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Stress Responses in Alfalfa (*Medicago sativa* L.)

II. Purification, Characterization, and Induction of Phenylalanine Ammonia-Lyase Isoforms from Elicitor-Treated Cell Suspension Cultures

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ABSTRACT

L-Phenylalanine ammonia-lyase has been purified from elicitor-treated alfalfa (*Medicago sativa* L.) cell suspension cultures using two protocols based on different sequences of chromatofocusing and hydrophobic interaction chromatography. Three distinct forms of the intact enzyme were separated on the basis of affinity for Octyl-Sepharose, with isoelectric points in the range pH 5.1 to 5.4. The native enzyme was a tetramer of M_r 311,000; the intact subunit M_r was about 79,000, although polypeptides of M_r 71,000, 67,000 and 56,000, probably arising from degradation of the intact subunit, were observed in all preparations. Two-dimensional gel analysis revealed the presence of several subunit isoforms of differing isoelectric points. The purified isoforms of the native enzyme had different K_m values for L-phenylalanine in the range 40 to 110 micromolar, although mixtures of the forms in crude preparations exhibited apparent negative rate cooperativity. The enzyme activity was induced approximately 16-fold within 6 hours of exposure of alfalfa cells to a fungal elicitor or yeast extract. Analysis by hydrophobic interaction chromatography revealed different proportions of the different active enzyme isoforms, depending upon either time after elicitation or the elicitor used. The elicitor-induced increase in enzyme activity was associated with increased translatable phenylalanine ammonia-lyase mRNA activity in the polysomal fraction.

L-Phenylalanine ammonia-lyase (PAL[2], EC 4.3.1.5) catalyzes the first committed step in the biosynthesis of a range of phenylpropanoid secondary compounds from L-phenylalanine (reviewed in refs. 8 and 13). In legumes such as bean, soybean, and pea, rapid increases in PAL activity precede the accumulation of antimicrobial isoflavonoid phytoalexins in intact tissues or cell suspension cultures in response to fungal infection or treatment with microbial elicitors (7). In bean, increased PAL activity in response to elicitor has been shown to result from rapid transcriptional activation of PAL genes (19).

Bean PAL is encoded by a small family of three genes (4), which are differentially activated in response to fungal infection, environmental conditions, and developmental cues (20). At the polypeptide level this is reflected by extensive subunit polymorphism and variation in the corresponding isoforms of the active PAL tetramer (1). Elicitation results in the specific accumulation of PAL isoforms with a low K_m for the substrate L-phenylalanine (1). In other systems, however, PAL may exist in a single form (3, 11, 14), and the regulation of phenylpropanoid biosynthesis in these cases may be correspondingly less complex and flexible.

We have recently shown that suspension cultured alfalfa cells accumulate the isoflavonoid phytoalexin medicarpin and related compounds on exposure to elicitor released from autoclaved cell walls of the bean pathogen *Colletotrichum lindemuthianum* (5). Phytoalexin accumulation results from rapid and extensive increases in the extractable activities of PAL, cinnamic acid 4-hydroxylase, 4-coumarate: CoA ligase, chalcone synthase, chalcone isomerase, and isoflavone *O*-methyl transferase. The system is an attractive one for studying defense gene activation because, unlike bean, alfalfa is amenable to genetic transformation (27). The rate of increase in PAL activity has also recently been correlated with resistance of alfalfa callus lines to infection with the vascular wilt fungus *Verticillium albo-atrum* (17). In an attempt to define better the biochemical basis of induced defense in alfalfa, we here report on the complexity of PAL at the enzyme level and confirm the *de novo* synthesis of the enzyme in response to elicitor.

MATERIALS AND METHODS

Buffers

The following buffers were used: (A) 0.1 M Tris-HCl (pH 8.5) containing 20 mM DTT, 5 mM 1,10-phenanthroline, and 1 mM PMSF; (B) 0.1 M Tris-HCl (pH 8.5) containing 2 mM 2-mercaptoethanol; (C) 25 mM imidazole HCl (pH 7.4) containing 2 mM 2-mercaptoethanol; (D) Pharmacia 'Polybuffer 74' (pH 4.0) containing 2 mM 2-mercaptoethanol; (E) 25 mM histidine HCl (pH 6.2) containing 2 mM 2-mercaptoethanol; (F) 0.1 M Tris-HCl (pH 8.5) containing 2 M $(NH_4)_2SO_4$ and 2 mM 2-mercaptoethanol; (G) 0.5 M Tris-HCl (pH 8.5) containing 2 mM 2-mercaptoethanol; (H) 3-fold diluted Pharmacia 'Polybuffer 74' (pH 6.0) containing 2 mM 2-mercaptoethanol; (I) 0.1 M Tris-HCl (pH 8.5) containing 15% ethylene glycol and 2 mM 2-mercaptoethanol; (J) 25 mM Tris, 192 mM glycine (pH 8.3) containing 20% (v/v) methanol.

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[2] Abbreviations: PAL, L-phenylalanine ammonia-lyase (EC 4.3.1.5); IEF, isoelectric focusing; pI, isoelectric point.

Plant Cell Cultures

Cell suspension cultures of alfalfa (*Medicago sativa* cvs Apollo and Europe) were obtained from callus derived from alfalfa seeds. After sterilization in NaOCl (10-fold dilution of commercial Clorox) for 20 min and extensive washing with sterile distilled water, seeds were transferred to callus initiation medium (Schenk and Hildebrandt medium supplemented with 10^{-5} M *p*-chlorophenoxyacetic acid, 2×10^{-6} M 2,4-dichlorophenoxyacetic acid and 10^{-7} M kinetin [6]). Calli were grown at 25°C in the dark and subcultured every 3 to 4 weeks. Cell suspension cultures were initiated and grown in liquid medium, of the same composition as that used for callus growth, on orbital shakers at 140 rpm and 25°C. Cells were subcultured every 7 d and 4-d-old cell suspensions were used in all experiments.

