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Phenylalanine Ammonia-Lyase from Tomato Cell Cultures Inoculated with *Verticillium albo-atrum*[1]

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ABSTRACT

Tomato (*Lycopersicon esculentum* Mill.) cell suspension cultures accumulated wall-bound phenolic materials in response to inoculation with *Verticillium albo-atrum* Reinke et Berth. in a fashion analogous to that observed in whole plants. Both monomeric and polymeric materials were recovered. Deposition of phenolics into the cell walls of inoculated tomato cell cultures was inhibited by the phenylalanine ammonia-lyase (PAL) inhibitor, 2-amino-2-indanephosphate. Tomato PAL activity was induced over 12-fold by fungal inoculation, with a concomitant increase in the corresponding mRNA. The enzyme was purified >3400-fold, to apparent homogeneity, by anion-exchange chromatography, chromatofocusing, and gel filtration. The holoenzyme had a molecular mass of 280 to 320 kilodaltons, comprising 74-kilodalton subunits, and displayed an isoelectric point of 5.6 to 5.7. Induced PAL displayed apparent Michaelis-Menten kinetics (K_m = 116 micromolar) and was not appreciably inhibited by its product cinnamic acid. Chromatographic analysis did not reveal multiple forms of the enzyme in either inoculated or uninoculated cultures.

Tomato (*Lycopersicon esculentum* Mill.) responds to challenge by the vascular wilt pathogen *Verticillium albo-atrum* Reinke et Berth. by invoking a variety of biochemical defense mechanisms. One of the most prominent responses involves the synthesis and deposition of cell wall-coating materials within the vascular system of infected plants. Street *et al.* (25) first demonstrated the phenylpropanoid nature of this coating material using the PAL[3] inhibitor AOPP. Kolattukudy and Robb (unpublished work cited in ref. 16) later reported the composition of the coating material to be consistent with that of suberin, a complex macromolecule comprising both phenolic and aliphatic moieties. Furthermore, the induction of a suberin-associated peroxidase in tomato cell cultures treated with an elicitor preparation from *V. albo-atrum* has been reported (20). The induction of tomato PAL, both at the enzyme and the mRNA levels, in petioles infected with *V. albo-atrum* has recently been reported (J. Robb, personal communication). Based on these and other reports (see ref. 1 for a review), it has become clear that phenylpropanoid metabolism plays an integral role in the host response of tomatoes to *V. albo-atrum* infection.

In an effort to further understand the role of phenylpropanoid metabolism in the interaction between tomato and *V. albo-atrum*, we have developed an *in vitro* cocultivation system (3) in which tomato cell suspension cultures are inoculated with spores of *V. albo-atrum*. Our initial reports focused on the induction of phenylpropanoid biosynthetic activity and the accumulation of specific soluble phenolic metabolites (3, 4). We report here the accumulation of phenylpropanoid-derived metabolites in the cell walls of *Verticillium*-inoculated tomato suspension cultures as correlated with the induction of PAL both at the enzyme and mRNA levels, and the purification of tomato PAL to apparent homogeneity.

MATERIALS AND METHODS

Tomato and Fungal Cultures

Tomato (*Lycopersicon esculentum* Mill.) and fungal cultures were grown as previously described (3). For the establishment of the cocultivation system, 10-d-old suspension cultures were transferred to fresh medium and inoculated 4 d later.

Analysis of Wall-Bound Phenolics

The *de novo* synthesis of phenolic metabolites was inhibited *in vivo* with the PAL inhibitor AIP at a final concentration of 10 μM in the culture medium as described previously (4).

Soluble phenolics were removed from tissue samples (500 mg) by extracting twice for 1 h with boiling 50% (v/v) methanol and washing once with 100% methanol. The amount of wall-bound phenolics in the alcohol-insoluble residue was measured by the thioglycolic acid assay essentially as described (7), except that all reagent volumes were reduced to allow the assays to be carried out in microfuge tubes. In some experiments, samples were base hydrolyzed (1 N NaOH, 3 h, room temperature) prior to extraction with thioglycolic acid. Base hydrolyzates were analyzed by HPLC as described (3).

