). In healthy cultures the activity of both species appears to increase as a function of culture density. In 3- to 6-d elicitor-treated cultures, levels of A1 increase markedly after the addition of elicitor, whereas the activity of A2 is essentially unaffected by

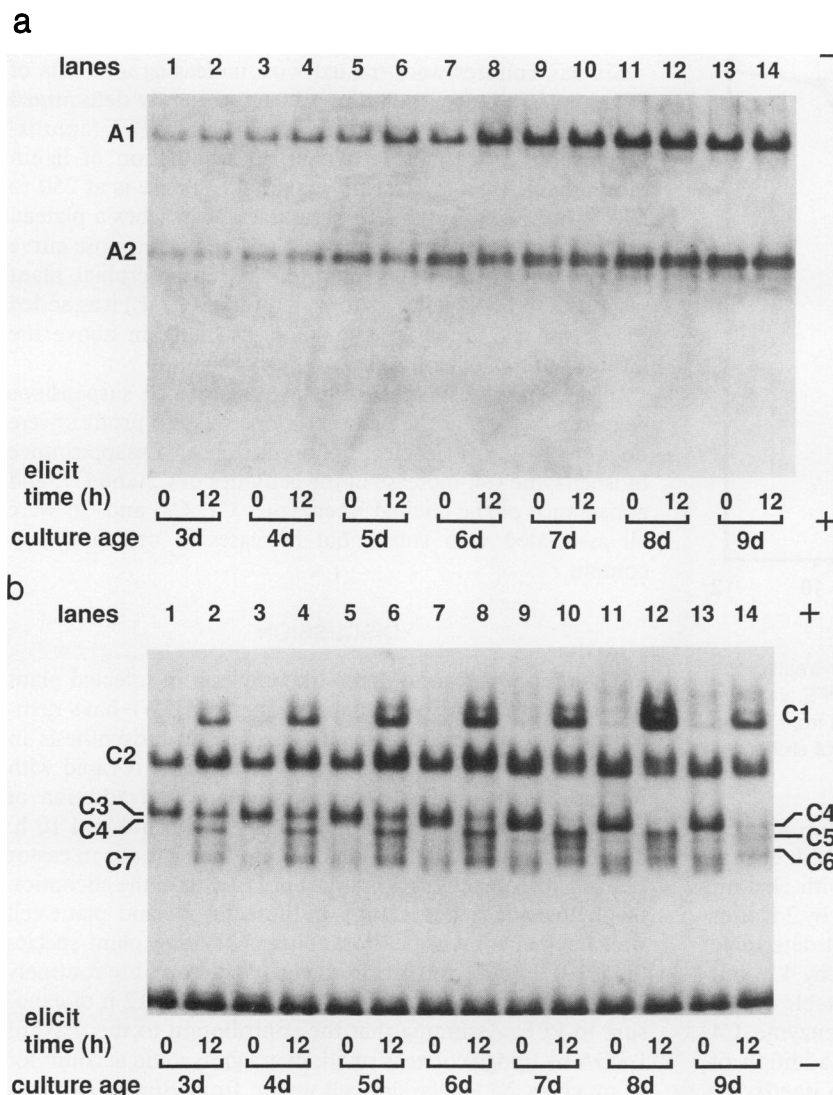


Figure 4. Influence of culture age and elicitor treatment on extracellular isoenzymes of peroxidase. PFE was added to cultures of the indicated age, and extracellular enzyme samples were prepared at zero time (control, odd-numbered lanes) and 12 h (elicited, even-numbered lanes). Samples were subjected to either anodic (a) or cathodic (b) native gel electrophoresis and stained for peroxidase activity. Sample volumes were not adjusted for culture growth (cell density) or for displacement of medium volume by cells. The positions of isoperoxidase bands are indicated at the sides of the outermost lanes. The visible band at the bottom of the cathodic gel is Cyt c, added as a tracking protein.

