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L-Phenylalanine ammonia-lyase from French bean (*Phaseolus vulgaris* L.)

Characterization and differential expression of antigenic multiple M_r forms

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L-Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) purified from suspension-cultured cells of French bean (*Phaseolus vulgaris*) has been further characterized. A number of techniques, including use of an antiserum and affinity probes, have established that all the antigenic polypeptides represent polymorphic M_r forms of the enzyme. These peptides include an apparently higher-M_r (83000) form which shows different kinetics of induction from the M_r-77000 forms that have been extensively characterized previously. The larger subunit appeared to be PAL by the following criteria: (*a*) binding to specific affinity and antibody matrices; (*b*) peptide mapping; (*c*) active-site labelling; and (*d*) amino acid composition. The increased M_r of the larger subunit was not completely attributable to glycosylation, although some sugar residues were detected in this M_r-83000 form but not in the other M_r forms. M_r-83000 subunits were also immunoprecipitated from translations *in vitro* of mRNA from cells that had been stressed for a long period. They were also detected in leaf tissues that were not yet undergoing an extensive wound response. This form of the enzyme may be constitutive and involved in the low-level accumulation of phenolics in most cell types. By contrast, the M_r-77000 forms of PAL were rapidly induced during elicitor action, wounding or cytokinin-induced xylogenesis as a key regulatory enzyme involved in the synthesis of phenolics under stress conditions or during differentiation.

INTRODUCTION

Differing types of plant phenolic products accumulate in cells and tissues during development and morphogenesis and in response to stress. Thus lignin is a major constituent of the secondary walls of vascular tissue, whereas flavonoids function as pigments and probably protectants against u.v. light in aerial organs. After wounding or pathogenic attack, lignin-like material accumulates at the lesion site of most species, whereas isoflavonoid phytoalexins are produced additionally in leguminous species. All these compounds are synthesized through pathways for which phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) is an initial key regulatory enzyme (Jones, 1984).

It is therefore important to understand the way in which the tissue-specific accumulation of particular phenolic species is governed by differential gene expression and accumulation of gene products. Cloning of a number of inducible PAL genes from French bean (*Phaseolus vulgaris*) and parsley (*Petroselinum crispum*) (Cramer *et al.*, 1989; Lois *et al.*, 1989) and study of PAL-gene expression in cell-suspension cultures has led to some understanding of the regulation of these processes. Combining these studies with those at the post-translational level has led to some understanding of the phenylpropanoid pathway as a whole, molecular studies having only recently been extended to intact tissue (Dixon *et al.*, 1986; Scheel *et al.*, 1987; Liang *et al.*, 1989*a*). The differential accumulation of inducible gene-specific mRNAs and protein subunits has been further analysed in French bean (Liang *et al.*, 1989*a*). Insight into the role of controlling elements in gene expression has been increased by work with transgenic plants (Liang *et al.*, 1989*b*; Bevan *et al.*, 1989).

However, although much of this work has been focused at the level of gene expression, certain aspects of control at the protein level indicate that regulation at this level is also complex. PAL is known to be subject to considerable post-translational processing, since the number of subunits of the M_r-77000 form observed in two-dimensional gel analysis exceeds the number of direct gene products (Bolwell *et al.*, 1985), and the molecular basis of multiple M_r forms has been investigated in the present study. The significance of the M_r polymorphism observed has been examined by differential expression studies in elicitor-treated cell-suspension cultures, hypocotyls and leaf tissue. Evidence is presented for an M_r-83000 form that accumulates preferentially in cells maintained in cultures and which is turned over in leaf tissue, as opposed to the M_r-77000 forms that are rapidly induced by wounding, elicitor, dilution or cytokinin action.

MATERIALS AND METHODS

Plant material

French bean (*Phaseolus vulgaris* L. var. The Prince) plants were grown in a growth chamber under a 16 h light (10000 lx)/8 h dark regime for 10 days. First leaves were harvested at this time, and wounding of the hypocotyl was carried out by excision of the epicotyl just below the cotyledons. Samples of the cut sites were taken 16 h later. Suspension cultures of the same variety of bean were derived, maintained and elicited as described previously (Dixon & Lamb, 1979). Suspension cultures were subjected to cytokinin treatments as described by Bolwell & Northcote (1983).

