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EXPRESSION OF POPLAR PHENYLALANINE AMMONIA-LYASE IN INSECT CELL CULTURES

GRANT R. MCKEGNEY,* STEFANIE L. BUTLAND,* DAVID THEILMANN*† and BRIAN E. ELLIS*‡

*Department of Plant Science, University of British Columbia, Vancouver, BC, Canada; †Pacific Agricultural Research Centre, Agriculture and Agri-food Canada, Vancouver, BC, Canada V6T 1Z4

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Abstract—A cDNA encoding one of the phenylalanine ammonia-lyase genes from *Populus trichocarpa* × *deltoides* was inserted into a baculovirus expression vector and the PAL protein was successfully expressed in insect cell cultures. High levels of active holoenzyme were obtained that could be purified in a single chromatographic step. Site-directed mutagenesis and expression of the mutant enzyme confirmed that conversion of the putative active site serine^{202} residue to alanine is sufficient to destroy the catalytic activity of PAL.

INTRODUCTION

L-Phenylalanine ammonia-lyase [E.C. 4.3.1.5], and the related enzyme, L-histidine ammonia-lyase [E.C. 4.3.1.3], constitute a unique class of homotetrameric enzymes that catalyse non-oxidative elimination of ammonia from their substrates without employing pyridoxal phosphate. Instead, the active sites of PAL and HAL appear to contain a dehydroalanine residue [1]. Active site-directed reagents capable of reacting with dehydroalanine side-chain residues have yielded products consistent with its presence at the active site [2], and radiolabelling studies have supported the hypothesis that a serine residue is post-translationally modified to yield dehydroalanine [3]. Site-directed mutagenesis of rat [4] and *Pseudomonas* [5, 6] HAL, and parsley PAL [7], cDNA clones has recently pointed to one fully conserved serine as the likely candidate for the active site residue. Despite considerable indirect evidence, however, neither dehydroalanine nor its structural context have been directly identified in either PAL or HAL proteins. It is also not known how the proposed dehydration of the serine residue is accomplished. Since both HAL and PAL have been obtained as fully active recombinant proteins in bacterial expression systems using host cells that don't normally produce either enzyme [7–13], it appears that the post-translational modification at the active site may be largely autocatalytic. It is uncertain whether this process is unimolecular, or involves cooperation between subunits.

PAL, in particular, plays a important role in plant metabolism, where it channels a substantial proportion of photosynthetically fixed carbon toward synthesis of phenylpropanoid products such as lignin. Despite its importance, and the many studies describing its properties and responses to environmental stimuli, there is remarkably little known about the detailed structure of PAL, or the nature of the active sites. PAL is a large and relatively unstable enzyme, and has been difficult to purify without accompanying degradation of its subunits. In order to develop a better understanding of the biochemistry underlying both the biogenesis and the mode of action of PAL it is essential to have access to larger amounts of purified active protein. Heterologous protein expression systems provide such a capability, and PAL genes from both parsley and yeast have been expressed in this fashion in bacterial cells [9–13]. However, since PAL is restricted to plants and fungi, and is thus potentially processed through eukaryotic-specific processes, it is preferable to use a eukaryotic expression system to generate recombinant PAL. We have therefore chosen a baculovirus vector for this purpose. We also demonstrate that elimination of serine^{202} within the poplar PAL sequence results in a complete loss of catalytic activity.

RESULTS AND DISCUSSION

In *Populus* tissues, PAL activity is essential for production of flavonoids, phenolic esters and glycosides, as well as for synthesis of the phenylpropanoid monomers that will be converted to lignin, which forms a major part of the biomass of a mature tree. PAL is encoded by at least two discrete genes within the *Populus* genome, both of which show a high degree of similarity to other angiosperm PAL genes [14]. The relationship between

‡Author to whom correspondence should be addressed.