Polymeric materials were extracted from base-hydrolyzed cell wall preparations with 2,4-dioxane:H_2O (96:4, v/v) for 1 week at 25°C. After removal of solvent *in vacuo*, the dioxane-soluble material was redissolved in 90% acetic acid and dispersed in a 10-fold greater volume of H_2O. The flocculent

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[3] Abbreviations: PAL, phenylalanine ammonia-lyase; AOPP, α-aminooxy-β-phenylpropionic acid; AIP, 2-amino-2-indanephosphate; IC_{50}, concentration inhibiting 50% of enzyme activity.

precipitate was collected by centrifugation (5000*g*, 30 min, room temperature) and either dissolved in 0.1 N NaOH for size exclusion chromatography or suspended in 50 mM Mes buffer (pH 5.5) containing 0.2 mg mL^{-1} cellulase (Onazuka R4) and incubated at 30°C overnight. The insoluble material was again collected by centrifugation and redissolved in 0.1 N NaOH for size exclusion chromatography on a Superose 12 gel filtration column (HR 10/30; Pharmacia). Samples (500 μL) were eluted isocratically with 0.1 N NaOH at a flow rate of 0.2 mL min^{-1}. The eluent was monitored at 280 nm. Blue Dextran 2000 ($M_r = 2.0 \times 10^6$) was used to estimate the void volume, whereas *p*-coumaric acid was used to estimate the elution volume of typical phenolic monomers.

Assay and Purification of Tomato Phenylalanine Ammonia-Lyase

Assay Conditions

PAL was assayed radiometrically as previously described (3). Active PAL enzyme was located on nondenaturing PAGE gels (see below) by assaying directly in gel slices. After removal of the molecular weight marker lane plus 0.5 cm of sample lane for staining, 0.5 cm horizontal slices were cut across the width of the gel. Each gel slice was macerated with a Teflon pestle in a 2-mL microfuge tube containing 500 μL of PAL assay buffer. The protein was allowed to equilibrate with the buffer for 1 h, with occasional stirring. Aliquots (3 × 150 μL) of gel slurry were then transferred to clean microfuge tubes and assayed for PAL activity.

Purification of Tomato Phenylalanine Ammonia-Lyase

All purification steps were carried out at 4°C as far as possible, and all buffers contained freshly added β-mercaptoethanol (5 mM). PMSF (dissolved in acetone and diluted to 1.0 mM final concentration) was added to the extraction buffer. Chromatographic steps were performed on a Pharmacia Fast Protein Liquid Chromatography system, monitoring the eluent at 280 nm. Column fractions (130 μL) were assayed for PAL activity after pH adjustment with 20 μL of 1 M potassium borate buffer (pH 8.7).

Frozen tissue (up to 500 g) was thawed in ice-cold extraction buffer (200 mM Tris-HCl, pH 7.5; 1 to 2 mL g^{-1} fresh weight) containing buffer-soaked PVP (1 g wet weight g^{-1} fresh weight) and incubated on ice for 20 min with occasional stirring. The homogenate was squeezed through four layers of buffer-washed Miracloth (Calbiochem), brought to 0.1% (w/v) protamine sulfate, and stirred for 20 min. After centrifugation (10,000*g*, 10 min, 4°C), the clear supernatant was stirred with Amberlite XAD-7 resin (Sigma; previously washed with methanol and glass distilled H_2O and equilibrated with extraction buffer; up to 0.5 g resin mL^{-1} extract) for 5 min. The XAD-7 suspension was filtered through Miracloth, and the filtrate was brought to 60% saturation with solid ammonium sulfate. Insoluble proteins were collected by centrifugation (10,000*g*, 20 min, 4°C), redissolved in a minimum volume of Mono-Q start buffer (20 mM Tris-HCl, pH 7.5), and desalted into the same buffer on a Sephadex G-25 (coarse) column (160 mL bed volume) at a flow rate of 2.5 mL min^{-1}.

The desalted protein extract was loaded onto a Mono-Q HR10/10 (semi-preparative) anion-exchange column (Pharmacia) and eluted with a steep salt gradient (0–300 mM sodium chloride over 45 min) at a flow rate of 2 mL min^{-1}. Fractions were collected at 1-min intervals.

Pooled active fractions from semi-preparative Mono-Q chromatography were exchanged into Mono-P (chromatofocusing) start buffer (25 mM L-histidine, pH 6.2) and loaded onto a Mono-P HR5/10 column (Pharmacia). Proteins were eluted over a pH range of 6.2 to 5.0 with 10× diluted Polybuffer 74 (pH 5.0) (Pharmacia) at a flow rate of 0.75 mL min^{-1}. Fractions were collected at 1-min intervals.

Pooled active fractions from Mono-P chromatography were exchanged into Mono-Q (analytical) start buffer (20 mM Tris-HCl, pH 7.5) and loaded onto an analytical Mono-Q HR5/5 column (Pharmacia). Proteins were eluted with a shallow salt gradient (0–200 mM sodium chloride over 60 min) at a flow rate of 1 mL min^{-1}. Fractions were collected at 1-min intervals.

