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Fungal Elicitor-Mediated Responses in Pine Cell Cultures[1]

III. Purification and Characterization of Phenylalanine Ammonia-Lyase

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ABSTRACT

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is involved in the lignification of pine suspension cultures in response to an elicitor prepared from an ectomycorrhizal fungus. To elucidate the molecular basis of this response, PAL was purified to homogeneity from jack pine (*Pinus banksiana*) suspension cultures using anion-exchange and chromatofocussing fast protein liquid chromatography. Physical characterization of the enzyme revealed that pine PAL was similar to PAL from other plant sources. Pine PAL had a pH optimum of 8.8, an isoelectric point of 5.75, and a native molecular mass of 340 kilodaltons. The enzyme appears to be a tetramer composed of 77 kilodalton subunits. Chromatographic and western blot analyses were used to identify possible isoenzymic changes in pine PAL in response to elicitation and to determine the nature of the increase in PAL activity associated with inducible lignification in these cultures. Only one species of PAL was detected in *P. banksiana* cell cultures and increased quantities of this protein were correlated with the enhanced enzyme activity observed in elicited cultures. *P. banksiana* PAL was not feedback-inhibited by a wide range of phenolic compounds at micromolar concentrations, including the reaction product cinnamic acid. Our data suggest that a different set of metabolic and molecular controls must be in place for the regulation of PAL in pine.

PAL[2] (EC 4.3.1.5) is the entrypoint enzyme into phenylpropanoid metabolism and thus regulates the production of the monomers necessary for lignin biosynthesis and other phenolic compounds in plant cells. Phenolics accumulate at the site of fungal ingress during ectomycorrhizal development (18), and an increase in phenylpropanoid metabolism, dominated by cell wall lignification, has been observed in cultured pine cells after treatment with elicitor from an ectomycorrhizal fungus (4, 5). This pattern differs from known pine defense and stress responses that include localized cell death and production of putative phytoalexins such as pinosylvin (12, 14, 16).

We have recently demonstrated that PAL plays a key role in the lignification response in elicited pine cultures (4, 5). In elicited pine cell cultures, PAL activity increases to levels approximately 10-fold greater than the corresponding controls by 24 h postelicitation, coinciding with the initiation of cell wall lignification (4). The activity of PAL was rapidly induced in elicited pine cell cultures (a significant increase over the activity observed in control cultures was evident by 6 h postelicitation), but this increase was transient and returned to control levels by 72 h postelicitation (4). Treatment of pine cultures with the PAL inhibitor AIP and feeding with radiolabeled phenylalanine has revealed the central importance of PAL in the lignification response (5). These data suggest that the contact between fungal symbiont and the plant may modulate the expression of PAL activity. Control of phenylpropanoid metabolism through the modulation of PAL activity has been well documented for other plant-microbe interactions (10, 11). As part of our investigation of the regulatory role of PAL in elicited pine suspension cultures, we have purified pine PAL to homogeneity and characterized the enzyme.

MATERIALS AND METHODS

Plant Material

Suspension cultures of jack pine (*Pinus banksiana* Lamb.) were established from root-derived callus cultures. Cultures were maintained on a 10-d subculture cycle in modified Murashige and Skoog medium as described by Campbell and Ellis (4).

Fungal Elicitor

Thelephora terrestris Fr. was maintained in modified Melin's medium as liquid still cultures at 25°C in the dark. Elicitor was prepared by collecting 3-week-old mycelia on Miracloth under suction. Mycelia were rinsed with water, weighed, resuspended in sterile distilled water to a final concentration of 50 mg fungal mycelium/mL, and blended in a Virtis blender at full speed for 1 min. A one-tenth volume of water

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[2] Abbreviations: PAL, phenylalanine ammonia-lyase; AIP, 2-aminoindane-2-phosphonate; AOPP, L-α-aminooxy-β-phenylpropionic acid; FPLC, fast protein liquid chromatography; TBS, Tris-buffered saline; IC_{50}, concentration for 50% inhibition of activity.

was added to the slurry and the mixture was autoclaved for 5 min. The resultant preparation is referred to as "elicitor."

Elicitation of Pine Cell Cultures

After transfer to fresh medium, pine cell cultures were incubated for 6 d prior to inoculation with 1 mL of elicitor preparation. Controls were inoculated with sterile distilled water. Incubation continued at 25°C for the indicated time periods.

Determination of PAL Activity

The PAL assay was as described by Campbell and Ellis (4). Briefly, PAL activity was determined by monitoring the conversion of L-[U-^{14}C] phenylalanine to cinnamate in cell protein extracts. During PAL purification, the assay was modified by incubating the enzyme for only 15 min. Protein determinations were made according to Bradford (1) using γ-globulin as a standard.

Purification of PAL from *P. banksiana*

The following purification protocol was completed within 16 h, with all steps, including chromatography, performed at 4°C. All solutions used during the purification contained fresh 5 mM 2-mercaptoethanol.