Other materials

Anti-(bean PAL) serum and purified anti-PAL IgG were derived as described by Bolwell *et al.* (1986*a*). Trans ^{35}S-Label (30TBq/mmol) was from ICN, High Wycombe, Bucks., U.K.

Polypeptide analysis and immunodetection techniques

Isolation of subunits of PAL by conventional column chromatography and ^{3}H-labelling of the active site were performed as described previously (Bolwell *et al.*, 1985, 1986*b*). Immunopurification of PAL subunits was done as described by

Abbreviations used: GRP, glycine-rich protein; PAL, phenylalanine ammonia-lyase; PVDF, poly(vinylidine difluoride).

Bolwell *et al.* (1986*a*); affinity purification was done as described by Dixon *et al.* (1987). Radiolabelling of polypeptides was carried out (*a*) by incubation of elicitor-treated suspension-cultured cells with Trans ^{35}S-label (74 kBq/ml) for between 3 and 4 h after addition of elicitor, (*b*) for hypocotyl-wound-site treatments by application of 74 kBq of Translabel in 20 μl of pure water to each cut site for 16 h before harvesting the top 0.5 cm of each hypocotyl, (*c*) for cytokinin-treated cultures by incubating cells with Translabel (74 kBq/ml) for 1 h before harvesting or (*d*) for first leaves, which were cut into 1 cm × 1 cm pieces to limit the wound response at the edge relative to the size of the explant, vacuum-infiltrated and floated on pure water containing 74 kBq/ml for up to 8 h under a continuous light regime (1000 lx). Immunoprecipitations of extracts of radiolabelled cells were then carried out at titre levels (< 1:20) at which radiolabelled M_r-83000 subunit would be observed (Bolwell *et al.*, 1986*a*).

Specific ^{3}H-labelling of the active site, using the active site protection method, was as described by Bolwell *et al.* (1986*b*). One-dimensional peptide ('Cleveland') mapping was carried out as described by Gooderham (1987), whereas two-dimensional separation was carried out as previously described (Bolwell *et al.*, 1986*a*). *N*-Terminal amino acid sequencing was carried out on the 83000-, 77000- and 70000-M_r peptides after blotting on to glass-fibre paper (Aebersold *et al.*, 1986) or poly(vinylidine difluoride) (PVDF) membranes (Legendre & Matsudaira, 1988) using an ABI gas-phase sequencer, model 470. Amino acid analysis was also carried out on the blotted bands after hydrolysis in 6 M-HCl on an LKB Alpha-plus amino acid analyser using sodium buffers and ninhydrin detection in accordance with the manufacturer's instructions. Sugar analysis of the blotted bands was carried out on methanolic hydrolysates, followed by derivatization by the method of Chambers & Clamp (1971) and analysis by capillary g.l.c. on a Varian 3300 gas chromatograph.