Pooled active fractions from Mono-Q were either loaded directly onto a Superose 6 gel filtration column (see below) or first concentrated over a bed of Aquacide IIA (Calbiochem) using dialysis membranes with a 10^4 D molecular mass cut-off.

Isoform Analysis

Samples (10 g) of tomato tissue were harvested at various times postinoculation, ground in liquid N_2, and stored at −70°C until analyzed. Proteins were extracted as described above, concentrated by precipitation with ammonium sulfate (60% saturation), and exchanged into the appropriate chromatography buffer (see below). Isoforms of tomato PAL were resolved either by a single anion-exchange chromatography step (Mono-Q HR 5/5; Pharmacia) with a shallow salt gradient (0–300 mM NaCl over 45 min) at a flow rate of 1 mL min^{-1} or by chromatofocusing (Mono-P HR 5/10; Pharmacia) over a narrow (6.2–5.0) pH gradient, at a flow rate of 0.75 mL min^{-1}.

Molecular Mass Determination

The molecular mass of native tomato PAL was determined by calibrated gel filtration chromatography and nondenaturing PAGE. For gel filtration, 500 μL of partially purified PAL (*e.g.* after analytical Mono-Q fractionation) was loaded onto a Superose 6 HR10/30 column (Pharmacia; effective molecular mass cut-off = 10^6 D) preequilibrated with 20 mM Tris-HCl, pH 7.5, 150 mM sodium chloride and eluted isocratically with the same buffer at 0.25 mL min^{-1}. Fractions were collected at 2-min intervals. The pH of aliquots of alternate fractions was adjusted with 1.0 M potassium borate buffer, pH 8.7, and assayed for PAL activity. A mixture of high molecular mass protein standards, including thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), and aldolase (158 kD), was used to calibrate the Superose 6 column. Blue Dextran 2000 ($M_r = 2 \times 10^6$) was used to estimate the void volume.

For nondenaturing-PAGE, 6, 7, and 8% (w/v) acrylamide gels were prepared and run with the Tris-Tricine buffer system described (8) in which proteins are resolved at a pH of 7.5.

The relative mobility of PAL at each acrylamide concentration was compared with that of ferritin (440 kD), catalase (232 kD), lactate dehydrogenase (140 kD), and BSA (67 kD).

Subunit molecular mass was determined by SDS-PAGE essentially as described (17). The relative mobility of denatured PAL on either 8 or 10% (w/v) acrylamide gels was compared with that of phosphorylase b (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD).

Kinetic Analysis

A partially purified PAL preparation (after Mono-Q shallow gradient fractionation) was used to determine the K_m for L-phenylalanine and to test for inhibition by a variety of metabolites, including cinnamic, caffeic, quinic, and chlorogenic acids, as well as by two synthetic substrate analogs, AOPP and AIP. For K_m determinations, 100-μL aliquots of enzyme preparation were incubated with 1 μM to 25 mM L-phenylalanine (approximately 10^5 dpm/assay) for 30 min. For K_i determinations, 100-μL aliquots of enzyme preparation were preincubated with inhibitor (0.1–100 μM) for 30 min on ice prior to the addition of substrate.

RNA Extraction and Analysis

Total RNA was extracted from tomato cell cultures (10 g samples) at various times postinoculation according to Parsons *et al.* (22) and separated in a formaldehyde gel (10 μg/lane) (19). The RNA was capillary-blotted onto Hybond-N paper (Amersham) overnight (19), baked for 1 h at 80°C, and prehybridized with denatured salmon sperm DNA for 1 h prior to the addition of the ^{32}P-labeled potato PAL cDNA probe (see below).

The PAL cDNA clone used (from *Solanum tuberosum*) (10) was obtained from C. Douglas (University of British Columbia, Vancouver BC) and was labeled with deoxycytidine-5′-[α-^{32}P]triphosphate (Amersham) with the Bethesda Research Laboratories random primer labeling kit. The blot was probed overnight at 65°C and washed twice for 10 min at room temperature and once for 1 h at 65°C. All probing and washing steps were performed at a stringency of 2 × SSC. The hybridized blot was exposed to Kodak X-OMAT AR film at −70°C for 14 d.