Tissue from pine suspension cultures was harvested by suction filtration onto Miracloth and then frozen in liquid nitrogen. Frozen tissue (250 g) was extracted with 500 mL 200 mM Tris acetate, pH 7.5, for 20 min with stirring, and filtered through Miracloth. Amberlite XAD-7 resin (50 g) was added to the filtrate and the mixture was stirred for 2 min. The resin was removed by filtration through Miracloth and the filtrate was brought to 0.1% (v/v) in protamine sulfate, using a 2% (v/v) solution of neutralized protamine sulfate. The mixture was stirred for 20 min and then centrifuged for 10 min at 12,000*g*. The protamine sulfate supernatant was brought to 50% saturation with ammonium sulfate (0.314 g ammonium sulfate/mL of supernatant). The solution was stirred for 20 min and then centrifuged at 12,000*g* for 20 min. The supernatant was decanted and the pellet redissolved in 20 mL 0.050 M Tris acetate, pH 7.5, containing 1 M NaCl. The redissolved pellet was desalted by passage through a bed of Biogel P-6DG resin according to the centrifugal method of Penefsky (20) into 0.050 M Tris acetate, pH 7.5.

The desalted extract was subjected to semi-preparative anion-exchange FPLC on an HR 10/10 Mono Q column (Pharmacia). Solvent A: 0.050 M Tris acetate, pH 7.5; solvent B: 500 mM NaCl in 0.050 M Tris acetate, pH 7.5. Gradient conditions: 0% B for 5 min; 0 to 30% B over 30 min; 30 to 100% B over 5 min; 100% B for 5 min. Flow rate: 4 mL/min. Fractions were collected at 1 min intervals, mixed by gentle inversion, and stored at 4°C. The PAL activity was determined for a 50 μL aliquot of each fraction and the most active fractions were pooled and desalted into 0.025 M histidine HCl, pH 6.0.

The pooled and desalted Mono Q fractions were chromatofocused between pH 6 and 5 on FPLC with an HR 5/20 Mono P column (Pharmacia). Solvent A: 0.025 M histidine HCl, pH 6.0; solvent B: 10% (v/v) Polybuffer 74 (Pharmacia) in distilled water. Gradient conditions: 0% B for 10.7 min; 100% B immediately; 100% B for 49.3 min. Flow rate: 0.75 mL/min. Fractions were collected at 1 min intervals, spiked with 100 μL 2 M Tris acetate, pH 7.5, mixed by gentle inversion, and stored at 4°C. The PAL activity was determined for a 50 μL aliquot of each fraction, and the most active fractions were pooled and desalted into 0.025 M NaCl in 0.050 M Tris acetate, pH 7.5. The pH of the eluate was monitored using a Pharmacia flow-through electrode.

The pooled, desalted Mono P fractions were subjected to anion-exchange FPLC on an analytical HR 5/5 Mono Q column (Pharmacia). Solvent A: 0.050 M Tris acetate, pH 7.5; solvent B: 500 mM NaCl in 0.050 M Tris acetate, pH 7.5. Gradient conditions: 5% B for 5 min; 5 to 25% B over 30 min; 25 to 100% B over 5 min; 100% B for 5 min. Flow rate: 1 mL/min. Fractions were collected at 1 min intervals, mixed by gentle inversion, and stored at 4°C. The PAL activity was determined for a 50 μL aliquot of each fraction and the most active fractions set aside for characterization. Total protein was assessed during each chromatographic separation by monitoring absorbance at 280 nm.

SDS-PAGE of PAL

SDS-PAGE gels were run using the protocol of Laemmli (17). All gels contained 8% acrylamide and were silver stained to visualize the protein.

Determination of Native Mol Wt on Nondenaturing PAGE

PAL and high mol wt standards were run in native PAGE gels of either 6, 8, or 10% acrylamide. Nondenaturing PAGE gels were run according to the protocol of Laemmli (17) on minigel apparatus, but with SDS omitted from the loading and running buffers. Migration relative to the marker front *versus* percentage of acrylamide was plotted for each standard and for PAL. The slope for each standard was determined and plotted against the log of its M_r to generate a Ferguson plot. The calculated slope for PAL was used to interpolate the relative M_r from the regression line of the Ferguson plot.

PAL activity was measured in the native PAGE gel by eluting 5 mm gel slices from one lane of the gel under standard PAL assay conditions, except that assays of PAL activity in gel slices were incubated at 30°C for 2 h.

Determination of Native Mol Wt on Calibrated Superose 6

Mol wt standards were run on an HR 10/30 Superose 6 column running in 0.05 M Tris acetate, pH 7.5, containing 0.25 M NaCl. The column was run at 4°C at a flow rate of 0.25 mL/min, monitoring the eluate at 280 nm. The elution volume (V_e) relative to the void volume (V_o) *versus* the log of the M_r was plotted. A M_r for PAL was determined by interpolating from the regression line of the plot using the V_e/V_o value for PAL obtained by running 200 μL of the pooled

purified enzyme under the conditions outlined above on the calibrated column. The elution time for PAL was determined both by monitoring the A_{280} and by measuring PAL activity in the eluate.

Investigation of PAL Isoforms via Chromatofocusing on Mono P

Tissue was buffer extracted and the extract subjected to clarification with Amberlite XAD-7 and 0.1% protamine 4sulfate, followed by concentration using a 0 to 50% ammonium sulfate precipitation as outlined in the PAL purification above.