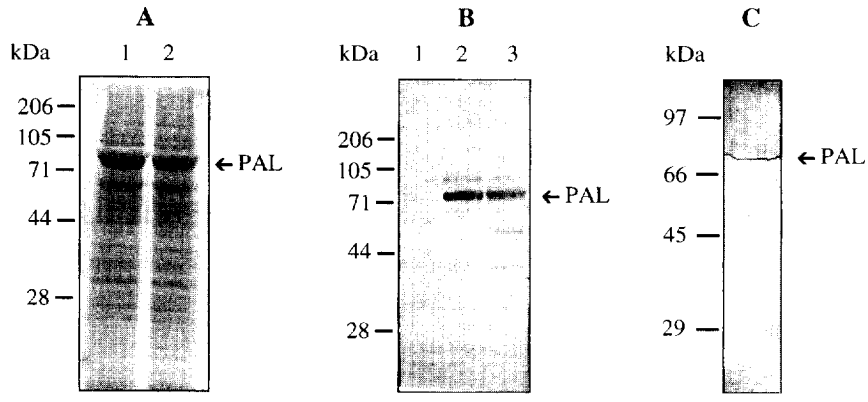

Fig. 1. (A) SDS-PAGE separation of the proteins extracted from cultured insect cells infected with recombinant AcNPV-PAL (lane 1), or recombinant virus containing the S202A mutant form of AcNPV (lane 2). Each lane was loaded with 10 μg protein, and after running, the gel was stained with Coomassie Brilliant Blue G250. (B) Western blot of SDS-PAGE-separated proteins obtained from insect cultures transfected with wild-type baculovirus (AcNPV) (Lane 1), recombinant AcNPV-PAL (Lane 2), and recombinant virus containing the S202A mutant form of AcNPV-PAL (Lane 3). (C) Western blot of SDS-PAGE-separated proteins obtained from developing xylem of *P. trichocarpa x deltoides* (15 μg protein). Primary antibody dilution for A; 1 : 10000; for C, 1 : 1000. The locations of the molecular mass standards are indicated on the left margins.

possible PAL isoforms and the gene family structure in poplar remains undefined, since the enzyme from poplar has never been fully purified and characterized.

A full-length cDNA representing the product of poplar PAL gene 2 [14] was cloned into a baculovirus transfer vector. This construct, together with the positive selection *BaculoGold Autographica californica* nuclear polyhedrosis virus (AcNPV) DNA, was used to co-transfect cultured *S. frugiperda* cells. Use of *Baculogold* viral DNA permits recombination efficiency close to 100%, thus ensuring a highly effective recovery of recombinant products from the plated cells. In these recombinant virions, transcription of the poplar PAL cDNA is driven by the strong polyhedrin promoter, and translation of the resulting mRNA is initiated and terminated at the PAL gene's *start* and *stop* codons, respectively.

Ten plaque-purified recombinant viruses were chosen and the proteins of seven day-old cells infected with each virus were examined by SDS-PAGE. The cell extract from each culture yielded a protein profile that contained a major polypeptide whose estimated M_r (78 000) corresponded to that predicted for the poplar PAL subunit coded for by the PopPAL2 cDNA (M_r 77 900). The intensity of this band varied between recombinant lines, but was estimated to make up about 20% of the total protein in the cell extracts (Fig. 1A, lane 1).

One of the recombinant viruses that yielded high levels of the M_r 78 000 polypeptide was chosen for further analysis. Assay of buffer extracts from *S. frugiperda* cells infected with recombinant AcNPV-PAL showed that high levels of PAL activity were present 60 hr after infection was initiated. A time-course analysis of the production pattern revealed that the most rapid accumulation of active PAL occurred between 24 hr and 48 hr post-infection, with the maximum yield being obtained at 60 hr post-infection (Fig. 2).