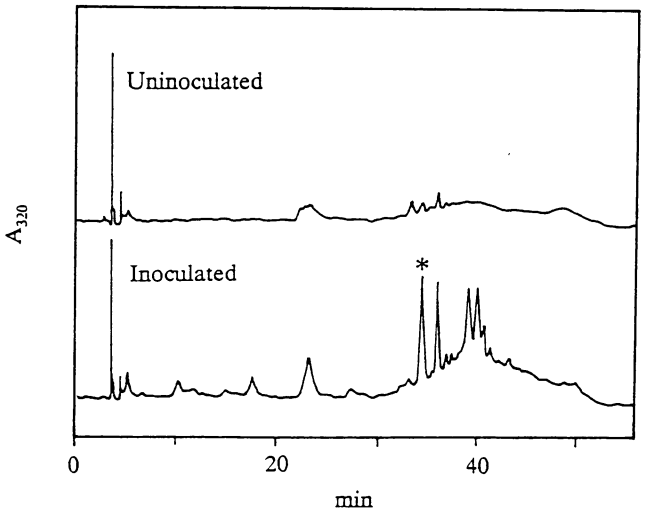

Figure 1. HPLC analysis of phenolic compounds released from the cell walls of tomato cell cultures by base hydrolysis. Compounds were released from cell wall preparations by mild base hydrolysis as described in "Materials and Methods." The labeled peak (*) coelutes with sinapic acid.

Table I. *Accumulation of Cell Wall-Bound Phenolics in Verticillium albo-atrum-Inoculated Tomato Cell Suspension Cultures*

Phenolics were quantified by the thioglycolic acid assay of Bruce and West (7). Values listed represent the average value obtained from the number of independent measurements indicated by the numbers in parentheses.

Time Postinoculation	Treatment	Phenolic Accumulation	
		Uninoculated	Inoculated
h		A_{280}/g fr wt	
24		0.49 (2)	0.50 (2)
36		0.30 (3)	1.73 (3)
	+ AIP[a]	0.35 (3)	0.75 (3)
	Hydrolysis[b]	0.39 (1)	0.77 (1)
48		0.36 (2)	2.25 (2)

[a] 10 μM AIP in culture medium added at 12-h intervals postinoculation. [b] Cell wall preparations were base hydrolyzed (1 M NaOH, 3 h, room temperature) to remove esterified phenolics prior to thioglycolic acid extraction.

RESULTS

Wall-Bound Phenolics

Tomato cell cultures inoculated with *V. albo-atrum* accumulated up to fivefold higher levels of wall-bound phenolics than were found in uninoculated control cultures as measured by the thioglycolic acid assay (Table I). This assay is believed to provide a quantitative measure of the amounts of wall-bound phenolics, including monomeric and polymeric metabolites. Base hydrolysis of the wall preparations before thioglycolic acid extraction released an array of phenolic compounds from wall preparations of inoculated cell cultures, whereas walls from uninoculated cells yielded only traces of esterified phenolics. A major component in this hydrolyzable phenolic population coeluted with sinapic acid (Fig. 1). Treatment of cell cultures with the PAL inhibitor AIP during fungal challenge reduced both the number and amount of compounds released by base hydrolysis (Table I and data not shown).

Size exclusion chromatography of dioxane-soluble material extracted from base-hydrolyzed cell wall preparations revealed a single major peak of UV-absorbing material with an elution volume between that of Blue Dextran 2000 ($M_r = 2 \times 10^6$) and *p*-coumaric acid. Treatment of the dioxane-soluble material with cellulase did not alter its elution volume. Alkaline nitrobenzene oxidation of the dioxane-soluble material, how-

ever, did not yield detectable amounts of any of the "classic" lignin phenolic monomers (data not shown).

Induction of Phenylalanine Ammonia-Lyase

PAL was induced in the tomato cultures, both at the enzyme and mRNA level, by *V. albo-atrum* inoculation (Fig. 2). Maximum enzyme activity occurred by 36 to 48 h postinoculation, concomitant with a marked accumulation of PAL mRNA. A low level of PAL mRNA was detectable in RNA extracted from uninoculated cell cultures, consistent with the measured levels of constitutive enzyme activity.

Purification of Tomato Phenylalanine Ammonia-Lyase

Phenylalanine ammonia-lyase was purified more than 3400-fold, to apparent homogeneity, from *V. albo-atrum*-inoculated tomato cell suspension cultures (Table II). Calibrated gel filtration chromatography yielded a holoenzyme molecular mass of 280 kD, whereas nondenaturing PAGE yielded a holoenzyme molecular mass of 320 kD (data not shown). All measurable PAL activity comigrated with the only band present in a silver-stained nondenaturing PAGE gel (data not shown). Denaturing PAGE analysis of the purified PAL preparation revealed three bands on silver staining (Fig. 3). The main band migrated as a 74 kD protein, with minor bands at 100 and 48 kD.