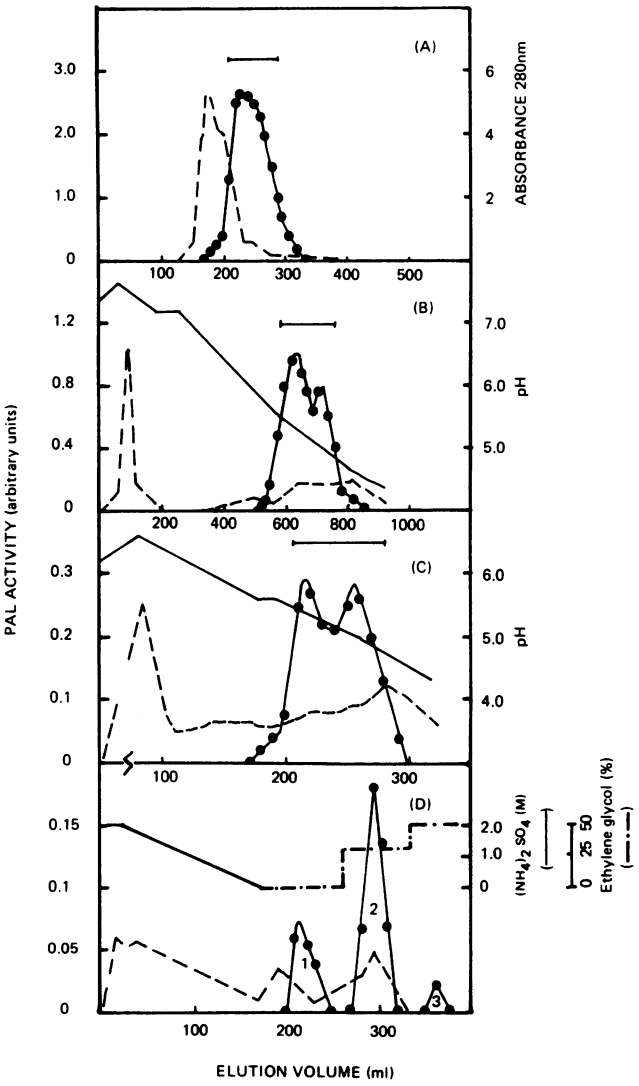

Figure 1. Purification of PAL from elicitor-treated alfalfa cell suspension cultures, using protocol A (see "Materials and Methods"). (A) Gel filtration on Sephacryl S-300; (B) chromatofocusing on Pharmacia PBE 94 (pH 7.0–4.0); (C) chromatofocusing on Pharmacia PBE 94 (pH 6.0–4.0); (D) hydrophobic interaction chromatography on Octyl-Sepharose CL4B. (●——●) = PAL activity (arbitrary units are $\Delta A_{290}h^{-1}$ per 100 μL of column fraction); (-----) = A_{280nm}; (——) = pH or $(NH_4)_2SO_4$ gradient; (–·–) = ethylene glycol gradient.

Preparation of Fungal Elicitor

Colletotrichum lindemuthianum was grown for 20 d in the dark at 20°C in Mathur medium (21) modified by the addition of 15 g L^{-1} of glucose. The cell wall elicitor was obtained by autoclaving mycelial cell walls prepared as previously described (9).

Treatment of Cell Cultures

Cell suspensions were treated with yeast extract (Bacto yeast extract, Difco Laboratories) or cell wall elicitor from *C. lindemuthianum* at concentrations of 7 mg mL^{-1} of medium and 60 μg glucose equivalent mL^{-1} of medium, respectively. After different times of treatment, cells were collected on filter paper (Whatman No. 1) by vacuum filtration, frozen in liquid nitrogen, and stored at −75°C until used.

Enzyme Extraction and Assay

Extraction and assay of PAL was carried out as previously described (1). Protein was determined by a modification of the method of Bradford (25). PAL activity is expressed as μkat kg^{-1} of protein. PAL activity values were linear both with respect to time (up to 90 min) and protein concentration in both crude and purified preparations. Measurement of activity in crude preparations by spectrophotometric assay gave a 15% underestimation of the activity as measured directly by conversion of [^{14}C]phenylalanine to *trans*-cinnamic acid (and determination by TLC).

Inhibitor Studies

PAL was assayed spectrophotometrically in the presence of a range of different phenolic compounds, with different concentrations of inhibitor and substrate. Phenolics were dissolved in EtOH, the presence of which (up to 10% v/v) in the assay mixture did not inhibit PAL activity.

Purification of PAL

Frozen cells (a mixture of 250 g of cells treated with elicitor from *C. lindemuthianum* and 350 g of yeast extract-treated cells) were thawed and homogenized in 1.5 L of buffer A plus 5% (w/v) polyvinylpolypyrrolidone in a Polytron homogenizer. Following filtration through Miracloth (Calbiochem), streptomycin sulfate (Sigma) was added to a final concentration of 1 mg mL^{-1} and the homogenate centrifuged at 25,000*g* for 15 min. The supernatant was fractionated with $(NH_4)_2SO_4$ (20–50% saturation), and the pellet was resuspended in 40 mL buffer B and applied to a gel-filtration column (Pharmacia C 26/100) filled with 470 mL of Sephacryl S-300 equilibrated and eluted with buffer B. Fractions containing high PAL activity were pooled and proteins precipitated with $(NH_4)_2SO_4$ (60% saturation). The enzyme was then subjected to two different sequences of purification. In the first (protocol A) the $(NH_4)_2SO_4$ precipitate was redissolved in 20 mL buffer C, dialyzed for 2 h against 2 L of buffer C, and proteins were