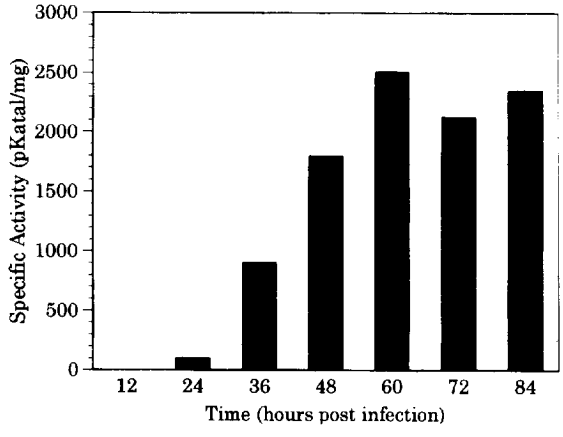

Fig. 2. Time-course of expression of PAL activity in extracts of insect cell cultures after transfection of the cultures with AcNPV-PAL.

The PAL activity detected in the buffer extracts eluted as a single peak during Mono Q anion exchange chromatography (Fig. 3). SDS-PAGE analysis of the active fractions demonstrated that the enzyme activity maximum corresponded to protein fractions that contained essentially only the M_r 78 000 polypeptide (Fig. 3 inset). A single chromatographic step was thus sufficient to purify the recombinant PAL. Non-denaturing PAGE analysis of the same crude cell homogenates showed that the protein migrated predominantly as a single band with an approximate M_r 320 000. This was sometimes accompanied by small amounts of a faster migrating band whose estimated molecular mass was half that of the main band (Fig. 4). Since the main band of native protein is presumed to be tetrameric PAL, it is possible that the faster migrating species represents a dimeric form of the

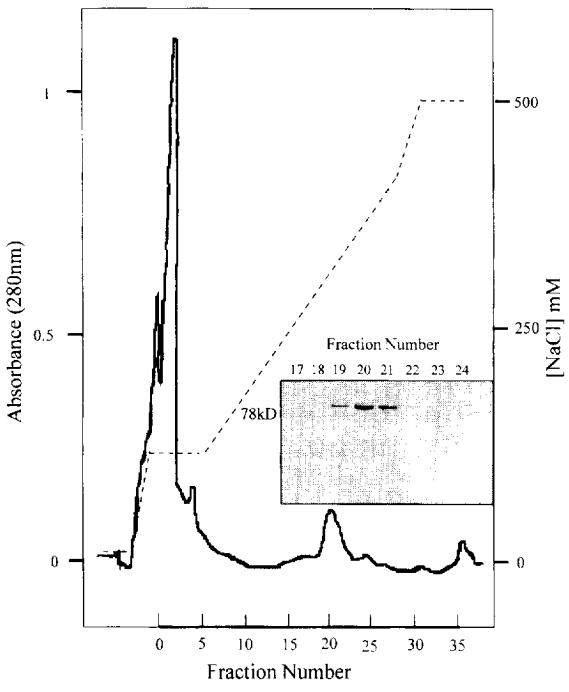

Fig. 3. Anion-exchange chromatography (Mono Q) of the proteins extracted from insect cell cultures 48 hr after transfection with AcNPV-PAL. The solid line is the UV absorption profile of the eluate, measured at 280 nm; the dashed line shows the 0–500 mM NaCl gradient profile. Significant PAL activity was only detected in fractions 19–23 of the eluate. Figure inset shows a Coomassie Blue-stained SDS-PAGE gel after electrophoresis of the fractions containing the highest PAL activity.