Initially, a wide pH range was utilized so as not to miss any potential isoforms. In this case, the ammonium sulfate pellet was redissolved in 1 mL 0.025 M Bis-Tris/HCl, pH 7.0, containing 500 mM NaCl and then desalted into 0.025 M Bis-Tris/HCl, pH 7.0. The desalted extract was loaded onto an HR 5/20 Mono P column (Pharmacia) preequilibrated with 0.025 M Bis-Tris/HCl, pH 7.0, and subjected to chromatofocusing between pH 7 and 4. Solvent A: 0.025 M Bis-Tris/HCl, pH 7.0; solvent B: 10% (v/v) Polybuffer 74/water, pH 4.0. Gradient conditions: 0% B for 8 min; 100% B immediately; 100% B for 42 min. Solvent flow: 0.75 mL/min.

For analysis of potential PAL isoforms over a narrow pH range, the ammonium sulfate pellet was redissolved in 1 mL 0.025 M histidine HCl, pH 6.0, containing 500 mM NaCl and then desalted into 0.025 M histidine HCl, pH 6.0. The desalted extract was loaded onto an HR 5/20 Mono P column (Pharmacia) preequilibrated with 0.025 M histidine HCl, pH 6.0, and subjected to chromatofocusing between pH 6 and 5. Solvent A: 0.025 M histidine HCl, pH 6.0; solvent B: 10% (v/v) Polybuffer 74 (Pharmacia) in distilled water. Gradient conditions: 0% B for 10.7 min; 100% B immediately; 100% B for 49.3 min. Solvent flow: 0.75 mL/min.

Fractions were collected at 1 min intervals for all chromatographic analyses, spiked with 100 μL 2 M Tris acetate, pH 7.5, mixed by gentle inversion, and stored at 4°C. The PAL activity was determined for a 50 μL aliquot of each fraction, and the most active fractions were pooled and desalted into 100 mM potassium borate, pH 8.8.

In each case, equivalent amounts of protein were loaded. Protein was detected during chromatography by monitoring absorbance at 280 nm, and the pH of the eluate was monitored using a Pharmacia flow-through electrode. All solutions utilized during chromatographic analysis of PAL contained fresh 5 mM 2-mercaptoethanol.

Western Blot of PAL

The most active fractions from the Mono P (pH 6–5) separation described above were pooled and 20 μL pooled extract was diluted 1:1 with Laemmli's gel loading buffer (17) and applied to a 0.75 mm PAGE minigel. The gel was run under denaturing conditions (17), equilibrated in Tris-glycine buffer, pH 8.3 (25 mM Tris-HCl, 190 mM glycine, 20% [v/v] methanol), and then electroblotted (250 mA, constant current, 2.5 h, 4°C) in Tris-glycine buffer onto polyvinylidene

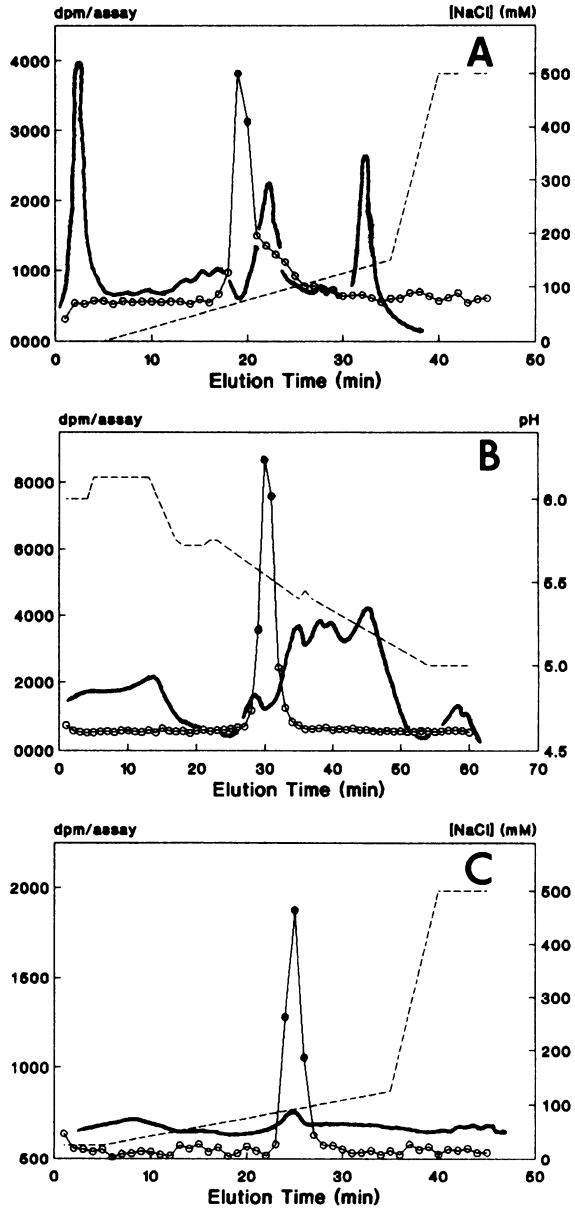

Figure 1. Purification of PAL from *P. banksiana* cell cultures. PAL was purified by sequential application to (A) semi-preparative anion-exchange, (B) chromatofocusing, and (C) analytical anion-exchange FPLC. The most active fractions (●) at each stage were pooled and used for the subsequent analyses. PAL activity (○), NaCl or pH gradient (– – –), and protein (—) were monitored throughout.