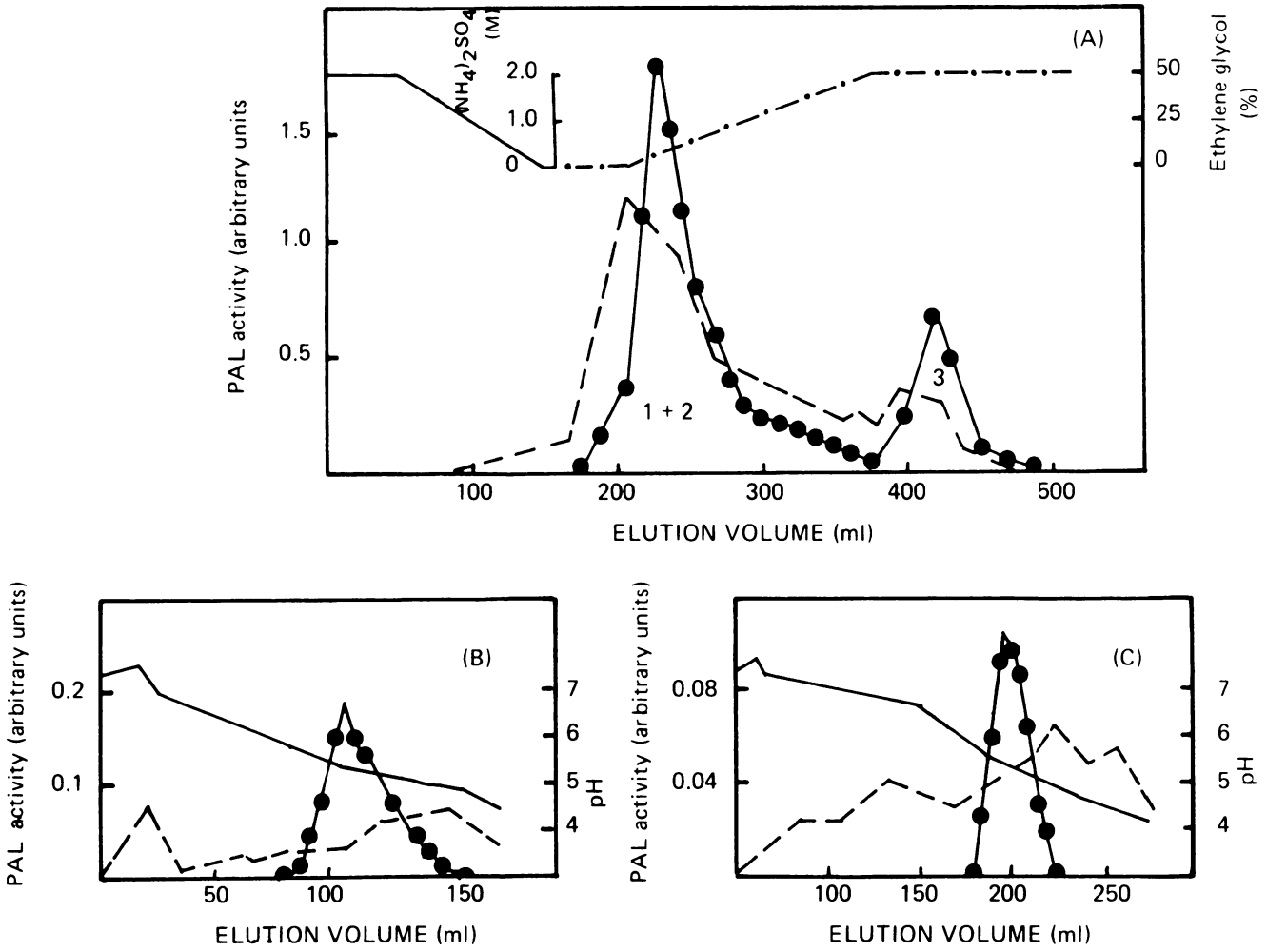

Figure 2. Purification of PAL from elicitor-treated alfalfa cell suspension cultures, using protocol B (see "Materials and Methods"). (A) Hydrophobic interaction chromatography on Octyl-Sepharose CL4B; (B) chromatofocusing on Pharmacia PBE94 of PAL peaks 1 + 2 from (A); (C) chromatofocusing on Pharmacia PBE 94 of PAL peak 3 from (A). (●——●) = PAL activity (arbitrary units are $\Delta A_{290}h^{-1}$ per 100 μL of column fraction); (-----) = A_{280nm}; (——) = pH or $(NH_4)_2SO_4$ gradient; (-·-) = ethylene glycol gradient.

chromatofocused on a Pharmacia C 16/40 column (filled with 70 mL of Polybuffer Exchanger PBE 94 equilibrated in buffer C), eluting with buffer D. Fractions with high PAL activity were pooled and proteins precipitated with $(NH_4)_2SO_4$ (80% saturation). The precipitate was resuspended in 25 mL buffer E, dialyzed against 2 L of the same buffer for 1 h and the solution rechromatofocused on a Pharmacia C 20/40 column (filled with 26 mL of PBE 94 equilibrated with buffer E), eluting with buffer D. Fractions with high PAL activity were collected and proteins precipitated with $(NH_4)_2SO_4$ (80% saturation). This precipitate was resuspended in 10 mL of buffer F and applied to a column (Pharmacia C 10/20) filled with 20 mL of Octyl-Sepharose CL4B equilibrated in buffer F. Proteins were eluted with 1 volume of buffer F followed by a decreasing gradient of ammonium sulfate (2.0–0.0 M) in buffer B and then with an increasing ethylene glycol gradient (0–50%) in buffer F.

The steps in the second sequence of purification (protocol B) consisted of gel filtration and $(NH_4)_2SO_4$ precipitation, hydrophobic interaction chromatography on Octyl-Sepharose CL4B, dialysis of PAL-containing fractions against buffer B, precipitation of proteins with $(NH_4)_2SO_4$ (80% saturation), dialysis against buffer C, and chromatofocusing on Pharmacia Polybuffer Exchanger PBE 94, using buffer D as eluant.

Induction of PAL Isoforms

Extracts from alfalfa cells (3–5 g) treated with elicitor from *C. lindemuthianum* or yeast extract for 4, 10, and 24 h were partially purified by $(NH_4)_2SO_4$ fractionation (20–60% saturation) and then subjected to chromatography on Octyl-Sepharose CL4B in disposable poly-prep chromatography columns (Bio-Rad) containing 4 mL of gel. PAL was eluted with a discontinuous (0%, 20%, 40% v/v) ethylene glycol gradient in buffer B.