RESULTS

Characterization of the M_r -83000 antigenic polypeptides

PAL was purified from suspension-cultured bean cells as far as the final chromatofocusing step (Fig. 1). Elution profiles from elicitor-treated cells were different from those from untreated cells (Bolwell *et al.*, 1985; Liang *et al.*, 1989*a*). M_r-77000 subunit isoforms from elicitor-treated cells were eluted over the pH range 5.4–4.8, whereas only the pI-4.8 isoform is found in unelicited cells, as previously described (Bolwell *et al.*, 1985). The M_r-70000 subunit, which is a degradation product (Bolwell *et al.*, 1985), was eluted simultaneously. These M_r values are those obtained from SDS/PAGE analysis on 10%-acrylamide gels when the observed M_r of the 77000-M_r form corresponds to the predicted M_r from the gene sequences (Cramer *et al.*, 1989). PAL subunits migrate aberrantly on SDS/PAGE, so that the apparent M_r values decrease with lowering gel percentage. A Ferguson analysis of PAL migration (Hames, 1982) gave a Y_0 of 1.7, rather than 1.0, and a K_R of 0.73. Fig. 1(*a*) shows that further PAL activity can be eluted as a shoulder with average pI of about 4.5–4.6. The predominant subunit in this fraction was of M_r 83000, determined relative to the other PAL forms (Fig. 1*b*) and had a K_m of 0.433 mM, substantially higher than that determined for the M_r-77000 subunit isoforms. In these samples there was a small amount (7%) of the M_r-77000 form, the presence of which could not account for the bulk of the activity and did not substantially cause deviation from Michaelis–Menton kinetics, so the K_m probably represents that for the higher-M_r form. The M_r-83000 form was the predominant subunit from suspension-cultured cells that were allowed to enter stationary phase and began to turn a brown colour. These cells were used as the source of the polypeptide in the composition studies. Fractions containing the subunits were subjected to blotting on to PVDF membrane (Legendre & Matsudaira, 1988), and the composition of each subunit isoform was determined and is shown in Table 1. The composition of these three forms deviate somewhat from those determined from the gene sequences for *PAL2* and *PAL3* (Cramer *et al.*, 1989) for some of the residues, whereas others are in close agreement. However, interpretation of the significance of these differences can only be limited, since the sequence of the third gene, *PAL1*, is incomplete, and this represents the predominant M_r form from elicited cells (Bolwell *et al.*, 1986*a*; Liang *et al.*, 1989*a*). However, some comparisons can be drawn. The M_r-70000 form has previously been shown by pulse–chase experiments and two-dimensional gel analysis to be a breakdown product of the M_r-77000 form (Bolwell *et al.*, 1986*a*). Within the limitations of acquiring compositions from blotted subunits (Ozals, 1990), inspection of those that have been obtained in comparison with the relative abundance of each amino acid at the *N*- and *C*-terminus of the gene sequences show that they are probably more consistent with the M_r-70000 subunit being a *C*-terminal cleavage product of the 77000-M_r subunit. The higher recoveries of amino acids that are relatively abundant at the *N*-terminus, such as leucine and isoleucine in the M_r-70000 subunit, support this conclusion, although the changes in the relative composition are clearly not quantitative and are subject to experimental error. This was further borne out when attempts to determine the *N*-terminal amino acid sequence with up to 100 pmol of subunit bands blotted on to either PVDF membrane (Matsudaira & Legendre, 1988) or glass-fibre paper (Aebersold

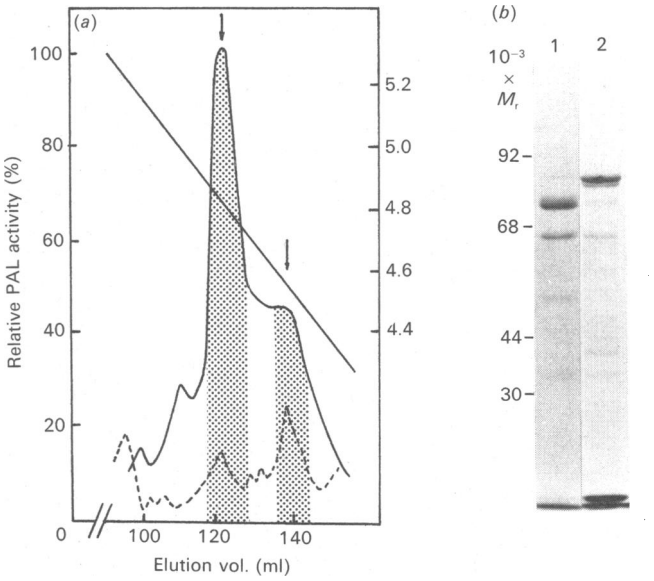

Fig. 1. Purification of PAL subunits from suspension cultures of bean

(*a*) Elution profile of PAL enzyme from chromatofocusing. PAL was purified by gel filtration on Sephacryl S300, DEAE-Sepharose and two rounds of chromatofocusing (Bolwell *et al.*, 1985). Fractions indicated by the stippled areas were pooled, dialysed and freeze-dried before SDS/PAGE analysis as shown in (*b*). ——, relative enzyme activity; – – – –, A_{280}. (*b*) SDS/PAGE analysis of purified PAL on 7.5%-acrylamide gels is shown for: 1, M_r-77000/70000 form from elicited suspension-cultured cells; the M_r-70000 subunit runs below the marker on 7.5% gels; 2, M_r-83000 form from stationary-phase suspension-cultured cells; each protein in the doublet at M_r 83000 has an identical amino acid composition when analysed after blotting on to PVDF paper and differ only in the extent of glycosylation. The M_r-77000 and -70000 subunits from 1 and the M_r-83000 form from 2 (as shown) were subjected to analysis after blotting on to PVDF membrane.