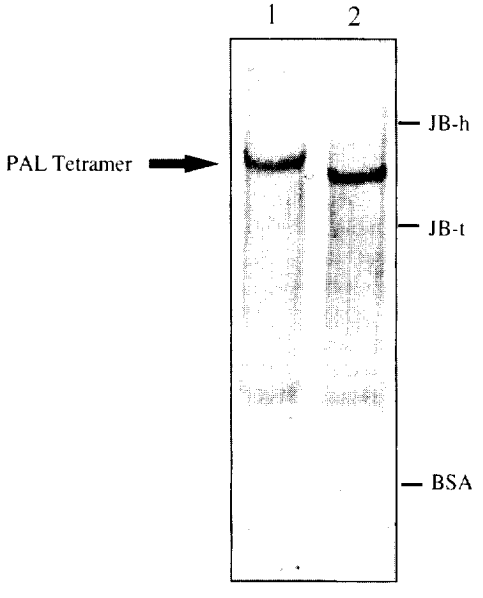

Fig. 4. Coomassie Blue-stained blot obtained from a non-denaturing PAGE gel after electrophoresis of cultured insect cells infected with recombinant AcNPV-PAL (Lane 1) and the S202A mutant AcNPV-PAL (Lane 2). Position of protein molecular mass standards is indicated on the right (JB-h: jack bean ureasehexamer M_r = 542000; JB-t: jack bean urease-trimer M_r = 272000; BSA: bovine serum albumin M_r = 66000).

protein. Elution of both bands from non-denaturing gels and assay for PAL activity showed that the main (tetrameric) band was catalytically active, whereas no activity could be detected in the other band (data not shown). Catalytically active non-tetrameric forms of PAL have been reported for the enzyme from yeast [15] and from sunflowers [16], but these appear to be exceptional cases.

Kinetic analysis of the wild-type recombinant protein after Mono Q purification showed that the enzyme displayed normal Michaelis–Menten kinetics, with a *Km* for L-phenylalanine of 450 μM. PAL from other sources has been shown to have *Km* values ranging from 16 μM to 5 mM L-phenylalanine. Different PAL preparations have been reported to display either Michaelis–Menten kinetics, or negative cooperativity [1]. It has been suggested that the apparent cooperativity response in some PAL preparations might be the result of the presence of a mixture of isoforms differing in their affinity for the substrate. When a mixture of alfalfa PAL isoforms displaying cooperativity was resolved into the individual components, the purified isoforms displayed normal Michealis–Menten kinetics [17]. The lack of cooperativity observed in the kinetic behaviour of the recombinant poplar PAL produced in insect cells is consistent with this concept, since only a single PAL gene is being expressed, and isoforms based on gene family differences are therefore excluded. Like most other angiosperm PAL species, recombinant poplar PAL was inhibited by its product, *trans*-cinnamic acid (Ki = 700 μM). It displayed low but detectable activity using L-tyrosine as substrate (<5% of L-phenylalanine activity).

Antisera raised against the recombinant enzyme proved to have a high affinity for PAL (Fig. 1B lane 2). Recombinant PAL (250 ng) can readily be detected on western blots at an antiserum dilution of 1:10,000. Using this antiserum, only a single protein (M_r 78 000) was detected on a western blot of buffer extracts from developing xylem or young leaf tissue of *P. trichocarpa* × *deltoides* (Fig. 1C). Attempts to obtain an *N*-terminal sequence from the native recombinant protein were unsuccessful, even with large quantities of protein, implying that a thorough post-translational modification of the *N*-terminal residue has taken place. *N*-terminal blockage of PAL polypeptides has also been reported for the enzyme purified from *Phaseolus vulgaris* cell cultures [18], but the nature of the modification remains unknown. HPLC-MS analysis, and micro-sequencing, of tryptic digests of the recombinant poplar PAL allowed a substantial number of PAL-specific peptides to be identified, but neither the amino terminus nor the putative active site peptides were recovered (R. Abersoeld, personal communication).

Surprisingly, analysis of both the crude extract and Mono Q-purified recombinant PAL on 2D-IEF/PAGE gels revealed the presence of apparent charge isoforms in these preparations (Fig. 5). Two major forms generally appear, with much smaller amounts of two or three other forms at higher and lower pI values. Since only a single gene product is involved, it appears that this protein must be post-translationally modified within the insect

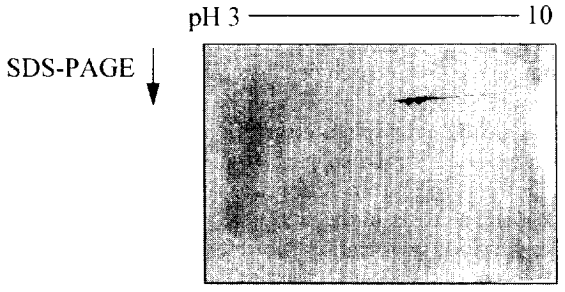

Fig. 5. Western blot obtained from a two-dimensional gel (IEF/SDS-PAGE) separation of Mono-Q-purified recombinant PopPAL2 produced in insect cell cultures.

cell milieu in such a fashion as to generate small charge differences. It remains to be seen whether similar charge isoforms appear in poplar PAL isolated from the plant.