Electrophoresis

PAL preparations were freeze-dried, redissolved in 50 mM Tris-HCl (pH 8.0), and subjected to one-dimensional gel electrophoresis. Nondenaturing PAGE was carried out with a

Table I. *Purification of PAL from Elicitor-Induced Alfalfa Cell Suspension Cultures*

Fraction	Volume	Protein	Activity	Specific Activity	Purification	Yield	Isoforms
	mL	*mg*	*nkat*	*μkat/kg protein*	*-fold*	%	
Crude supernatant	900	1620	92.7	57.2	1	100	1 + 2 + 3
Sephacryl S-300	95	84.2	52.9	628.3	11	57.1	1 + 2 + 3
PROTOCOL A:							
Chromatofocusing I (pH 7–4)	200	21.6	33.5	1551	27.1	36.1	1 + 2 + 3
Chromatofocusing II (pH 6–4)	70	14.5	15.6	1076	18.8	16.8	1 + 2 + 3
Octyl Sepharose CL4B:							
Total	80	1.3	3.1	2385	41.7	3.4	1 + 2 + 3
Buffer eluted	30	0.17	0.85	5000	87.4	0.9	1
30% Ethylene glycol-eluted	40	0.98	2.11	2160	37.8	2.3	2
50% Ethylene glycol-eluted	10	0.09	0.22	2444	42.7	0.2	3
PROTOCOL B:							
Octyl-Sepharose CL4B:							
10% Ethylene glycol-eluted	80	21.06	18.4	873.7	15.3	19.8	1 + 2
50% Ethylene glycol-eluted	75	11.3	4.6	407	7.0	4.9	3
Chromatofocusing pH 7–4:							
10% Ethylene glycol eluant	50	1.6	4.0	2500	43.7	4.3	1 + 2
50% Ethylene glycol eluant	30	0.3	1.2	4000	69.9	1.3	3

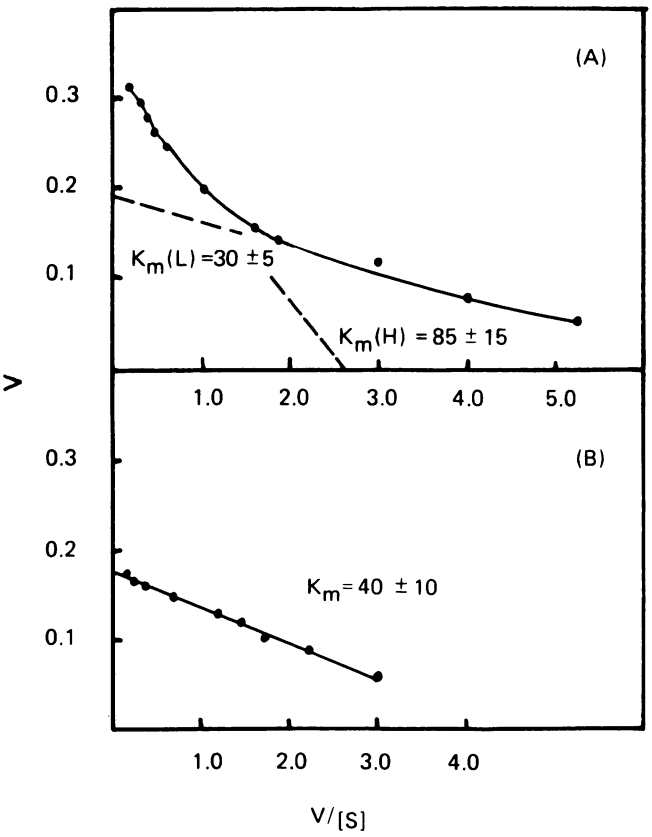

Figure 3. Eadie-Hofstee plots of initial rate data for alfalfa phenylalanine ammonia-lyase preparations. (A) Pooled fractions after Sephacryl S-300 chromatography, Figure 1A; (B) peak 1 from Octyl Sepharose CL4B, Figure 1D. $v = A_{290}h^{-1}$, [*S*] in mM.

5 to 20% (w/v) acrylamide gradient as previously described (12). SDS-PAGE (16) was performed with 10% (w/v) acrylamide in the resolving gel. Samples containing 20% (w/v) sucrose, 3% SDS, 7% (v/v) 2-mercaptoethanol, and 0.002% (w/v) bromphenol blue were heated at 90°C for 5 min before loading. Two-dimensional IEF/SDS-PAGE was performed according to the method of O'Farrell (23). Following electrophoresis, proteins were stained with Coomassie brilliant blue G-250 (22).

Molecular Weight Determination

The M_r of the native enzyme was estimated by gel filtration and PAGE. Gel filtration on Sephacryl S-300 (35 × 1.6 cm) was carried out on a partially purified PAL preparation (after gel filtration, Fig. 1) with different eluant systems (buffers B, F, H, and I). Thyroglobulin (M_r 669 × 10^3), apoferritin (M_r 443 × 10^3), β-amylase (M_r 200 × 10^3), alcohol dehydrogenase (M_r 150 × 10^3), BSA (dimer, M_r 132 × 10^3; monomer M_r 66 × 10^3), and carbonic anhydrase (M_r 29 × 10^3) were used as reference proteins.

Nondenaturing PAGE was carried out as described above and mol wt were calculated from the following reference proteins: urease (dimer, M_r 272 × 10^3; tetramer, M_r 545 × 10^3), BSA, ovalbumin (M_r 45 × 10^3), carbonic anhydrase (M_r 29 × 10^3), and α-lactalbumin (M_r 14.2 × 10^3).

The M_r of PAL subunits was determined by SDS-PAGE as described above with myosin (M_r 205 × 10^3), β-galactosidase (M_r 116 × 10^3), phosphorylase B (M_r 97.4 × 10^3), BSA, ovalbumin, and carbonic anhydrase as reference proteins.