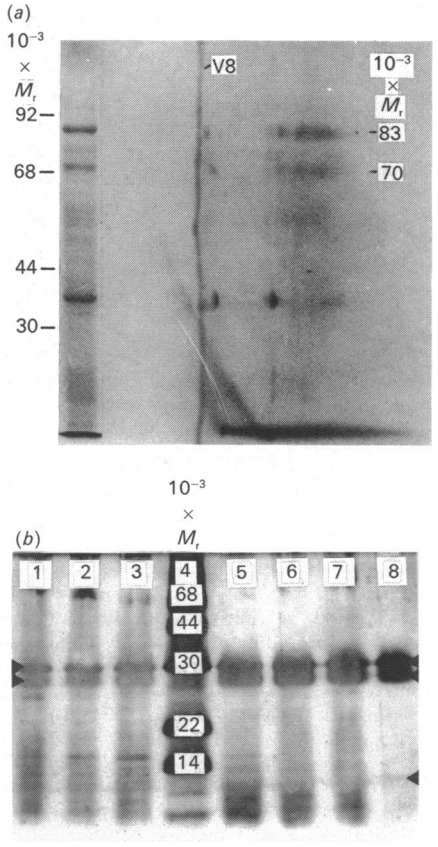

Fig. 2. Peptide mapping of PAL subunits

(*a*) A preparation of PAL taken to the first chromatofocusing stage was analysed by two-dimensional peptide mapping. Subunits were separated by SDS/PAGE in the first dimension on 10% acrylamide, followed by mapping in the second dimension using *Staphylococcus aureus* V8 proteinase ('V8') in a 15%-acrylamide gel. A duplicate track from the first-dimension run was aligned against the proteolytic gel and the composite photograph rotated through 90° for illustrative purposes. Both gels were stained with Coomassie Blue. (*b*) Cleveland mapping of purified PAL subunits on a 15%-acrylamide gel. Purified M_r-83000 (tracks 1 and 5), -77000 (2 and 6) and -70000 (3 and 7) subunits were subjected to proteolysis with V8 proteinase [tracks 1, 2 and 3 (100 ng/track) or tracks 5, 6 and 7 (1 μg/track)]. Track 4, M_r markers (14000, 20000, 30000, 44000, 68000 and 92000). Track 8, Proteinase-specific bands. The gel was silver-stained.

et al., 1986) led to no sequence being obtained. This indicates that, in common with many cytoplasmic proteins, the subunits may be *N*-terminally blocked, and the 70000-M_r subunit remains so after cleavage. Monosaccharides were detected in the M_r-83000 subunits, but not the 77000- and 70000-M_r subunits. The amounts detected were insufficient to account for the differences in M_r observed between the 77000- and 83000-M_r subunits.

The M_r-83000 subunit shows nearly identical patterns on one-dimensional or two-dimensional Cleveland mapping (Fig. 2). Fig. 2(*a*) shows a two-dimensional map of the M_r-83000 compared with the 70000-M_r form and stained with Coomassie Blue, whereas Fig. 2(*b*) shows an additional comparison with the 77000-M_r form in a one-dimensional format and silver-stained. In both cases, although the resolution of the digestion products is not complete, presumably because of the contribution of different isoforms (Bolwell *et al.*, 1985), the M_r forms only differ in the position of one or two peptides. The PAL sample used for the two-dimensional mapping differed from that shown in Fig. 1(*b*), since it was not purified to the same extent, the final chromatofocusing step being omitted. The polypeptide seen at

Table 1. Composition of PAL subunits blotted on to PVDF membrane

Compositions of M_r forms were determined as described in the text; for *PAL2* and *PAL3* products from gene sequences (Cramer *et al.*, 1989).