The catalytic mechanism proposed for both L-phenylalanine and L-histidine ammonia-lyases involves reaction of the substrate with a novel electrophile at the active site. Chemical modification analysis [19], and radiolabelling studies [2,3], have implicated a dehydroalanine residue as the electrophile, although this has yet to be confirmed through direct identification within the protein. The suggested origin of such a dehydroalanine moiety within the PAL/HAL primary structure is a serine residue, and only a small number of highly conserved serine residues are plausible candidates for this role. Site-directed mutagenesis studies have recently shown that an S → A conversion of ser^{254} in rat HAL [4] and ser^{143} in *Pseudomonas* HAL [5,6] completely eliminates catalytic activity. A similar alteration of ser^{202} in parsley PAL expressed in a bacterial expression vector also yielded inactive enzyme [8]. The availability of the poplar PAL cDNA provided an opportunity to modify its sequence through site-directed *in vitro* mutagenesis and extend these recent observations to a PAL derived from a woody species. $Serine^{202}$ within the poplar PAL amino acid sequence is found in the same highly conserved sequence context (... GTITASGDLV...) as the HAL and PAL serine residues identified in the above studies. Replacement of poplar PAL ser^{202} with alanine, and expression of the mutagenized protein in insect cell cultures yielded one major new protein in cell extracts (Fig. 1A, lane 2). This protein showed the same mobility as wild-type PAL on native and denaturing SDS-PAGE gels, and gave a strong positive reaction with the anti-PAL antiserum (Fig. 1B, lane 3). However, it possessed no detectable PAL activity, consistent with the hypothesis that the putative active site dehydroalanine residue in PAL is derived from the serine residue located within the sequence (... GTITASGDLV...).

Production of large quantities of both native and modified higher plant phenylalanine ammonia-lyase proteins in the baculovirus/insect cell culture system offers an excellent opportunity to address many unresolved questions concerning the formation, architecture and regulation of this important enzyme.

EXPERIMENTAL

PAL activity and protein assays. Enzyme activity was determined radiometrically as described previously [20]. Protein concentrations were measured using the Coomassie dye-binding assay [21] with BSA as standard.

Maintenance of Sf9 cells. Spodoptera frugiperda cells (Sf9) were routinely maintained in spinner flask cultures at 28°C, with 50 ml of TC-100 medium (Grace's complete, 10% fetal bovine serum, 10% lactalbumin/yeast hydrolysate) using a 5-day subculture regimen. Cells grew from a starting density of 5×10^5 cells ml^{-1} to about 4.0×10^6 cells ml^{-1} in this period.

Construction of recombinant baculovirus containing the PAL genes. The entire poplar PAL gene 2 cDNA [13] or the S202A mutant cDNA was subcloned into the baculovirus transfer vector pVL1393 [22] by the following method. Plasmid pPopPAL2H11-7 (in Bluescript SK^-) [13] was first digested with *Xho*I. The plasmid was then made blunt using the Klenow fragment of DNA polymerase and then digested with *Bam*HI, thus creating 5′ *Bam*HI and 3′ blunt ends. The 2.4 kb PAL cDNA containing both 5′ and 3′ untranslated regions was then purified and ligated into *Bam*HI-*Sma*I digested pVL1393, yielding plasmids pVL1393/PAL or pVL1393/S202A. This construct generates a non-fusion PAL protein, since translation is initiated at the PAL cDNA start codon.