Western Blotting

Western blotting of partially purified and final PAL preparations after nondenaturing PAGE, SDS-PAGE, and IEF/SDS-PAGE were carried out with a Trans Blot unit (Bio-Rad). After electrophoresis, gels were equilibrated in buffer J

Table II. *Inhibition of Alfalfa PAL by Phenolic Compounds*

A PAL preparation purified as far as the first chromatofocusing step (protocol A), and containing the three isoforms, was used. Activity is expressed as a percent of the activity in the absence of potential inhibitor. All phenolics were added at a final concentration of 50 μM.

Compound(s)	Concentration of L-Phenylalanine	Activity
	mM	%
Trans-cinnamic acid or 4-coumaric acid	0.1	58
Caffeic acid	0.1	68
Ferulic acid	0.1	71
Sinapic acid, *o*-hydroxycinnamic acid, *p*-hydroxybenzoic acid, gallic acid, chlorogenic acid, or catechin	0.1	74–78
Coniferyl alcohol or umbelliferone	0.1	100
Formonetin, daidzein or coumestrol	1.0	85–88
Daidzin, genistein, or genistin	1.0	76–78
Biochanin A, isoliquiritigenin, naringenin, 5-hydroxyisoflavone, or 7-hydroxyisoflavone	0.1	89–92
Apigenin	0.1	105

Table III. *Inhibition of Alfalfa PAL forms 1 and 2*

PAL isoforms were purified and separated by gel filtration, chromatofocusing and hydrophobic interaction chromatography as in Figure 1. Forms I and II (equal activities) were assayed in the presence of 500 μM L-phenylalanine and different concentrations of *trans*-cinnamic acid, chlorogenic acid, and genistein. PAL activity is expressed as a percent of the activity in the absence of potential inhibitor.

Inhibitor	Concentration	PAL Activity	
		Form 1	Form 2
	μM	%	
t-Cinnamic acid	100	28.0	41.0
	50	69.0	82.0
	10	85.0	96.0
Chlorogenic acid	100	74.0	93.0
	50	84.0	100.0
	10	93.0	100.0
Genistein	500	0.0	0.0
	100	89.0	100.0
	10	100.0	100.0

and electroblotted onto nitrocellulose sheets for 12 h at 150 mA. Transfer was efficient as assessed by the use of prestained SDS-PAGE standards (M_r range 17 × 10^3 to 130 × 10^3, Bio-Rad).

After blotting, the membrane was washed and incubated with anti-(bean PAL) serum (1) (1:3000 diluted) as described in the Bio-Rad Immuno-Blot Assay Kit. Protein antibody complexes were detected with goat anti-rabbit alkaline phosphatase conjugate (Bio-Rad) according to the manufacturer's instructions.

In Vitro Translation and Immunoprecipitation of PAL

Polysomal RNA was extracted from alfalfa cells (cv Europe) treated with elicitor from *C. lindemuthianum* (60 μg glucose equivalents mL^{-1}) for 1, 2, 3, 4, 6, and 20 h, by a modification (26) of the method of Palmiter (24). RNA was assayed spectrophotometrically at 260 nm. Isolated polysomal RNA was translated *in vitro* in the presence of [^{35}S]methionine with an mRNA-dependent rabbit reticulocyte lysate translation system (Promega).

In vitro translated PAL was immunoprecipitated indirectly with bean anti-PAL serum using Pansorbin cells (Calbiochem; to final concentration of 9 μg mL^{-1} serum) in place of protein A Sepharose according to a previously described method (18). Immunoprecipitated material was subjected to SDS-PAGE as described above. After fixing gels in glacial acetic/methanol/water (1:9:10), the distribution of radioactivity was analyzed by fluorography.

Extraction and Analysis of Phenolic Metabolites

Frozen cells were homogenized in 2 volumes of absolute ethanol. After evaporation of the ethanolic extract to dryness under vacuum, the residue was taken up in double-distilled H_2O and partitioned twice against ethyl acetate. The organic layers were pooled and taken to dryness under vacuum. The residue was dissolved in ethanol and used for HPLC separation and analysis of phenolic compounds according to the method of Koster *et al.* (15).

RESULTS AND DISCUSSION

Purification of PAL

The enzyme was purified from mixed batches of alfalfa suspension culture that had been separately exposed for 10 h to cell wall elicitor from *C. lindemuthianum* (60 μg glucose equivalents mL^{-1}) (250 g cells) and yeast extract (0.7 mg mL^{-1}) (350 g cells). After the gel filtration stage, two different protocols were used (See Materials and Methods). In protocol A, two rounds of chromatofocusing preceeded hydrophobic interaction chromatography on Octyl Sepharose CL4B (Fig. 1). In protocol B, peaks resolved on hydrophobic interaction chromatography were individually subjected to chromatofocusing (Fig. 2). Chromatofocusing revealed two major but unresolved peaks of PAL activity, which were resolved into