Table I. *Purification of Phenylalanine Ammonia-Lyase from Pinus banksiana Cell Cultures*
Purification was as outlined in "Materials and Methods."

Purification Stage	Protein	Activity	Specific Activity	Recovery of Activity	Purification
	mg	*pKat*	*pKat mg*$^{-1}$	%	*-fold*
Crude	420	7889	19		
Post XAD-7	320	5730	18	73	0.9
Protamine Sulfate s/n	130	4822	37	61	1.9
0–50% Ammonium Sulfate pellet	87	2429	28	31	1.5
Desalting	77	1947	25	25	1.3
Mono Q I	5.3	645	122	8	6.5
Mono P	0.03	178	5,933	2	310.0
Mono Q II	0.0005[a]	89	178,000	1	9400.0

[a] Estimated from silver-stained gel.

fluoride membrane (Westran, Schleicher & Schuell). After electroblotting, the membrane was stained for total protein using 0.2% (w/v) Ponceau S in 3% (w/v) TCA and the mol wt markers recorded in pencil on the blot. The blot was destained with several 20 mL washes of TBS (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) and then blocked with 5% nonfat dry milk (Boehringer Mannheim) in TBS for 2 h at room temperature with gentle agitation. The blot was then probed with rabbit anti-alfalfa PAL polyclonal antibodies (15) (courtesy of RA Dixon, Samuel Roberts Noble Foundation) diluted 1:2000 in 5% nonfat dry milk in TBS for 12 h at room temperature. Unbound antibody was removed with four 20 mL washes of TBS and the blot was incubated for 2 h at room temperature in goat anti-rabbit immunoglobulin G-alkaline phophatase conjugate (Sigma) diluted 1:1000 in 5% nonfat dry milk in TBS. Unbound secondary antibody was removed with four 20 mL washes of TBS. Bound alkaline phosphatase activity was detected on the blot using the reagents and the manufacturer's instructions for the Boehringer Mannheim Non-radioactive DNA Detection System.

Effect of Phenolic Compounds and Inhibitors on PAL Activity

The effects of various phenolic compounds on the *in vitro* activity of PAL were tested on enzyme that had been partially purified by chromatofocusing (pH 6.5) as outlined above for isozyme analysis. Phenolic compounds were initially dissolved at 100 mM concentrations in 50% (v/v) methanol. These stock solutions were diluted 1:100 in 100 mM potassium borate, pH 8.8, and the latter solution was diluted in the PAL

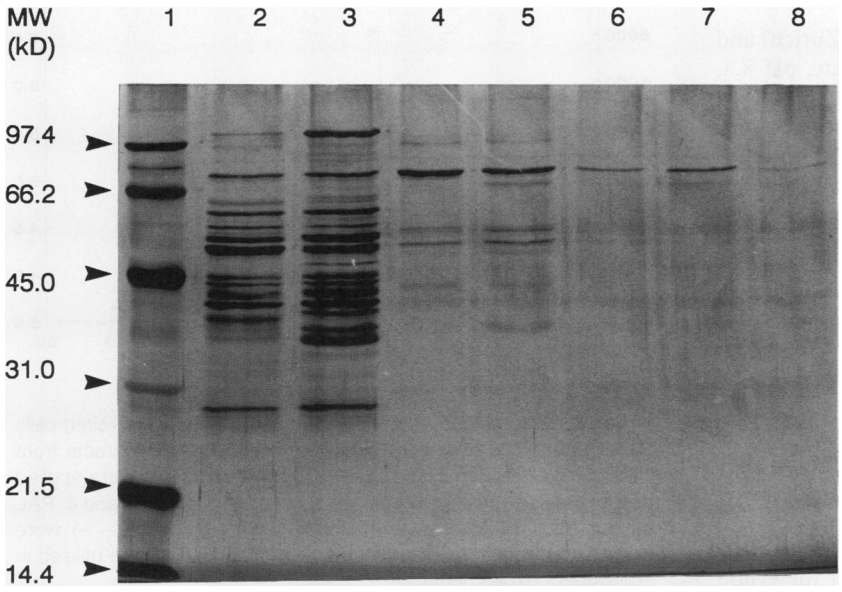

Figure 2. SDS-PAGE documenting the sequential purification of PAL from *P. banksiana*. Equivalent aliquots of fractions 19 and 20 from semi-preparative anion-exchange (lanes 2 and 3, respectively), fractions 30 and 31 from chromatofocusing (lanes 4 and 5, respectively), and fractions 24, 25, and 26 from analytical anion-exchange (lanes 6, 7, and 8, respectively) were separated according to Laemmli (17) and the resultant gel silver stained. Low mol wt standards were run for reference (lane 1).