elicitor treatment. There are four cationic isoenzymes of peroxidase in healthy cultures (Fig. 4b). C2 is the most abundant; its control levels increase until day 7 after which they decline. Activity in C2 increases markedly in 3- to 6-d cultures after treatment with elicitor, but its activity seems to decrease following elicitor-treatment in 7- to 9-d cultures. Isoenzyme C3 is only abundant in early stages of culture growth (3–5 d). Conversely, isoenzyme C4 (slightly more mobile) is evident only in older cultures (5–9 d). Whereas C4 disappears in elicited cultures, at least some of the activity in C3 persists. The most mobile species is C7 which is possibly a pair of closely resolved bands. C7 activity is higher in elicited 3- to 6-d cultures.

In addition to the isoperoxidases detectable in healthy cultures, three additional cathodic isoenzyme species are present in readily detectable levels only after elicitation. Of these, C1 is the most abundant. Elicited activity in C1 peaks at 6 d in tandem with lignin biosynthesis and then again at 8 d when lignin biosynthesis is much lower. Estimates from serial dilution indicate that activity in C1 is at least 16-fold higher in

elicited 6-d cultures than in controls. Isoenzymes C5 and C6, in a pattern more closely matching that of lignin biosynthesis, are elicited maximally in 6- to 7-d lignifying cultures and to a lessening extent after 7 d.

Time Course of Lignin Biosynthesis and of Changes in Cathodic Isoperoxidase Activities in Elicited Cultures

Lignin contents were measured from AIR of 6-d cultures exposed to PFE for increasing lengths of time. The first detectable increase in lignin occurs between 2 and 3 h following addition of elicitor (Fig. 5). Lignin accumulates at a rapid and relatively constant rate during the 4 to 10 h interval after which its rate of appearance declines. Lignin levels continue to increase by approximately 0.15 absorbance units every 12 h during the 12 to 36 h time interval (data not shown).

The time course after elicitation of changes in extracellular cationic isoperoxidase activities is presented in Figure 6. No changes are observed in the isoperoxidase profile within the first hour after addition of elicitor (lanes 1–3), indicating that PFE has no immediate influence by itself on the detectable

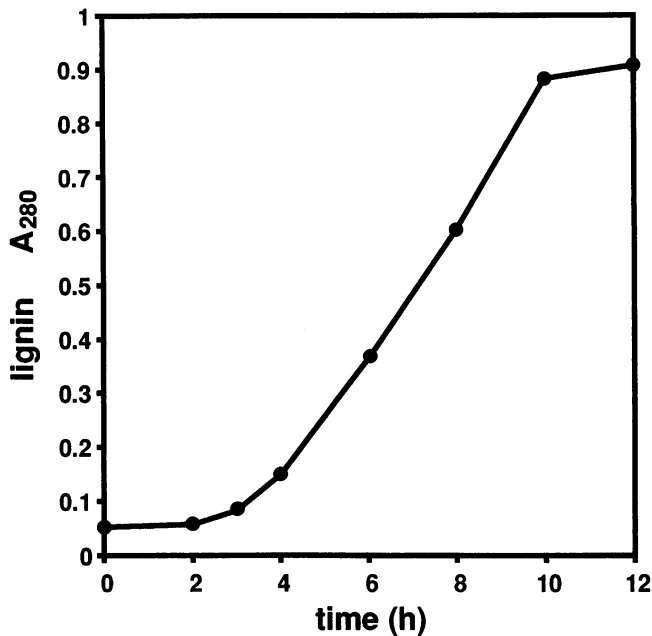


Figure 5. Time course of lignin biosynthesis in elicitor-treated cultures. Six-day suspension cultures were treated with PFE and then harvested at the indicated times. Lignin was measured from 50 mg of AIR. Cultures were 40% PCV at the time of addition of elicitor.