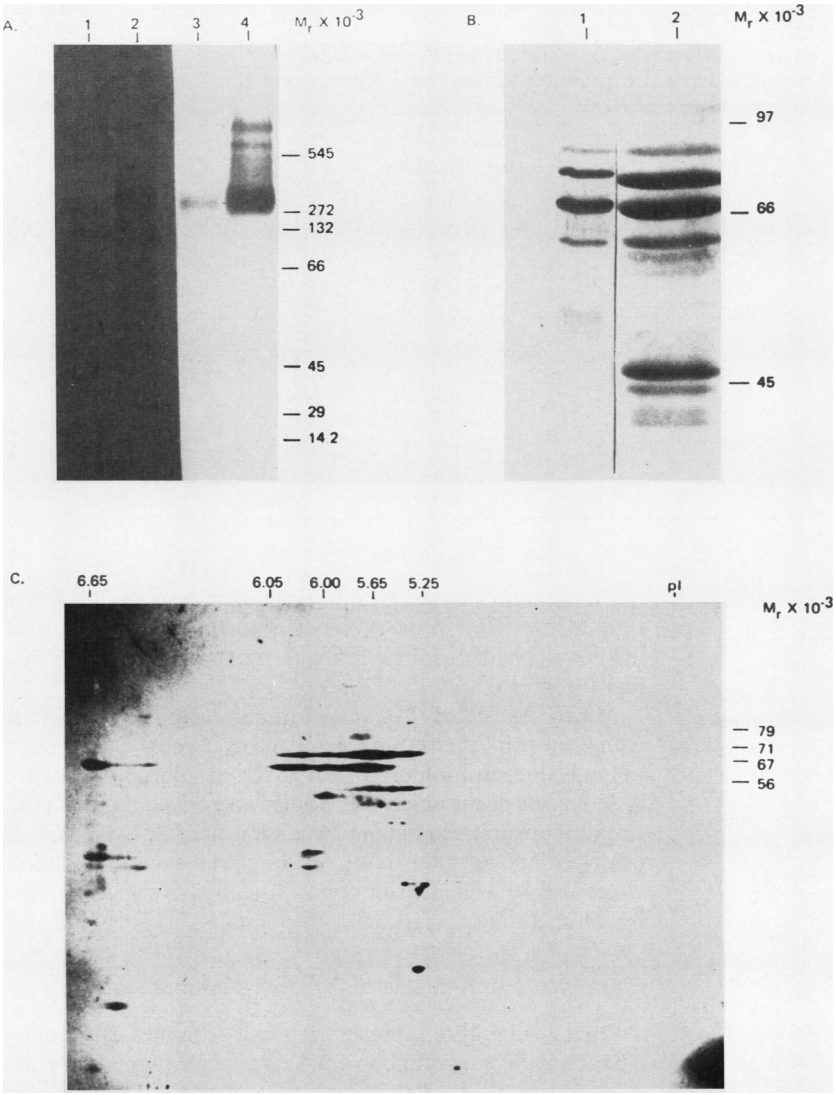

Figure 4. Gel electrophoretic analysis of alfalfa phenylalanine ammonia-lyase preparations. The enzyme was purified by gel filtration, hydrophobic interaction chromatography, and chromatofocusing to yield a mixture of forms 1 and 2 (as in Fig. 2B). (A) Nondenaturing PAGE. Tracks are: 1, M_r markers; 2, 15 μg protein; 3, Western blot of 2 μg protein; 4, Western blot of 15 μg protein. (B) SDS-PAGE of 20 μg protein. Track 2 is the gel separation, track 1 the Western blot. (C) Two-dimensional IEF/SDS-PAGE of 30 μg protein.

three peaks by hydrophobic interaction chromatography (Fig. 1, C and D). Hydrophobic interaction chromatography immediately after gel filtration failed to resolve fully the two, less hydrophobic PAL forms (Fig. 2A); subsequent chromatofocusing of forms 1 plus 2 showed that they must have isoelectric points of approximately 5.4 (peak) and 5.1 (shoulder) (Fig. 2B), whereas form 3 gave a single symmetrical chromatofocusing peak of pI 5.4 (Fig. 2C). The pI values of the individual PAL forms correspond to those observed for the mixed population of PAL forms in Figure 1C. Alfalfa PAL is clearly a very hydrophobic enzyme, as form 3 requires 50% ethylene glycol for elution from Octyl-Sepharose.

The final specific activities of the purified PAL isoforms varied from 2160 to 5000 μkat kg^{-1} (Table I). This is in the same range as for the PAL isoforms from elicitor-induced bean cell cultures (1). However, the final specific activity for identical isoforms differed depending on the purification protocol. This is most likely due to partial degradation, and thereby inactivation, of the enzyme during purification, as also reported for the enzyme from bean (2) (see below). From a number of separate purifications, we observed that the highest final specific activities were obtained when the enzyme was purified as quickly as possible.

Kinetic Properties

Previous reports have suggested that PAL may exhibit negative rate cooperativity for its substrate L-phenylalanine (8, 13), although this may simply reflect the combined kinetics of a number of isoforms each exhibiting classical Michaelis-Menten kinetics (1). In most cases where a single PAL isozyme was studied, the Hill coefficient approximated to unity (8, 14). A similar situation was observed for alfalfa PAL (Fig. 3). Eadie-Hofstee plots of initial rate data for mixtures of forms 1, 2, and 3 were non-linear, with maximum and minimum K_m values calculated by extrapolation to be 85 and 30 μM, respectively (Fig. 3A). Values of 135 and 65 μM were obtained with a mixture containing forms 1 and 2 (not shown). In contrast, individual alfalfa PAL isoforms gave linear Eadie-Hofstee plots (*i.e.* obeyed Michaelis-Menten kinetics) with calculated K_m values (μM) of 40 (Fig. 3B), 70, and 110 for