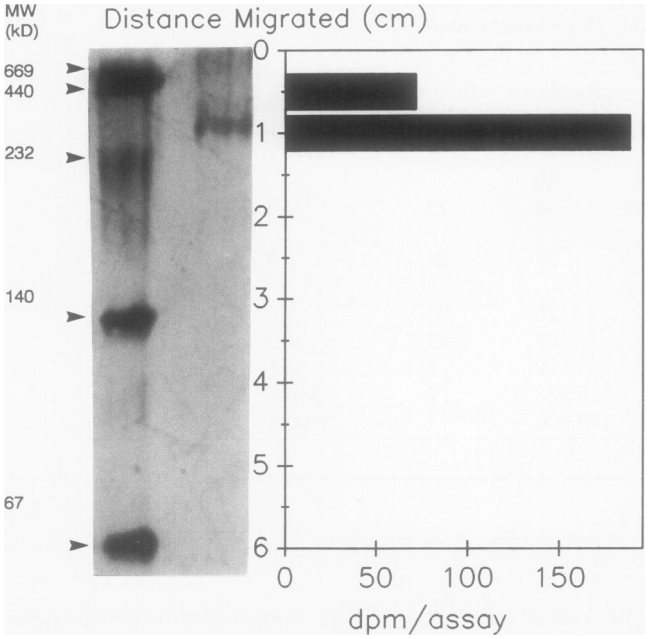

Figure 3. Nondenaturing PAGE analysis of purified PAL from *P. banksiana*. After nondenaturing PAGE, one lane containing purified pine PAL was sliced into 0.5 cm sections that were assayed for PAL activity (histogram). PAL (2) and high mol wt standards (1) were visualized in the remainder of the gel by silver staining.

assay mixture to give a final concentration of either 100 or 10 μM. Control assays were prepared with identical dilutions of 50% (v/v) methanol, which did not inhibit PAL activity. All compounds tested were obtained from Fluka except pinosylvin, pinosylvin monomethyl ether, pinobanksin, and pinocembrin. The latter were extracted from *P. banksiana* heartwood, purified by semi-preparative HPLC, and their identities verified by UV and NMR spectroscopy (our unpublished results).

The substrate analogs AIP (N Amrhein, ETH, Zurich) and AOPP were dissolved in 100 mM potassium borate, pH 8.8, and diluted to give a final concentration of 1, 10, or 100 μM in the PAL assay mixture. For cinnamate IC_{50} studies, cinnamate was dissolved in 2-methoxy ethanol and diluted to give 0.001, 0.002, 0.01, 0.02, 0.1, 0.2, 1, 2, 10, and 20 mM concentrations in the PAL assay mixture. Substrate was added after the enzyme had been incubated with the inhibitor for 5 min.

RESULTS

Purification of PAL from *P. banksiana*

Initial experiments determined that the pH optimum of *P. banksiana* PAL in crude extracts was 8.8 and that the assay was linear over 90 min.

The purification scheme developed for *P. banksiana* PAL (Fig. 1) resulted in a 9500-fold purification from the crude extract (Table I) within 16 h. This value must, however, be treated with caution because of potential inaccuracy associated with the estimation of the final protein concentration. Due to the speed of the protocol, the enzyme was not subjected to any freeze-thaw cycles and was always held at 4°C.

This protocol resulted in a single protein species as visualized by silver staining of the SDS-PAGE gel (Fig. 2). The stained band corresponded to a M_r of approximately 77,000. A single band of protein of this M_r was observed when using Coomassie blue staining after the chromatofocusing step (data not shown), but silver staining revealed the presence of significant contaminants (Fig. 2). Visual assessment of the relative quantity of PAL protein detected by silver staining in each lane of the SDS-PAGE gel indicated good correspondence with the relative amount of PAL activity in each fraction from the analytical anion-exchange chromatogram (compare

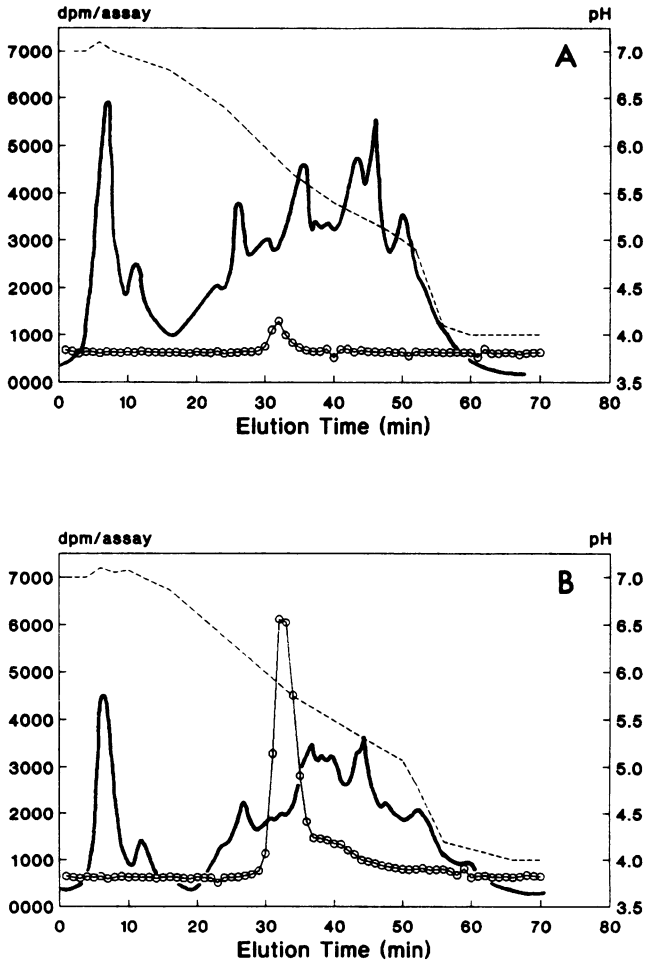

Figure 4. Comparison of pine PAL from elicited and unelicited cells via chromatofocusing over a wide pH range. Protein extracts from control (A) or elicited (B) cultures (24 h postelicitation) were applied to a Mono P column and chromatofocused between pH 7 and 4. PAL activity (○), total protein (—), and the pH gradient (– – –) were monitored throughout. The most active fractions (●) were utilized in subsequent experiments.