Amino acid or sugar	Subunit M_r...	Composition (mol of residue/100 mol of amino acid)				
		70000	77000	83000	*PAL2*	*PAL3*
Asx		8.4	9.6	8.0	9.4	8.3
Thr		2.4	3.3	3.9	5.7	5.6
Ser		10.3	8.6	9.4	7.1	7.3
Glx		15.6	14.9	14.7	10.9	11.4
Pro		3.1	8.2	7.7	4.2	5.1
Gly		8.9	8.1	8.2	7.3	5.9
Ala		5.6	5.7	7.6	10.1	8.0
Cys		3.2	4.9	3.0	1.9	0.9
Val		10.2	5.6	6.2	5.7	2.9
Met		0.5	0.5	0.5	1.8	1.8
Ile		10.6	3.9	4.9	5.7	5.8
Leu		10.1	8.1	9.9	11.2	12.3
Tyr		0.4	4.7	2.8	2.5	0.7
Phe		2.7	3.4	2.4	3.2	3.4
His		5.7	3.9	4.0	5.8	6.1
Arg		2.4	6.0	4.5	3.9	4.1
GlcNAc		0	0	0.5	–	–
Man		0	0	1.4	–	–

M_r 38000 is removed if the final step is included and certainly differs in its peptide map. Furthermore, it is not related to the M_r-46000 degradation product of PAL identified previously (Bolwell *et al.*, 1986*a*).

Immunological studies

The anti-PAL serum and the IgG fraction purified subsequently were that previously designated 'antiserum 1' (Bolwell *et al.*, 1986*a*). Immunopurification was also used to assess the extent of glycosylation. In these studies, inhibitors of glycosylation (tunicamycin and monensin) were included during the pulse-labelling of suspension cultures, or digestion of immunoprecipitated bands was performed with glycosidases specific for glycoprotein linkages. Neither treatment altered the relative abundance of the 83000- and 77000-M_r forms (L. Osborne & G. P. Bolwell, unpublished work). These M_r variations are thus not a product of a gross difference in the level of glycosylation, and this is further indicated by the fact that when PAL subunits are immunoprecipitated from elicited suspension-cultured cells exposed to radiolabelled sugars, little incorporation into the subunits is seen, unlike in the case of the potato (*Solanum tuberosum*) enzyme (Shaw *et al.*, 1990).

Fig. 3(*a*) shows that the M_r-83000 form can be subjected to active-site-protected ^{3}H-labelling. This M_r species is also immunoprecipitated from extracts radiolabelled *in vivo* and from translations *in vitro* of mRNA prepared from both uninduced and elicitor-treated tissue-cultured cells. Fig. 3(*b*) shows that, after elicitor induction, the kinetics of accumulation of newly synthesized M_r-83000 subunit differs from the M_r-77000 form in being much less rapidly induced. Although translation of the mRNA species coding for M_r-77000 PAL has previously been found to be poor, even with hybrid-release-generated mRNA, different patterns of transcript accumulation were also observed.

Fig. 4 shows that, of the polypeptides newly synthesized in elicitor-treated suspension-cultured cells, as demonstrated by radiolabelling, incorporation into the 77000-M_r subunit, as isolated on the basis of either affinity or antibody-binding, is highest. The M_r-83000 form also isolated by immuno- or affinity-