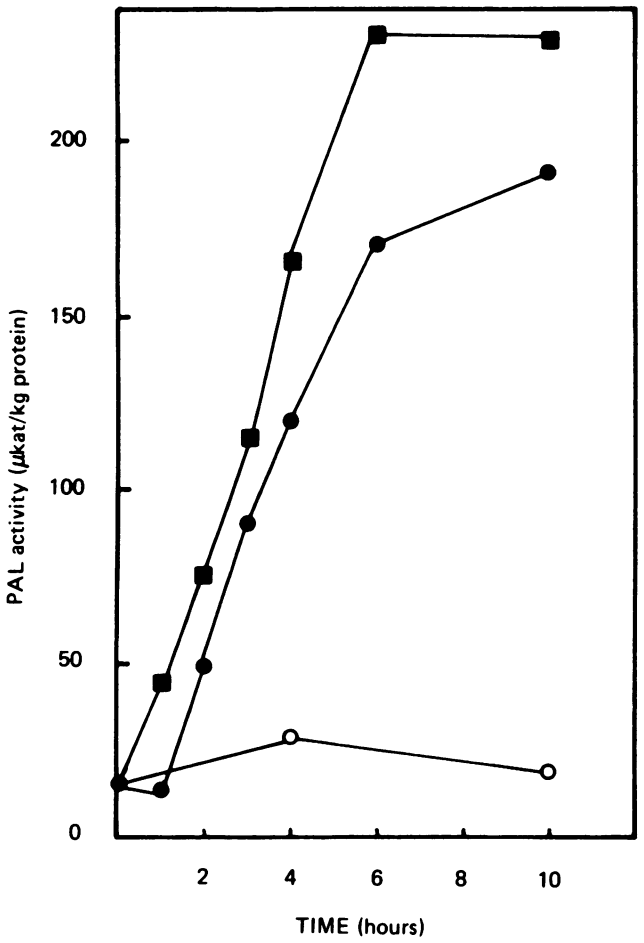

Figure 5. Changes in PAL activity in elicitor-treated and control alfalfa cell suspension cultures in response to cell wall elicitor from *C. lindemuthianum* (●—●, 60 μg glucose equivalents mL^{-1}) or yeast extract (■—■, 0.7 mg mL^{-1}). O—O = untreated cells.

forms 1, 2, and 3, respectively. These values are somewhat lower than observed with the PAL isoforms from cultured bean cells (77–302 μM).

Inhibitor Studies

In some species, isoforms of PAL are inhibited differently by different phenolic compounds (8). A number of phenolic compounds were therefore tested as potential inhibitors of PAL activity (a mixture of forms 1, 2, and 3) (Table II). At 50 μM, cinnamic and 4-coumaric acids were the only compounds to give more than 40% inhibition of activity (with substrate concentration of 100 μM). A number of isoflavonoids (formononetin, daidzein, genistein) inhibited by 80 to 100% at 500 μM with substrate concentration 1 mM (data not shown), but gave only about 10 to 20% inhibition at 50 μM with 1 mM substrate (Table II) or 0.1 mM substrate (not shown). At high concentrations, isoflavonoids may simply cause inactivation rather than classical inhibition. The effects of cinnamic acid, chlorogenic acid, and genistein were very similar on the individual PAL forms 1 and 2 (Table III).

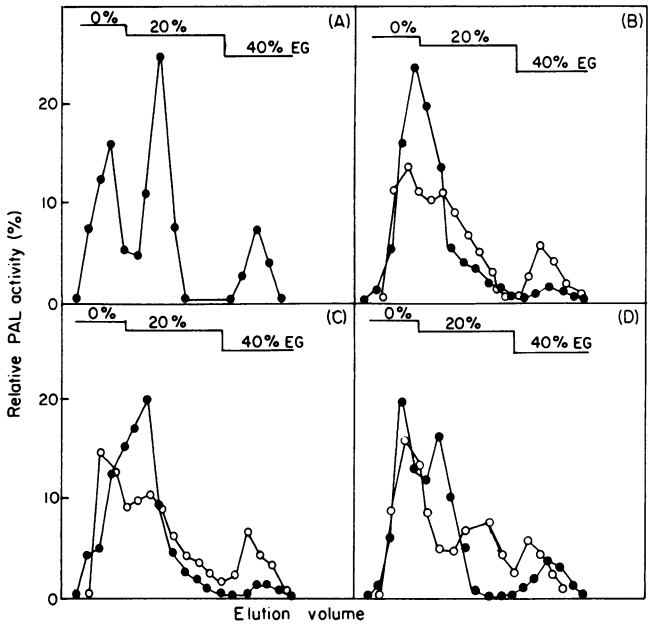

Figure 6. Hydrophobic interaction chromatography profiles for PAL forms induced in alfalfa cell suspension cultures by fungal elicitor or yeast extract. Cells were treated with elicitor from *C. lindemuthianum* (●—●) (60 μg glucose equivalents mL^{-1}) or yeast extract (O—O) (0.7 mg mL^{-1}) for 0 h (A), 4 h (B), 10 h (C), or 24 h (D). PAL was partially purified by $(NH_4)_2SO_4$ precipitation prior to chromatography on Octyl-Sepharose CL4B, eluting with a stepped gradient of ethylene glycol (EG). Elution profiles are normalized (total eluted activity = 100%).

Table IV. *Differential Induction of PAL Isoform 3 by Yeast Extract and Fungal Elicitor*

Elicitor Time	Yeast Extract			*Colletotrichum* Elicitor		
	Total PAL	PAL3	PAL3	Total PAL	PAL3	PAL3
h	*pkat*		*% of total PAL*	*pkat*		*% of total PAL*
0	110	15.4	14.0	110	15.4	14.0
4	2,700	351	13.0	1,444	58	4.0
10	3,100	481	15.5	2,930	85	2.9
24	1,100	165	15.0	720	72	10.0

Molecular Weights of Native Enzyme and Subunits

Gel filtration on a calibrated Sephacryl S-300 column gave an M_r of 311,000 for the intact, active PAL enzyme. To ensure that the isoforms observed on chromatofocusing or hydrophobic interaction chromatography were not simply dissociated dimers or monomers, gel filtration was repeated with preequilibration and elution in ampholyte polybuffer, ammonium sulfate, or ethylene glycol, as used in these later techniques. In each case, the M_r was identical at 311,000 (data not shown). A similar M_r of 316,000 was also determined by nondenaturing gradient PAGE (Fig. 4A). Minor higher aggregation products which, like the major 316,000 component, reacted with anti-(bean PAL) serum on western blots, were also observed on nondenaturing gels (Fig. 4A).