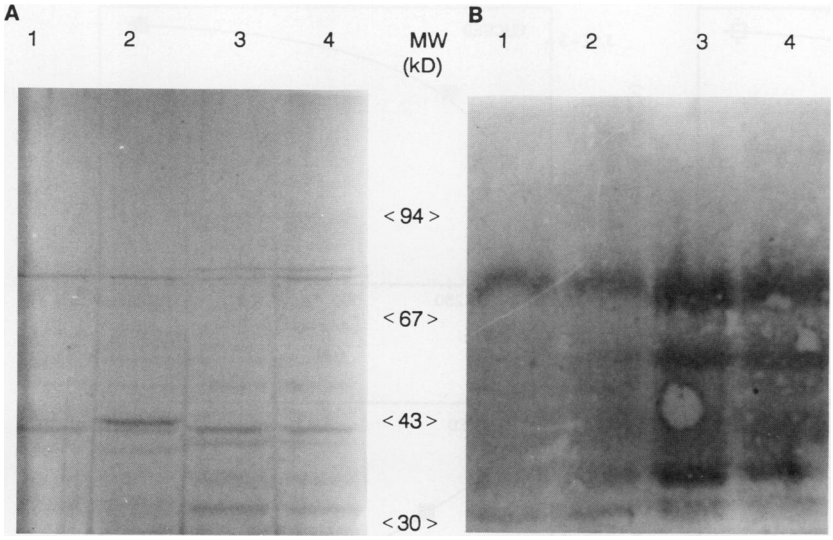

Figure 5. Western blot of partially purified PAL from *P. banksiana*. SDS-PAGE analysis was conducted on the two most active sequential fractions of chromatofocused PAL from either control (lanes 1 and 2) or elicited (lanes 3 and 4) cells 24 h postelicitation, using duplicate gels. One gel was subsequently silver stained (panel A) and the other was subjected to a western blotting protocol with anti-alfalfa PAL polyclonal antibodies (panel B). Relative mol wts were as indicated.

filled circles from Fig. 1C to amount of protein in lanes 6–8).

A silver stained nondenaturing PAGE gel of purified PAL revealed one band of protein (Fig. 3) and assays of eluted gel slices for PAL activity demonstrated that this band corresponded to the location of PAL activity (Fig. 3).

Native Mol Wt

The PAL native M_r was determined to be 354,000, based on interpolation of a Ferguson plot established for various mol wt standards on nondenaturing PAGE. Calibrated Superose 6 gel permeation chromatography revealed a similar native M_r (324,000) for the enzyme.

Search for Isozymic Variants of PAL by Chromatofocusing FPLC

Single discrete peaks of PAL activity were observed in both control and elicited protein extracts independent of whether the extract was subjected to chromatofocusing over a wide pH range (Fig. 4), or a narrow pH range (data not shown). PAL from control cultures cochromatographed with PAL from elicited cultures at an isoelectric point of 5.75.

Western Blot of PAL from Control and Elicited Cultures

PAL from elicited and control cultures (24 h postelicitation) was partially purified by chromatofocusing (Fig. 4) and the four most active fractions were subjected to SDS-PAGE for western blot analysis. Anti-alfalfa PAL antibodies were used to probe the blotted protein extracts from both elicited and control cell cultures of *P. banksiana,* which resulted in detection of a major band in the region corresponding to the subunit M_r of PAL, 77,000 (Fig. 5). A much stronger reaction was observed on blots of the elicited extracts (Fig. 5). There was also a substantial amount of cross-reactivity with proteins at M_r 67,000, 47,000, 40,000, and 37,000 in both extracts, but again this was stronger in the elicited extracts (Fig. 5).

Catalytic Properties of Pine PAL

Partially purified *P. banksiana* PAL (Fig. 4) from both elicited or control cultures displayed normal Michaelis-Menten kinetics with no suggestion of cooperativity or multiple K_m values (Fig. 6). K_m values were calculated for PAL based on both Lineweaver-Burk (not shown) and Eadie-Hofstee plots (Fig. 6). PAL obtained from elicited and control cultures had a K_m for L-phenylalanine of approximately 90 μM. PAL from *P. banksiana* displayed no activity against L-tyrosine.

Inhibition of PAL from *P. banksiana*

Regardless of the tissue source (elicited or control cultures), the *in vitro* activity of PAL from *P. banksiana* was essentially unaffected by phenolic compounds at concentrations as high as 100 μM. Cinnamate, 4-coumarate, and caffeate had a slight inhibitory effect (<10%), whereas the remainder of the phenolic compounds, including the major stilbenes (pinosylvin, pinosylvin monomethyl ether) and flavonoids (pinobanksin, pinocembrin) from *P. banksiana* were not inhibitory.