activity of any of these species. Isoenzymes C2, C3, and C7 all increase slowly in healthy cultures during the 12-h elicitation interval (lanes 12–14). In cultures treated with elicitor, increase in the activity of isoenzyme C1 is evident by 2 h after addition, whereas isoenzymes C5 and C6 are not detectable until 4 h. The level of C2 is increased noticeably by 4 h, but small increases before this time may not be detectable due to the abundance of C2 in healthy cultures. Isoenzyme C4 disappears during the interval of 1 to 5 h after addition of elicitor and, as it does, the band corresponding to isoenzyme C3 (slightly lower mobility) becomes visible. Because of the presence of C4, it is difficult to determine whether levels of C3 change after addition of elicitor. The activity of isoenzyme C7 begins to increase by 6 h.

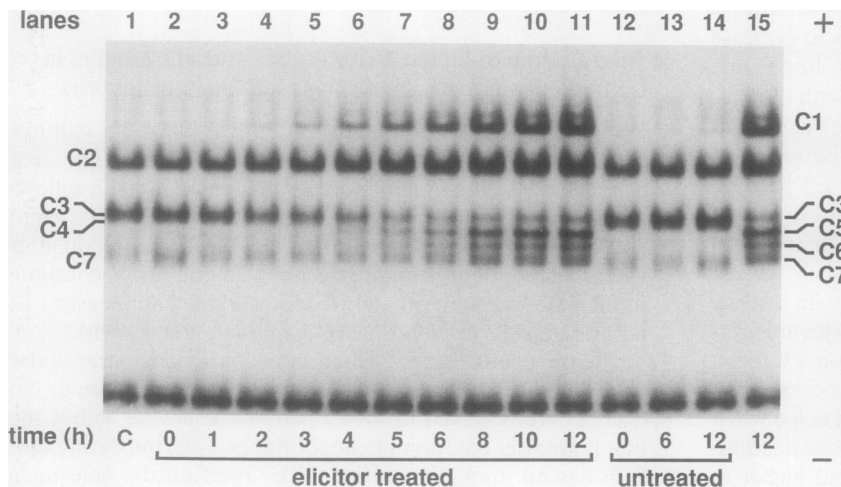


Figure 6. Time course of changes in cathodic peroxidase isoenzymes in elicitor-treated cultures. PFE was added to 6-d cultures and samples of cell suspension were removed at the specified times. Extracellular enzyme extracts from these samples were subjected to cathodic native gel electrophoresis. Lane 1 is prior to addition of elicitor, lanes 2 through 11 and 12 represent elicitor-treated cultures, and lanes 12 through 14 are untreated control cultures for comparison. Cultures were 34% PCV at the time of addition of elicitor.

Dose Response

Six-day cultures were treated with increasing amounts of PFE and lignin accumulation was subsequently determined (Fig. 7). There appears to be a threshold level of PFE (approximately 50 $\mu\text{g}/\text{mL}$) below which no stimulation of lignin biosynthesis is observed. Half maximal response is at 250 to 300 $\mu\text{g}/\text{mL}$. The response is saturable and reaches a plateau at elicitor levels higher than 500 $\mu\text{g}/\text{mL}$. This response curve is very similar to others reported for antimicrobial plant responses to PFE. Cultures to which water (1 mL) was added instead of elicitor showed no increase in lignin above the levels of untreated controls.

In an analogous experiment, aliquots of cell suspensions were removed, and the cathodic isoperoxidase profiles were obtained by native gel electrophoresis (Fig. 8). Disappearance of isoenzyme C4, increase in the activities of C2 and C7, and appearance of the elicited isoenzymes C1, C5, and C6 were all associated with substantial increases in cellular lignin content.