Transfection and virus purification. Spodoptera frugiperda Sf9 cells were co-transfected with pVL1393/PAL or pVL1393/S202A (2 μg) and AcNPV viral DNA (BaculoGold, Pharmingen) (0.5 μg), and recombinant virus (designated AcNPV-PAL or AcNPV-S202A) was plaque-purified [23].

Large-scale production and purification of recombinant PAL. Sf9 cells were grown from a density of 5×10^5 cells ml^{-1} to 2×10^6 cells ml^{-1} in a 50 ml spinner flask and then infected at a multiplicity of infection (MOI) of 1. At 48 hr p.i., the cultures were centrifuged (1500 g, 10 min; 4°). The medium was decanted and the cell pellet washed in 10 ml cold PBS. The cells were centrifuged again as before, resuspended in 5 ml 50 mM Tris-acetate pH 7.5 containing 5 mM 2-mercaptoethanol, lysed (Dounce homogenizer; 20 strokes) and then vortexed for 1 min. This crude lysate was centrifuged (10000 *g*), filtered (0.45 μ) and subjected to anion-exchange FPLC using an analytical HR 5/5 Mono Q column. Solvent A: 50 mM Tris-acetate pH 7.5; solvent B: 500 mM NaCl in 50 mM Tris-acetate pH 7.5, both containing 5 mM 2-mercaptoethanol. Gradient conditions: 0% B for 10 min; 20% B for 20 min; 20 to 60% B over 60 min; 100% B for 10 min. Flow rate 0.5 ml min^{-1}. Fractions were collected at 3 min intervals. Protein levels were measured immediately and samples were assayed for activity, or were frozen at −20°.

Antibody generation. Polyclonal antibodies were raised in New Zealand White rabbits to post-Mono-Q PAL. RIBI adjuvant was reconstituted in PBS and mixed 1:1 with the PAL protein. An aliquot (1 ml) of this mixture

containing 150 μg post-Mono-Q PAL was injected subcutaneously. Booster injections (intramuscular) were given at 4-week intervals.

Gel electrophoresis and immunoblotting. SDS-PAGE was performed according to Laemmli [24] using 10% acrylamide separating gels and Gibco pre-stained protein MW standards. Native PAGE (4 hr; 4°; 10 mA) was carried out using 7.5% acrylamide gels without SDS. 2D-PAGE was accomplished using the Biorad Mini Gel apparatus and protocols. Proteins were stained with Coomassie Brilliant Blue R-250. Western blots were carried out using PVDF-blotted proteins from 10% SDS-PAGE gels using a semi-dry electroblotter and a transfer buffer of 39 mM glycine, 48 mM Tris and 20% methanol (0.8 mA/cm^2 gel (75 mA/two gels); 1 hr). The blot was blocked with 10 ml 5% non-fat milk powder in PBS (blocking buffer) for 1 hr on a gyratory shaker. It was then probed with rabbit anti-PAL polyclonal antibodies (1:10000) in 10 ml blocking buffer (1 hr; RT). The blot was washed using 3 × 10 ml blocking buffer washes of 10 min each, and then incubated for 1 hr (1:2500 dilution in blocking buffer) with goat anti-rabbit IgG-alkaline phosphatase conjugate. Excess second antibody was removed with four 10 ml washes of blocking buffer. Alkaline phosphatase activity was detected on the blot using Nitroblue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt.

In vitro *mutagenesis.* The S202A mutation was introduced into pPopPAL2H11-7 by oligonucleotide-directed mutagenesis [25]. Uracil-containing ssDNA was isolated by R408 helper phage rescue [26] from *E. coli* strain RZ1032 transformed with pPopPAL2 H11-7. The mutagenic oligonucleotide, 5′-CACTGCTGCAG-GTGATTTAGT-3′ binds to the antisense strand of pPopPAL2H11-7. Mutants were identified by the presence of a new *Pst*I restriction site created by the S202A mutation. To verify that only a single nucleotide had been changed in the coding region, the open reading frame of the mutant DNA was sequenced from a series of nested deletions. DNA was sequenced using the AmpliTaq DyeDeoxy Terminator Cycle Sequencing protocol.

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