The substrate analogs AOPP and AIP were both effective at inhibiting PAL activity *in vitro*. Although 0.1 and 1.0 μM concentrations of AOPP appeared to have a somewhat stimulatory effect of PAL activity (<15%), 10 μM AOPP resulted in inhibition in PAL activity by approximately 80%. AIP was effective at concentrations as low as 0.1 μM but was not as effective at 10 μM as AOPP.

To better evaluate the effect of cinnamate on PAL activity, an IC_{50} curve was established. PAL from elicited or unelicited pine suspension cultures was relatively unaffected by cinnamate at concentrations lower than 200 μM, but, at concentrations above this range the activity dropped off. The IC_{50} value for cinnamate interpolated from the plot was approximately 4 mM.

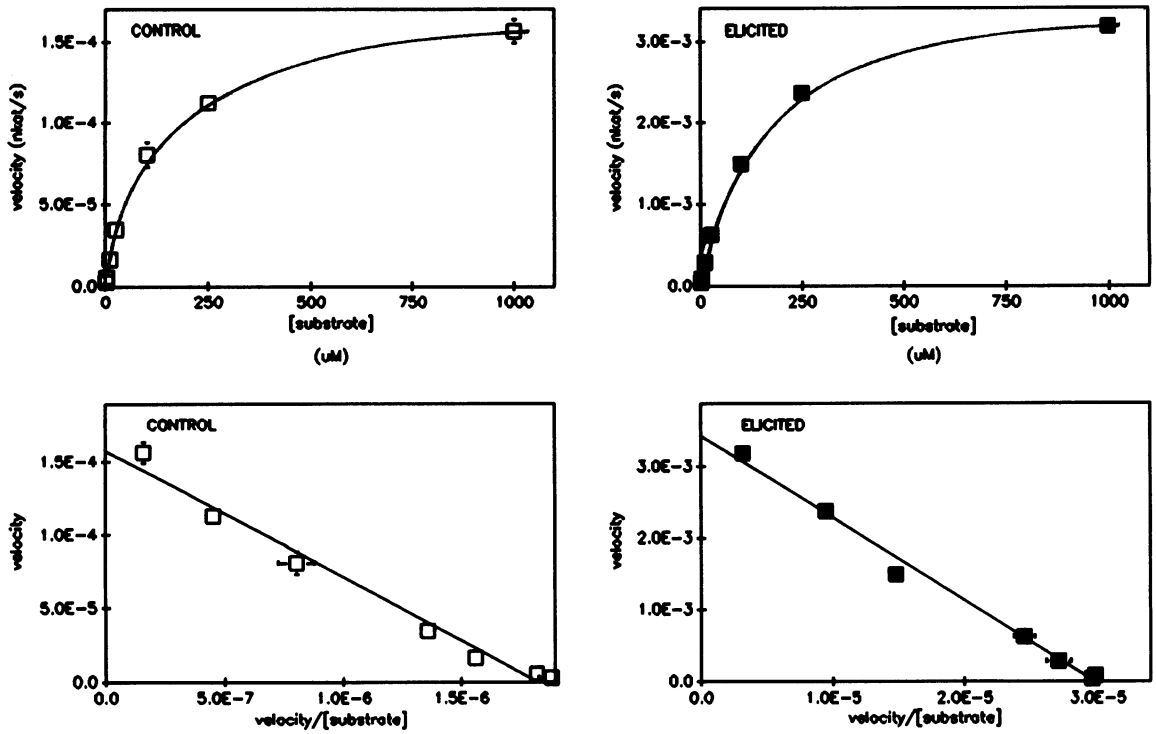

Figure 6. Comparison of kinetic properties of partially purified pine PAL from elicited and unelicited cells. Chromatofocused PAL from either control (□, left panels) or elicited (■, right panels) cells were analyzed. Data were plotted on Michaelis-Menten (top panels) or Eadie-Hofstee (bottom panels) plots. Error bars represent one SD.

DISCUSSION

Elicitor preparations from the ectomycorrhizal fungus *T. terrestris* induce rapid accumulation of typical gymnosperm lignin in the cell walls of suspension cultured cells of its host *P. banksiana* (4, 5). Concomitant with lignification of the elicited cultures was a rapid and transient increase in the activity of PAL (4). The pattern and time frame of PAL induction in pine cell cultures is consistent with that which has been documented in elicited cell cultures of angiosperms (6, 10). Beyond this, however, nothing is known about how the molecular regulation of PAL in a gymnosperm compares with the extensive data for angiosperm PAL.

To gain further insight into the molecular control of inducible lignification in conifers, PAL was purified to homogeneity from *P. banksiana* suspension cultures (Figs. 1–3). To the best of our knowledge, this represents the first detailed investigation of PAL from a gymnosperm species. The enzyme was found to have an isoelectric point of 5.75, a native M_r of approximately 340,000, and a subunit M_r of approximately 77,000, suggesting that the holoenzyme consists of a tetramer of similar or identical subunits. These data are consistent with values for PAL from other sources, including parsley (24), alfalfa (15), bean (2), strawberry (13), and bamboo (7).