As in the case of bean PAL (2), it was not possible to obtain a single band on SDS-PAGE of purified alfalfa PAL prepa-

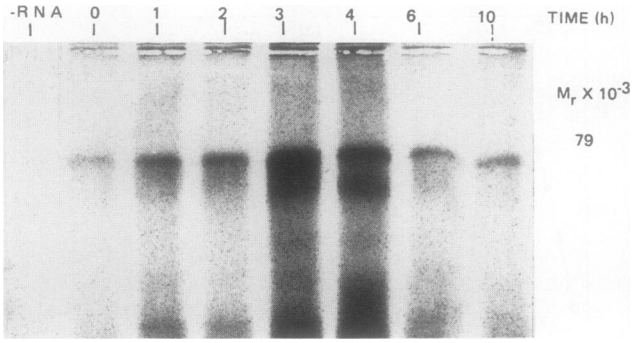

Figure 7. Changes in translatable PAL mRNA activity in elicitor-treated cell suspension cultures of alfalfa cv Europe. SDS-PAGE of polysomal mRNA *in vitro* translation products labeled with [^{35}S] methionine and immunoprecipitated with anti-(bean PAL) serum.

rations (Fig. 4B). In bean, detailed peptide mapping and interconversion studies have shown that the intact M_r 78,000 PAL subunit is unstable and degrades to distinct partials of M_r 70,000 and 45,000 (2). Components of M_r 79,000, 71,000, 67,000, and 56,000 were observed in all purified alfalfa PAL preparations, and as they all reacted with anti-(bean PAL) serum (Fig. 4B), it is likely that they represent PAL and its degradation products. Other bands, including a major component of M_r 48,000 that did not react with anti-(bean PAL) serum, are probably contaminants.

Two-dimensional IEF followed by SDS-PAGE of purified alfalfa PAL preparations revealed a number of poorly resolved subunit charge isoforms in the pI range 5.1 to 6.1 (Fig. 4C). In bean, at least 11 charge isoforms of the intact PAL subunit have been observed by two-dimensional gel analysis of immunoprecipitates of polypeptides labeled with [^{35}S]methionine *in vivo* (1).

Elicitor Induction of PAL Activity

In uninduced alfalfa cell cultures, PAL activity increased approximately 50% between 60 and 80 h after subculture (data not shown). This coincided with the period of most rapid growth rate and was followed by a steady decline in basal activity. Addition of either cell wall elicitor from *C. lindemuthianum* or yeast extract to cells 72 h after subculture resulted in a rapid increase above the basal PAL activity (Fig. 5).

Elicitor Induction of PAL Isoforms and Phenylpropanoid Compounds

In bean, elicitation leads to a preferential induction of the two PAL isoforms with the lowest K_m values (1). This may exert a metabolic priority for entry of phenylalanine into the phenylpropanoid pathway specifically under stress conditions. To investigate whether the active PAL isoforms were differentially regulated in alfalfa cell cultures, cells were harvested at 0, 4, 10, and 24 h after exposure to either elicitor from *C. lindemuthianum* or yeast extract, and the isoforms analyzed by hydrophobic interaction chromatography (Fig. 6). Although forms 1 and 2 are not fully resolved on analysis of extracts containing high protein concentrations, it was clear that isoform 1 was preferentially induced during the first 4 h of exposure to elicitor from *C. lindemuthianum*. The relative proportions of the three forms changed less during induction by yeast extract. These findings were confirmed in a separate experiment in which the relative proportions of the PAL isoforms were determined after resolution by chromatofocusing (data not shown). The behavior of isoform 3 (the most hydrophobic form with the highest K_m value) was easier to analyze as it was better resolved and its total activity could be calculated. During the 28.2-fold induction of PAL activity by yeast extract, the proportion of PAL 3 remained approximately constant at between 13 and 15.5% of the total activity (Table IV). In contrast, PAL 3 only accounted for 2.9% of the total activity 10 h after exposure to elicitor from *C. lindemuthianum*.

To test whether induction of different PAL isoforms resulted in altered patterns of phenolic metabolites, extracts from elicitor-treated alfalfa cells were analyzed by HPLC. At least 26 compounds, including isoflavones, hydroxycinnamic acids, and the pterocarpan phytoalexin medicarpin were resolved (data not shown). Identification of many of these compounds is still in progress (5). Most of the less polar compounds with retention times over 15.4 min (including medicarpin) were induced by both elicitors (the increase being apparent within 11 h), and the extent of induction was greatest with the fungal elicitor. There were, however, no absolute qualitative differences in the patterns of phenolics in response to the two elicitors. The different kinetic properties of the PAL pools, resulting from different rates of appearance of the isoforms, therefore, only appear to affect phenylpropanoid metabolism in a quantitative manner in these cells.

Elicitor Induction of PAL mRNA Activity

Increased activity of phytoalexin biosynthetic enzymes in cultured plant cells or intact tissues of the whole plant invariably arises as a result of increased translatable mRNA activity due to transcriptional activation in response to elicitor or other microbial signals (10, 18, 19). Analysis of *in vitro* translation products from polysomal mRNA, by immunoprecipitation with anti-(bean PAL) serum followed by SDS-PAGE, indicated that the elicitor-induced PAL activity increase corresponded to an increase in the translational activity of an mRNA species encoding a polypeptide subunit or M_r 78,000 and recognized by the bean PAL antibody (Fig. 7). Maximum PAL mRNA activity was observed 3 h post-elicitation.

CONCLUDING REMARKS

We have demonstrated that alfalfa PAL exists in a number of isoenzymic forms exhibiting significant differences in hydrophobicity. This suggests that the enzyme may be encoded by a multigene family, as recently demonstrated in bean (4). Of particular interest are the clear differences in activity levels of the different isoforms following elicitation. With elicitor from *C. lindemuthianum*, PAL induction results in a large relative increase in the form with the lowest K_m value for phenylalanine, as observed in bean cells treated with this elicitor (1). In contrast, the pattern of PAL forms induced by

yeast extract would result in less change in the overall kinetic properties of the PAL pool. It is interesting that this difference is also associated with different levels of accumulation of phenylpropanoid secondary products, although the differences are quantitative rather than qualitative, and different PAL forms did not appear to be differentially inhibited by any of the phenylpropanoids tested. A fuller understanding of the significance of the multiple PAL forms must await the cloning of the PAL gene(s) from alfalfa.

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