DISCUSSION

The accumulation of lignin-like material in infected plant tissue is a widely reported phenomenon (34). We have demonstrated that pectic fragments elicit lignin biosynthesis in castor bean suspension cultures. This response is rapid with increases in lignin first detectable by 3 h after addition of elicitor and maximal rates of synthesis between 4 and 10 h. The time course of elicitation of lignin biosynthesis in castor bean suspension cultures is as fast or faster than the elicitation of phytoalexin accumulation by both fungal and plant cell wall fragments in suspension cultures of other plant species (3, 5, 19). Lignin contents from elicited cultures are routinely 10-fold higher than from control cultures after 12 h of exposure to PFE. Assuming that the contribution to the mass of LTGA by thioglycolate is small, then lignin could account for as much as 2% of the dry cell weight from elicited cultures. Curiously, castor bean seedlings, when treated with PFE in a typical split seedling bioassay (23), did not accumulate lignin (data not shown). The plant tissue used in the split seedling assay is predominantly endosperm, which perhaps displays a

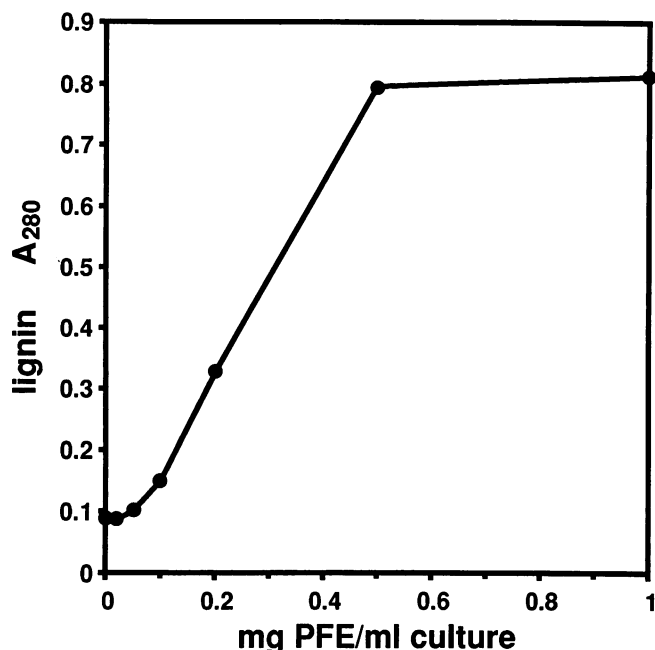


Figure 7. Dose-response curve for elicitation of lignin biosynthesis. Six-day cultures were treated with increasing amounts of elicitor and cells were harvested for lignin assay 12 h later. Lignin was measured from 50 mg of AIR. Cultures were 32% PCV at the time of addition of elicitor. A_{280} of lignin from cultures treated with 1 mL H_2O : 0.081.

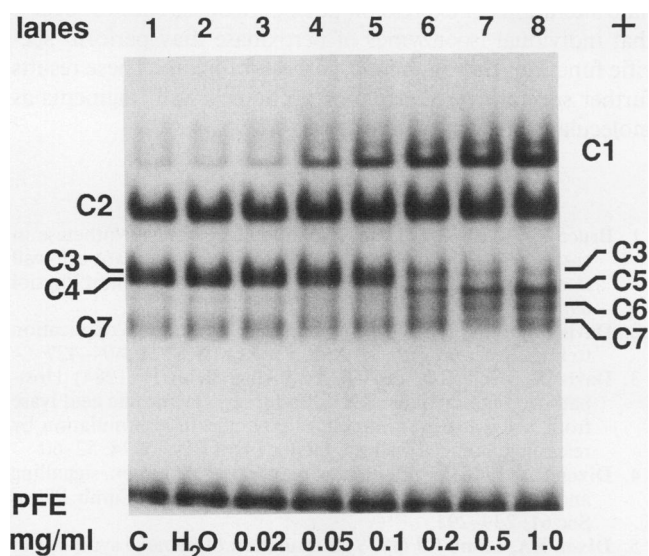


Figure 8. Dose dependence of changes in cathodic isoenzymes of peroxidase. Extracellular enzyme extracts were prepared from cultures treated with elicitor for 12 h as in Figure 7. Samples of these extracts were subjected to cathodic native gel electrophoresis. Lane 1 is from untreated cultures, lane 2 from cultures treated with H_2O (1 mL), and lanes 3 through 8 from cultures elicited with increasing quantities of PFE. Cultures were 38% PCV at the time of addition of elicitor (6 d).

different biochemical pattern of response than observed in suspension cultured cells.