Kinetic and Isoform Analyses

Tomato PAL, partially purified from fungal-challenged cell cultures by anion-exchange chromatography, showed apparent Michaelis-Menten saturation kinetics and yielded a K_m value of 116 μM for L-phenylalanine (Fig. 4a). Tomato PAL was not inhibited by cinnamic, caffeic, quinic, or chlorogenic acids, even at concentrations as high as 100 μM (Fig. 4b). By contrast, two known PAL inhibitors, AIP and AOPP, were very effective against tomato PAL (IC_{50} = 1.0 and 2.3 μM, respectively).

Tomato cultures showed no evidence for induction of multiple PAL isoforms in response to challenge with *V. albo-atrum*, whether analyzed by anion-exchange chromatography (Fig. 5a) or chromatofocusing (Fig. 5b). Similarly, in unchallenged cells, only one constitutive form of PAL was evident (Fig. 5a). A protein extract prepared from *V. albo-atrum* cultures and fractionated by anion-exchange chromatography under the same chromatographic conditions as used for the tomato isoform analysis revealed a single, late-eluting peak of *V. albo-atrum* PAL activity. The beginning of the elution of

Table II. *Purification of Phenylalanine Ammonia-Lyase from Verticillium albo-atrum-Inoculated Tomato Cell Suspension Cultures*

Purification Step	Protein	Activity		Purification
		Total	Specific	
	mg	*nkat*	*nkat mg^{-1}*	*-fold*
Crude extract	2770	88.2	3.2×10^{-2}	
Protamine sulfate	1530	82.5	5.4×10^{-2}	1.7
XAD-7 (filtrate)	706	49.8	7.1×10^{-2}	2.2
60% $(NH_4)_2SO_4$ pellet[a]	370	53.6	1.4×10^{-1}	4.5
Mono-Q (semi-prep)[a]	102	25.9	2.5×10^{-1}	7.9
Mono-P (pH 6.2–5.0)[a]	2	5.4	2.7	85
Mono-Q (analytical)	0.03[b]	1.0	31[b]	980[b]
Superose 6	0.002[b]	0.18	111[b]	3470[b]

[a] Measurements made after buffer exchange. [b] Estimated values. Protein content derived from comparison with BSA and ovalbumin protein standards on a silver-stained SDS-PAGE gel.