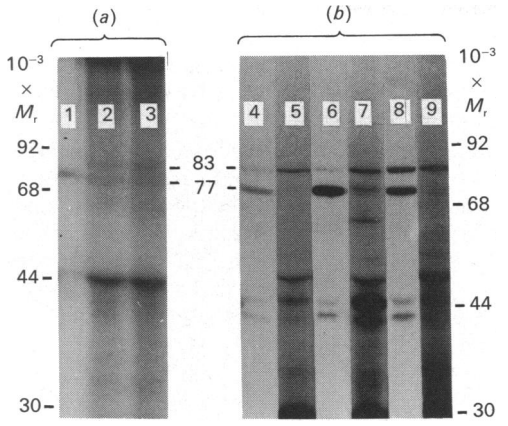

Fig. 3. Analysis of immunopurified PAL subunits from suspension-cultured cells

(*a*) Immunopurified PAL subunits from suspension-cultured plant cells. Protein A–Sepharose 4B was used to precipitate antigen–antibody complexes. Track 1, [^{35}S]methionine-labelled M_r-77000 marker PAL band; track 2, ^{3}H-active-site-labelled PAL preparation 1; track 3, as 2 (a second preparation). (*b*) Tracks 4–9 contain [^{35}S]methionine-labelled subunits produced by pulse-labelling *in vivo* (4, 6 and 8) or by translation *in vitro* (5, 7 and 9); track 4, unelicited control cells pulse-labelled for 1 h; track 5, translated from RNA extracted from control unelicited cells; track 6, cells pulse-labelled 3–4 h after elicitation; track 7, RNA from cells 4 h after elicitation; track 8, cells pulse-labelled between 9 and 10 h after elicitation; track 9, RNA 10 h after elicitation.

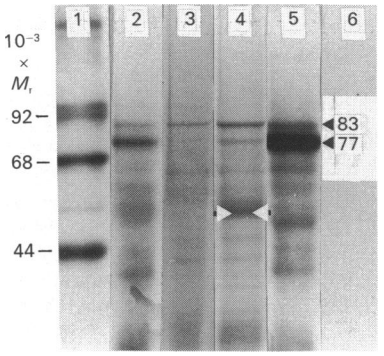

Fig. 4. Comparison of immuno- and affinity-purified PAL subunits from suspension-cultured bean cells

(*a*) Suspension-cultured cells were exposed to Translabel between 2 and 10 h after addition of elicitor. PAL was then purified from 30 g of cells as far as the Sephacryl S300 stage (Bolwell *et al.*, 1985), followed by chromatography using the (1, 2) affinity matrix (Dixon *et al.*, 1987) or (3, 4) immobilized antibody (Bolwell *et al.*, 1986*a*). After the requisite washing proceedures, PAL subunits were released from the respective matrices by boiling for 2 min in 40 mM-Tris /HCl buffer (pH 6.8)/2% SDS/50 mM-dithiothreitol/10% (v/v) glycerol/0.005% Bromophenol Blue. Subunits were then analysed by SDS/PAGE on 8%-acrylamide gels. Track 1, M_r markers; 2, affinity-purified, fluorography; 3, affinity-purified, Coomassie Blue-stained; 4, immunopurified, Coomassie Blue-stained (arrowheads show IgG subunits); 5, immunopurified, fluorography; 6, position of M_r -83000 and -77000 bands.

purification, is not so highly labelled, but stains more intensely. This polypeptide is evidently turning over at a lower rate. Taken together, these results are consistent with the existence of a relatively-less-inducible form of PAL (83000 M_r).

Differential expression

Because of the difficulties in detecting PAL subunits when crude extracts of induced tissue are subjected to Western analysis,

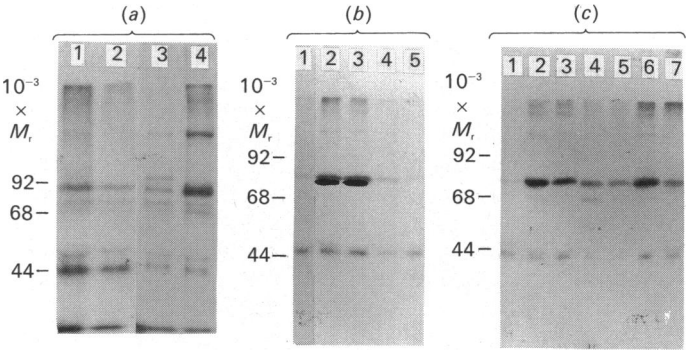

Fig. 5. A comparison of newly synthesized M_r forms in wounded hypocotyl and elicitor-, dilution- and cytokinin-treated suspension-cultured cells