The thioglycolate procedure has been reported (9) to measure the lignin component of suberin, a lamellar, polymeric

matrix composed of alternating domains of lignin and fatty acid-derived polyester (17). Suberin biosynthesis has been reported in plants following infection (34), although its appearance is more commonly observed during wound healing (17). The histochemical stains Sudan black B and Nile blue partition into lipids and would be expected to stain the polyester component of suberized cells (13). No differences in the intensity of staining of cultured cells by either of these dyes were observed after elicitor treatment. These results suggest that lignin, as opposed to suberin, is rapidly synthesized in elicitor-treated cultures.

Coincident with lignification in castor bean suspension cultures is a striking pattern of alteration in the isoenzyme profile of extracellular peroxidase. These changes can be classified into four categories: (a) appearance of new isoenzymes of peroxidase not present at readily detectable levels in healthy cultures (C1, C5, and C6), (b) increase in activity during elicitation of specific peroxidase isoenzymes which were already present at significant levels in healthy cultures (A1, C2, and C7), (c) disappearance of the detectable activity of particular isoperoxidase species (C4), and (d) no appreciable change in isoenzymatic activity (A2 and C3).

Large increases in extractable peroxidase activity are frequently associated with fungal infection in plants, although the increases do not always correlate with race/cultivar specificity. The search for such a correlation is complicated by the fact that the rate and extent of spread of an invading fungus are usually much greater in compatible interactions. The appearance of new isoenzymes of peroxidase in infected tissue has also been previously reported (15, 31, 33, 37). It is not usually clear, however, whether the new enzymes are of plant or fungal origin. This question has added importance in light of the probable involvement of fungal peroxidase and H_2O_2 in the enzymatic degradation of lignin by wood-degrading fungi (21). When elicitors are used instead of infection to induce the accumulation of new peroxidase isoenzymes, as in the present work and also as demonstrated in wheat (25), the new enzymes are by necessity of plant origin.

The experimental techniques which have been used in many laboratories for the separation and detection of plant peroxidase are frequently incapable of providing a complete and unambiguous picture of the isoenzyme profile. This is due primarily to the extreme range of isoelectric pH values common to this group of proteins. Peroxidase isozymes from horseradish have been reported to migrate off both ends of wide pH range isoelectric focusing gels (12) and can therefore escape detection. Anodic native gel electrophoresis is also commonly employed as the sole experimental technique. Many basic peroxidases, however, do not migrate into such a gel. The combination of both anodic and cathodic native gel electrophoresis will accurately portray the isoperoxidase profile. The only potential complication of a two-gel technique such as we have used is that some proteins (isoelectric point range 3.8–8.3) will migrate into both gels. In our case, however, none of the isoperoxidases from castor bean suspension cultures enter the resolving region of both gel systems.

Extraction of intact cells with $CaCl_2$ was necessary to obtain good resolution and accurate representation of some of the ionically bound cathodic isoperoxidases. Calcium salts are

efficient extractants for ionically bound cell wall peroxidase (32). Calcium ions displace the bound enzymes from their interaction with pectic cell wall components and also eliminate their interaction with pectic elicitor, thus allowing their free migration into gels. When CaCl_2 is not used in the isolation procedure, isoperoxidases C4, C5, C6, and C7 are no longer detectable on cathodic gels, and the species C2 and C3 migrate irregularly. C1 appears to have little or no affinity for cell walls, nor do either of the anionic isoenzymes.