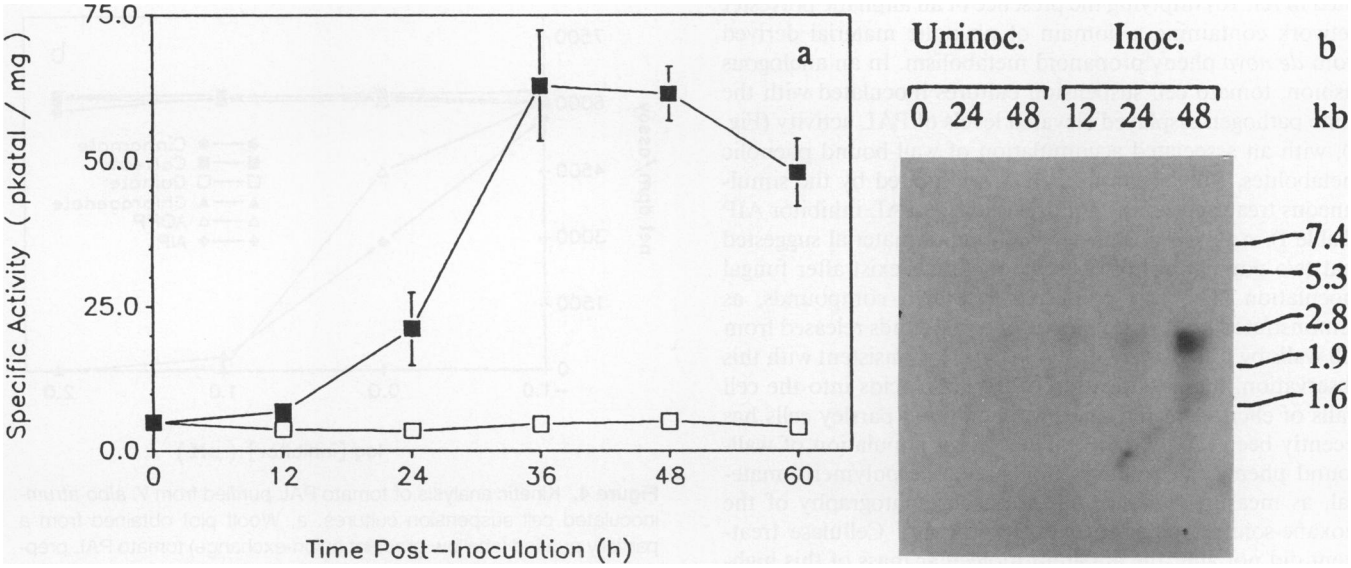

Figure 2. Induction of phenylalanine ammonia-lyase in *V. albo-atrum*-inoculated tomato cell suspension cultures. a, Time course of PAL activity in mock- (□) and fungal-inoculated (■) tomato cell suspension cultures. Values represent the average of triplicate measurements at each time point. Bars = 1 SD. b, Northern blot of total RNA extracted from tomato suspension cultures and probed with a potato PAL cDNA as described in "Materials and Methods." Equal quantities of total RNA (10 μg) were loaded in each lane.

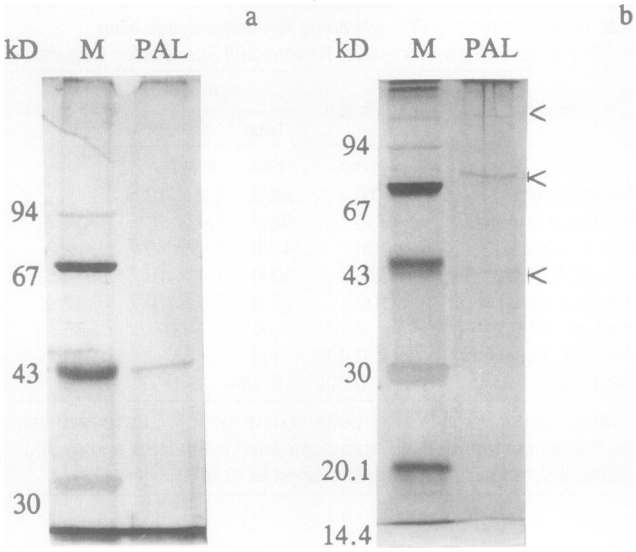

Figure 3. Subunit molecular mass determination of purified tomato PAL. Tomato PAL was purified both in the absence (a) and presence (b) of PMSF and electrophoresed under denaturing (SDS) conditions in either 8 (a) or 10% (b) (w/v) acrylamide gels. M, molecular mass markers (see "Materials and Methods"); PAL, sample of PAL after the last step of purification.

the fungal-derived PAL activity was several fractions after the last of the tomato PAL activity had eluted (data not shown).

DISCUSSION

Tomato petiole segments inoculated with *V. albo-atrum* synthesize and deposit cell-wall coating material within their vascular system in a PAL-dependent fashion (25). The coating material is reportedly suberin-like (Kolattukudy and Robb, cited in ref. 16) implying the presence of an aliphatic polyester network containing a domain of phenolic material derived from *de novo* phenylpropanoid metabolism. In an analogous fashion, tomato cell suspension cultures inoculated with the same pathogen displayed elevated levels of PAL activity (Fig. 2), with an associated accumulation of wall-bound phenolic metabolites. This accumulation was inhibited by the simultaneous treatment of the cultures with the PAL inhibitor AIP (Table I). Analysis of this cell wall-bound material suggested that two populations of phenolic materials exist after fungal inoculation. The first comprises esterified compounds, as demonstrated by HPLC analysis of compounds released from cell walls by mild base hydrolysis (Fig. 1). Consistent with this observation, the esterification of phenolic acids into the cell walls of elicitor-treated suspension-cultured parsley cells has recently been reported (13). The second population of wall-bound phenolics comprises nonbase-labile polymeric material, as measured by size exclusion chromatography of the dioxane-soluble material (data not shown). Cellulase treatment did not alter the apparent molecular mass of this high-mol wt material, although this does not rule out the possibility of phenolic-carbohydrate complexes.