(*a*) PAL subunits were immunoprecipitated from extracts of cells from: (1) the hypocotyl wound site treated with Translabel (74 kBq/site) for 0–16 h after wounding; (2) suspension-cultured cells labelled 3–4 h after addition of elicitor when the M_r-83000 subunit is not being synthesized; (3) leaf explants labelled 0–4 h after excision; (4) leaf explants labelled 4–8 h after excision. Tracks 1 and 2 were analysed on polyacrylamide gels different from that in 3 and 4 and have been aligned accordingly. (*b*) Subunits immunoprecipitated from suspension-cultured cells after subculture into maintenance media (dilution effect). Cells were labelled for 3 h before harvesting at (1) 3 h, (2) 75 h, (3) 123 h, (4) 171 h and (5) 243 h after subculture. (*c*) Subunits immunoprecipitated from cells subcultured into a media that induces xylogenesis. Cells were labelled for 3 h before harvesting at (1) 3 h, (2) 75 h, (3) 99 h, (4) 123 h, (5) 147 h, (6) 171 h or (7) 243 h after subculture. (*b*) forms the control for this experiment and was performed at the same time on the same batch of cells.

it is probable that they are in relatively low abundance. Immunoprecipitation of radiolabelled subunits can be relatively more sensitive. We have reassessed the inducibility of the 83000-M_r form under conditions where the synthesis of the 77000-M_r form is induced. Fig. 5(*a*) shows immunoprecipitates of newly synthesized PAL subunits in wounded hypocotyl compared with elicitor-induced tissue-cultured cells. In both cases the induced PAL which accumulated was of M_r 77000, whereas newly synthesized 83000-M_r form was not observed. The band at 46000-M_r which accumulated over a protracted time course was shown previously to be a degradation product of PAL (Bolwell *et al.*, 1986*a*). In the case where the stress (Fig. 5*b*) was due to dilution of cells into new media, only the 77000-M_r form was newly synthesized. All the PAL genes coding for 77000-M_r subunits are induced by stress (Liang *et al.*, 1989*a*). The accumulation of this M_r form only is also observed in cytokinin-treated cell cultures that are undergoing some xylogenesis (Fig. 5*c*). However, in vascular tissue the induced gene is known to be *PAL2* only, which codes for an M_r-77000 peptide (Lamb *et al.*, 1989; Liang *et al.*, 1989*b*; Bevan *et al.*, 1989). Finally, in leaf tissue (Fig. 5*a*), when the cells are pulsed-labelled over a short period immediately after excision and vacuum-infiltration, some M_r-83000 subunit is observed after immunoprecipitation, whereas over a more protracted time course the M_r-77000 subunit, characteristic of the wound response, is the only form observed.

DISCUSSION

Purification of PAL from French bean shows that a number of isoforms of differing pI, but similar M_r of 77000, can be separated and correspond to the products of members of the *PAL* gene family (Bolwell *et al.*, 1985; Liang *et al.*, 1989*a*). The present study shows that a higher-M_r (83000) form can be isolated which shows distinct differences in composition and extent of

glycosylation. Its identity as a form of PAL was confirmed by peptide mapping, specific active-site labelling, antigenicity and affinity properties.

Depending on the immunodetection method and the tissue under investigation, the anti-PAL antiserum initially raised against the highly purified M_r-77000 subunit recognizes a number of antigenic polypeptides. PAL subunits are detected with an M_r of 77000 in Western blots of partially purified preparations (Bolwell *et al.*, 1986*a*). It has been shown previously that in many preparations the level of the 77000-M_r form recovered, even after being rapidly prepared under conditions that minimize degradation, was found to be relatively low and was accompanied by additional peptides, notably those of M_r 70000, 53000 and 46000. These peptides have previously shown to be forms of PAL (Bolwell *et al.*, 1986*a*).

At high concentrations of antiserum relative to PAL antigen, the M_r-83000 polypeptide is also recognized. This is the M_r of PAL obtained from a number of tissues, including bean leaf (Da Cunha, 1988). From both leaf and cultured cells the 83000-M_r form has a low pI and higher K_m. It does not appear to be a glycosylated form of the M_r-77000 subunit, and its transcript shows differential kinetics of accumulation and translation to the inducible PAL products. The latter all have an M_r of 77000, as confirmed by gene analysis and sequence identity with other cloned genes (Cramer *et al.*, 1989; Liang *et al.*, 1989*a*; Lois *et al.*, 1989). This available evidence suggests that the M_r-83000 polypeptide is also a direct transcription product which has not yet been mapped to any gene.