Isoforms of PAL have been chromatographically resolved in extracts of a number of angiosperm species, in which different stresses appear to induce increases in specific isozyme forms (2, 15). In contrast, PAL from *P. banksiana* could not be resolved into distinct isoforms by anion-exchange or chromatofocusing FPLC (Fig. 1). Elicitor treatment of pine cell cultures did not result in the production of new, chromatographically distinct isoforms, but rather resulted in an increase in PAL activity that was chromatographically indistinguishable from that seen in control cultures (Fig. 4). In angiosperms in which PAL isoforms have been observed, partially purified PAL activity often displays complex, sigmoidal kinetics; however, once PAL isozymes have been chromatographically resolved, individual isoforms display normal Michaelis-Menten kinetics, although with very different K_m values (2, 15). In contrast, partially purified pine PAL displayed normal Michaelis-Menten kinetics (Fig. 6). Elicitor treatment did not result in a change in the enzyme kinetics or a significant change in the K_m of the enzyme in elicited *versus* control pine cultures (Fig. 6). These data suggest that *P. banksiana* PAL is constitutively present as a single form and that the levels of this form are elevated in the lignifying cultures.

Western blotting of partially purified pine PAL, using anti-alfalfa PAL polyclonal antiserum as a probe, resulted in the detection of a major band of immunoreactivity that corresponded to the 77,000 mol wt of the PAL subunit (Fig. 5). The stronger reaction observed for this band in the elicited extracts suggests that increased PAL activity in elicited pine cultures was a consequence of increased quantity of the enzyme.

The dectection of immunoreactive material at mol wts

corresponding to 67,000, 46,000, and 40,000 may have some interesting implications (Fig. 5). Potato PAL has recently been demonstrated to be a glycoprotein (22), which raises the possibility that the polyclonal antiserum used in the present study was cross-reacting with some glycoprotein contaminants, given the antigenicity and common epitope structure of many glycoprotein oligosaccharide side chains. However, it has also been established that PAL is sensitive to a PAL-specific proteolytic system (reviewed in ref. 9) that is apparently activated by the PAL reaction product cinnamate (3, 23). Because the initial increase in PAL activity would be expected to lead to an increase in the production of cinnamate, it would not be surprising to find a higher level of such proteolytic activity in elicited cultures. Rapid PAL turnover in elicited pine cultures is further suggested by the transient nature of the PAL activity increase in these lignifying cultures, which reaches its peak and returns to control values within 48 h postelicitation (4). The increased amount of immunoreactive material at the lower mol wts in the blots of elicited cell extracts, therefore, may be indicative of increased PAL proteolysis in these cells. Interestingly, western blots of PAL from *Pinus taeda,* using anti-*P. taeda* PAL polyclonal antibodies, also show immunoreactive bands at mol wts corresponding to those found in this study (R. Whetton, R. Sederoff, personal communication).

The fact that polyclonal antibodies raised against alfalfa PAL cross-reacted with pine PAL suggests that pine PAL shares significant homology with PAL found in herbaceous plant species (Fig. 5). In fact, DNA consensus sequences found in the PAL coding region from a number of plant species have been used to develop polymerase chain reaction primers that successfully amplified a PAL gene from *P. banksiana* (M.M. Campbell, J. Pitel, R. Rutledge, B.E. Ellis, unpublished results).

In addition to a PAL-specific proteolytic pathway, the activity of angiosperm PAL is proposed to be modulated by feedback inhibition mediated by various phenolic compounds, especially the reaction product cinnamate (21). However, *P. banksiana* PAL was relatively unaffected by phenolic compounds in the concentration ranges tested. Although cinnamate, 4-coumarate, and caffeate did have a minor effect on PAL activity, all of the remaining phenolic compounds, including common stilbenes and flavonoids from pine, did not. The effect of cinnamate is not substantial until millimolar concentrations are used. These data are in contrast with those for PAL from other species such as yeast, sweet potato, and pea (21), and alfalfa (15), in which phenolic compounds are effective at inhibiting PAL at concentrations as low as 100 μM. It may be that in pine, whose tissues are typically rich in phenolics and highly lignified, modulation of PAL activity by low concentrations of metabolites is not a metabolically feasible mechanism.

In contrast with the model that has been developed from extensive studies in angiosperm species, particularly legumes, the regulation of PAL activity in *P. banksiana* does not appear to occur via control of the quantitative and qualitative pattern of PAL isozymes. Furthermore, it would appear that any regulatory processes must operate at a level other than direct product inhibition of enzyme activity. Instead, our data suggest that regulation of PAL activity in *P. banksiana* must depend on other mechanisms, such as subcellular localization, enzyme turnover/inactivation, or signal-specific transcriptional control. Cell culture systems have led to a greater understanding of the molecular genetic mechanisms underlying the control of PAL activity (*e.g.* 8, 19), but, to date, an analogous system does not exist for gymnosperms. The pine cell culture/fungal elicitor system offers an excellent opportunity to clarify the molecular mechanisms involved in the regulation of a gymnosperm PAL, and to determine how they are integrated with control of the other processes required for inducible lignification.

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