The marked induction of tomato cell culture PAL by fungal inoculation was accompanied by the accumulation of PAL mRNA (Fig. 2). Induction of PAL by fungal or elicitor challenge has been reported for many plant species, including whole plant and tissue culture systems (reviewed in ref. 13). In contrast with these reports, which describe a transient PAL induction as early as 8 or 12 h postinoculation, the induction of PAL in suspensions of tomato cells would appear to be a relatively slow and sustained process, occurring 24 to 36 h postinoculation and lasting for up to 36 h. However, spore germination and hyphal extension probably form an essential part of the tomato-*V. albo-atrum* interaction *in vitro*, because heat-killed spores alone failed to elicit the same responses in the tomato cell cultures (data not shown). The 12 to 18 h required for germination and hyphal extension may thus delay the time between the addition of spores to a flask of tomato cells and initial contact between the two organisms. The true PAL induction time frame could, therefore, be 12 to 18 h. Moreover, the continual growth and sporulation of the fungus within the cocultivation system provides the tomato cultures

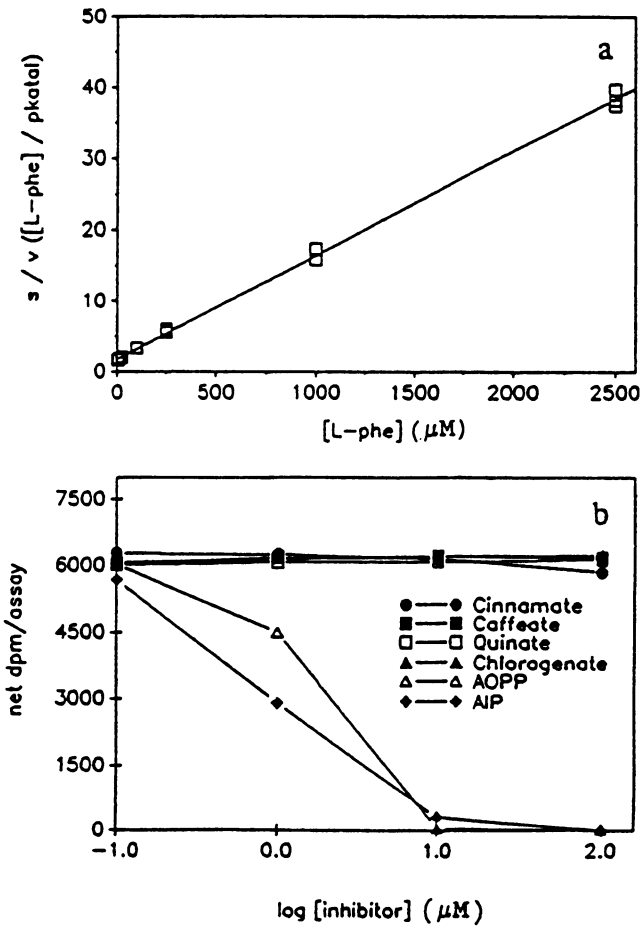

Figure 4. Kinetic analysis of tomato PAL purified from *V. albo-atrum*-inoculated cell suspension cultures. a, Woolf plot obtained from a partially purified (shallow gradient anion-exchange) tomato PAL preparation. b, Inhibition of tomato PAL by a range of biologically relevant compounds and two synthetic inhibitors. The maximum PAL activity in the absence of inhibitor was 6000 dpm assay^{-1}. Values represent the mean of triplicate measurements for each substrate and inhibitor concentration.

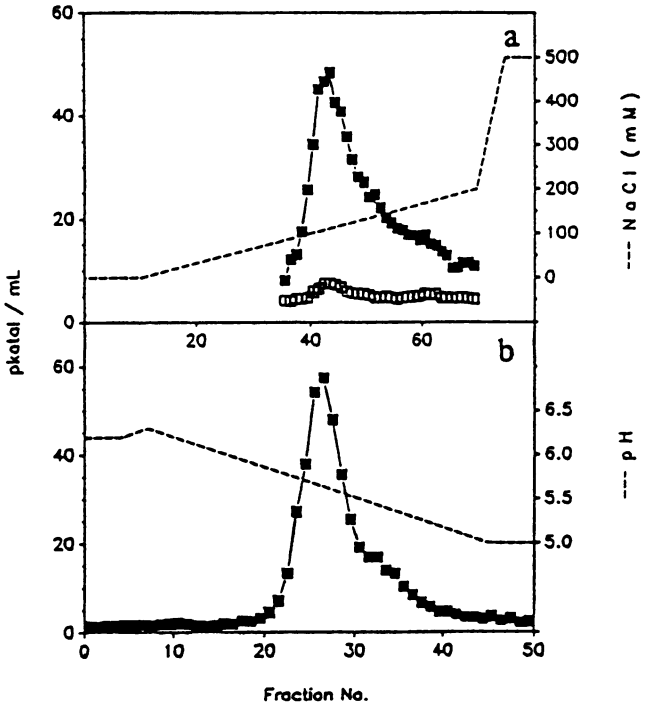

Figure 5. Isoform analysis of tomato PAL partially purified from *V. albo-atrum*-inoculated cell suspension cultures. a, Shallow gradient anion-exchange chromatography of tomato PAL extracted 0 (□) and 48 (■) h postinoculation. b, Chromatofocusing (pH range 6.2–5.0) of tomato PAL extracted 36 h postinoculation. Each activity profile represents the total activity extracted from equal amounts (*e.g.* 10 g) of tomato cell culture tissue.

with a continuous inoculum, unlike other systems in which either a one-time elicitation event or a hypersensitive host response limit the extent of the overall challenge to the plant tissue (13).