Attempts to confirm the identity of the M_r-83000 polypeptide by seeking sequence similarity from *N*-terminal sequencing led to no sequence being obtained. The available gene sequences indicate that the gene family is divergent at the *N*-terminus, so that this data, if obtained, may have been of limited value in the present study, especially since 5′ gene sequences are absent for two of the family, with full-length sequences existing for only *PAL2* and *PAL3*. Moreover, the known gene sequences represent inducible gene products. In this context, all three PAL genes that have been cloned so far and code for 77000-M_r polypeptides are expressed in wounded hypocotyls, whereas only *PAL1* and *PAL2* are expressed in unwounded shoots (Liang *et al.*, 1989*a*). mRNAs showing sequence similarity to *PAL3* are least down-regulated in response to cinnamic acid action (Bolwell *et al.*, 1988; D. J. Millar, personal communication) and are present in uninduced cells. However, *PAL3* codes for a high-pI form, the *PAL1* product is an intermediate-pI form and *PAL2* codes for the acidic form (Liang *et al.*, 1989*a*). So even though translation *in vitro* of mRNA from cells subjected to cinnamate treatment gave only the 83000-M_r form on immunoprecipitation (G. P. Bolwell, unpublished work) it is not yet possible to make any predictions as to possible sequence similarity between the gene coding for the 83000-M_r form and the PAL genes cloned, although on the basis of its pI and amino acid composition it is closest to *PAL2*.

The possible role of the M_r-83000 subunit, besides its expression in uninduced cultures and leaves, which both accumulate flavonoids at a low level, has been studied at the protein level. Accumulation of subunits has been studied in response to a number of stimuli, using pulse-labelling to very high specific radioactivity in order to detect subunits that might be turned over very slowly. Immunoprecipitations were carried out at titres in which radioactive M_r-83000 subunits would be expected to be detected if present (Bolwell *et al.*, 1986*a*). None were detected in response to wounding, elicitor or cytokinin action when these were reassessed in the present study. M_r-77000 PAL-gene products and those of stress-related gene expression specifically accumulate in the epidermis in response to wounding, light and infection, including chalcone synthase (Beerhues *et al.*, 1988; Cuypers *et al.*, 1988; Schmeltzer *et al.*, 1988, Liang *et al.*, 1989*a*). Tissue- and cell-specific patterns of distribution have also been determined for β-glucuronidase gene fusions linked to either the bean *PAL2* promoter (Bevan *et al.*, 1989; Liang *et al.*, 1989*b*) or glycine-rich-protein (GRP) promoter (Keller *et al.*, 1989). These two promoters are also switched on by stress, including wounding, and the similarity to the types of genes induced by differentiation, and thus probably cytokinin/auxin interaction, is striking (Bolwell, 1988). PAL activity can be induced by cytokinin treatments of cultured cells (Haddon & Northcote, 1976; Kuboi & Yamada, 1978; Nagai *et al.*, 1988). Synthesis of PAL protein of M_r 77000 has been shown to be experimentally induced by cytokinin in the present study. In addition to PAL and GRP, hydroxyproline-rich glycoproteins, chitinase and β-(1–3)-glucanase (Memelink *et al.*, 1987) can also be induced by cytokinin or mechanical stress, though not necessarily with the synthesis of the products of identical genes. Thus, although quantitative control of phenolic biosynthesis is dependent on the appearance of the synthetic systems, the actual type of product accumulated, lignin in vascular tissues and phytoalexins and lignin-like wall-bound phenolic compounds in stressed cells, is probably dependent on additional controls. However, PAL is a key regulatory enzyme for all these pathways.

In conclusion, therefore, the M_r-83000 subunit may be the form of PAL produced and slowly turned over in cells that have not been programmed or stimulated to produce phenolic compounds rapidly or in a tissue-specific way. It may represent a 'housekeeping' form of PAL involved in the long-term accumulation of characteristic phenolics that are found at relatively low levels in unstimulated cells. In this context, a number of the earlier purifications of PAL from bulk tissue had an M_r of 83000 rather than 77000 (Jones, 1984). This higher-M_r form is probably distinct from those produced by members of the gene family so far cloned (Cramer *et al.*, 1989).

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