Peroxidase catalyzes the conversion of cinnamyl alcohols into their corresponding free-radicals, which spontaneously polymerize into lignin (11). Peroxidase is also likely to be responsible for the generation of H_2O_2 required during this step from molecular oxygen and extracellular NADH (11). The dramatic changes we observed in the activity of some extracellular isoperoxidases during lignification suggest that individual isoenzymes of peroxidase may have unique functions during lignin biosynthesis. It is important to emphasize, however, that no specific function for any plant isoperoxidase species has yet been demonstrated. Furthermore, several other biochemical processes which have either been shown to occur, or may plausibly occur, during plant disease resistance also require extracellular peroxidase. These are (a) covalent insolubilization of hydroxyproline-rich glycoproteins within the cell wall, (b) crosslinking of wall-esterified *p*-coumaric or ferulic acids, (c) membrane lipid peroxidation, (d) oxidative degradation of auxin, *i.e.*, IAA oxidase activity, and (e) generation of H_2O_2 required for these events. We have not attempted to determine whether any of these events occur in elicited castor bean suspension cultures.

In cucumber seedlings, the systemic accumulation of an anionic isoenzyme of peroxidase has been associated with induced systemic protection against pathogen attack (10). It is possible that elicited isoenzymes of peroxidase in our suspension cultures may normally have a function in induced protection of castor bean plants, although the phenomenon of induced immunity has not been demonstrated in castor bean. It is also possible that elicited isoenzymes may have some secondary feature which makes them functionally superior to normal plant isoenzymes during attempted infection such as, for example, increased resistance to proteolytic inactivation.

During the culture interval examined, the capacity of elicited cells to synthesize lignin increases until 6 d of growth when the cultures have reached 30 to 40% PCV, or roughly half the density of stationary phase. After this time, the ability of cultures to synthesize lignin steadily declines. Dependence of the elicitation of phytoalexin production on the stage of culture growth has been reported for suspension cultures of other plant species (3, 18, 20). The increases in activity of isoperoxidases A1, C2, and C7, along with the appearance of isoenzymes C5 and C6, all parallel the amount of elicitable lignin with respect to culture age. Isoperoxidase C1, although very abundant in 6-d elicited cultures, is maximally elicitable at 8 d, a time when elicitation of lignin biosynthesis is markedly decreased. The most notable difference that we are aware of between isoperoxidases C1, C5, and C6 is in their cell wall-binding affinities, with C5 and C6 being entirely wall bound in lignifying cultures and C1 being essentially unbound (data

not shown). Although overall less abundant than C1, C5 is the most abundant of the tightly wall-bound isoperoxidases (C5, C6, and C7) in elicited cultures and is likely to be present at relatively high local concentrations within the plant cell wall. What exact influence the differential wall binding of isoperoxidases might have on lignin biosynthesis is still uncertain.

The molecular mechanisms responsible for increases in activity of isoperoxidase species in elicited castor bean suspension cultures have not yet been identified. The time lag of 2 to 4 h before the increases are first detectable is consistent with a requirement for *de novo* protein biosynthesis, possibly from elicitor-specific peroxidase mRNAs. Elicitor-activated alterations in the extracellular isoperoxidase profile of castor bean suspension cultures may also be the result of posttranslational events. Presecretory processing and glycosylation of peroxidase polypeptides could change upon elicitation and have major effects on observed isoenzymatic activity. Isoenzymes originally localized within or targeted toward particular organelles may be secreted. Previously existing extracellular isoperoxidases could also be activated or interconverted.

Deposition of lignin by plant cells is part of a coordinated pattern of biochemical responses which are intended to protect plants against microbial attack. We consider the elicitation of lignin biosynthesis by PFE in 6-d suspension cultures of castor bean to be a model of events which may occur in intact plants during attempted fungal infection. The coordinate alterations of expression of isoperoxidase species suggest that individual isoenzymes of peroxidase may perform specific functions during the biosynthesis of lignin. These results further substantiate the role of pectic cell wall fragments as molecular signals of plant disease.

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