PAL has been purified from a wide variety of plant, fungal, and bacterial sources (2, 11, 12, 14), but this is the first report of the purification of this enzyme from tomato tissue. The purified tomato PAL had a holoenzyme molecular mass of 280 to 320 kD. Denaturing PAGE analysis of the purified protein, however, showed three polypeptides. The main band migrated as a 74 kD protein, consistent with the molecular mass of PAL purified from other Solanaceous species (reviewed in ref. 14, 24). The main contaminating band migrated as a 48 kD protein and probably represents a degradation product of the main PAL subunit. This conclusion is supported by the fact that PAL purified from tomato cultures over a 4-d period, without the addition of PMSF to the extraction buffer, yielded only a single 48 kD band (Fig. 3a). By contrast, the PAL purification shown in Figure 3b was completed within 48 h and in the presence of PMSF. In either case, the enzyme was purified on the basis of activity, and the sample prepared for SDS-PAGE demonstrated measurable PAL activity. Phenylalanine ammonia-lyase degradation products in the range of 48 kD have also been reported for the enzyme from other species (6, 24). The minor high molecular mass band (approximately 100 kD) may represent aggregates of degraded PAL subunits or another contaminating protein.

PAL is regarded as a kinetically complex enzyme, displaying negative cooperativity toward its substrate, L-phenylalanine (14, 21). Most kinetic analyses report two K_m values for PAL, one high (*e.g.* >100 μM) and one low (*e.g.* 10–40 μM) (14, 24). Kinetic parameters have not been reported for tomato PAL, but the enzyme from potato displayed typical complex kinetics (two K_m values, 38 and 280 μM, and a Hill coefficient of 0.75) (14, 24). In contrast with these earlier reports, tomato PAL from inoculated cell cultures displayed normal Michaelis-Menten saturation kinetics and only a single K_m value (116 μM). Recently, simple enzyme kinetics were reported for individual PAL isoforms of alfalfa (*Medicago sativa* L.) (15), and a similar reversion of *Phaseolus vulgaris* PAL to simple Michaelis-Menten kinetics after individual isoforms were separated was reported for the enzyme from elicitor-treated cell cultures (5).

PAL is reportedly subject to feedback inhibition by one of its products, cinnamic acid (14, 23). For example, partially purified PAL from sweet potato displayed a K_i of 16 μM with respect to cinnamic acid *in vitro* (23). Tomato PAL, however, does not appear to be inhibited by cinnamic acid, even at concentrations as high as 100 μM. Similarly, neither chlorogenic acid (the major phenolic constituent of the tomato cell cultures used in this study) (4) nor its hydrolysis products (caffeic and quinic acids) had any effect on tomato PAL activity. By contrast, two known synthetic PAL inhibitors, AIP and AOPP, were very effective against tomato PAL (IC_{50} = 1.0 and 2.3 μM, respectively).

Multiple forms of PAL have been reported for a number of plant species and may arise from a family of PAL genes (9). Different members of the gene family are apparently transcribed in a stimulus-specific fashion (9). A preliminary report (18) suggested that a PAL gene family exists in tomato, and that inoculation of petioles with *V. albo-atrum* spores leads to a selective transcription of specific PAL genes. In tomato cell cultures, with or without fungal inoculation, however, only one distinct form of tomato PAL could be resolved chromatographically (Fig. 5). Both anion-exchange and chromatofocusing, however, have readily resolved PAL isoforms in legume species (5, 15). If other forms of PAL can potentially be expressed in tomato as suggested by the work in potato (Gilliat and Northcote, personal communication cited in ref. 5) and the report of a PAL gene family in the tomato genome (18), they may require stimuli other than *V. albo-atrum* inoculation for their induction.

Based on these results, the tomato cell culture-*V. albo-atrum* cocultivation system represents a good experimental system in which to explore the role of PAL and phenylpropanoid metabolism in this host-pathogen interaction. Further work will require characterization of the wall-bound phenolics and the generation of tomato PAL-specific antibodies and cDNA clones.

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