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PURIFICATION AND CHARACTERIZATION OF A PHENYLALANINE AMMONIA-LYASE FROM *OCIMUM BASILICUM*

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Key Word Index—*Ocimum basilicum*; Lamiaceae; basil; phenylalanine ammonia-lyase; 2-amino-indan-2-phosphonic acid; *trans*-cinnamic acid; *trans*-methyl cinnamate; *d*-labelling; feedback inhibition.

Abstract—A new phenylalanine ammonia-lyase (PAL) was purified from leaves of *Ocimum basilicum* L. (chemotype, methyl cinnamate). Separation techniques applied included anion exchange chromatography and preparative electroelution from a non-denaturing polyacrylamide gel. A 180-fold purification was obtained. The native enzyme was a homotetramer of M_r 152 000–153 000; the intact subunit M_r was *ca* 38 000. The enzyme catalysed the conversion of L-phenylalanine-d_8 into *trans*-cinnamic acid-d_7, as determined by GC–mass spectral analysis of silylated reaction products. The purified native enzyme had K_m and V_{max} values of 329 μM and 11.43 μmol min^{-1} mg^{-1} protein, respectively, for L-phenylalanine and was competitively inhibited by 2-amino-indan-2-phosphonic acid, *trans*-cinnamic acid and *trans*-methyl cinnamate with K_i values of 19 nM and 57 and 130 μM, respectively. Comparing the K_i values between *trans*-cinnamic acid and *trans*-methyl cinnamate for L-phenylalanine indicated that the regulation of PAL is not only related to the mechanism of feedback inhibition in the biosynthesis of *trans*-methyl cinnamate. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyses the elimination of ammonia and the *pro*-3*S* hydrogen from L-phenylalanine to yield *trans*-cinnamic acid [1]. This reaction is the first step in the biosynthesis of a large range of phenylpropanoid-derived secondary products in plants such as the lignins, flavonoid pigments, UV protectants, plant hormones and phytoalexins. Due to the central function of PAL at a branch point of metabolism, this enzyme has been thought to play a pivotal role in the regulation of the biosynthetic routes leading to the above compounds [1]. Although PALs have been purified and characterized from a number of plant tissues [2, 3], cell suspension cultures [4, 5] and fungi [6], their structures and kinetic properties for the biosynthesis of several phenylpropanoids important as aroma and flavour compounds remain unclear.

Our initial goal was to examine whether there is an association between PAL activity and the formation of aromatic volatile phenylpropanoids. As such, we developed chemotypes of basil (*Ocimum basilicum* L.), which varied only in the end products of the shikimate/arogenate and phenylpropanoid pathway such as eugenol, *trans*-methyl cinnamate and methyl chavicol [7, 8]. Basil was selected as the model species because of the unusually high accumulation of these compounds in the volatile oil. In this paper, we report the purification and characterization of a new PAL isolated from the leaves of methyl cinnamate basil (Purdue, SW-69). In addition, we provide evidence that this PAL can catalyse the conversion of the substrate labelled by stable isotopes, L-phenylalanine-d_8, into the product with corresponding isotopes, *trans*-cinnamic acid-d_7.

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RESULTS AND DISCUSSION

Purification of PAL

The purification protocol developed for PAL from leaves of *O. basilicum* L. is presented in Table 1. The fractions with PAL activity were sequentially traced by UV spectroscopic examination of the increase in absorbance at 290 nm [9]. The strongest activity was detected in the precipitates obtained between 25 and 55% of ammonium sulphate saturation. Anion exchange chromatography was repeated twice (Fig. 1, lanes 2 and 3, respectively). The second anion exchange was necessary to attain higher purity (Fig. 1, lane 3). In this study, experimental results indicated that preparative electroelution PAGE was an ideal method for preparing milligram quantities of PAL after

Table 1. Purification of PAL from *Ocimum basilicum* leaves

Fraction	Total protein (mg)	Specific activity (nkat mg^{-1})	Purification (fold)	Recovery (%)
Crude	36 000	0.86	1	100
25–55% $(NH_4)_2SO_4$	21 000	1.38	1.6	94
DEAE-Sephacel (1st separation)	390	28.2	32.5	35
DEAE-Sephacel (2nd separation)	94	49.4	57	15
Preparative PAGE	13.8	156	180	7

anion exchange separation because the PAL obtained by this method still exhibited higher specific activity.

Physicochemical properties of PAL

The M_r of the basil PAL subunit, estimated from the mobility on SDS–PAGE, was 38 000. The calculated M_r of the native protein on non-denaturing PAGE was 152 000. A similar M_r of 153 000 was also determined by gel filtration on Sephacryl S-300. Analysis of the purified protein preparations by SDS–PAGE and silver staining revealed the presence of a single protein band with a M_r of 38 000 (Fig. 1, lanes 4 and 5). Because most PALs are known to exist as hetero- or homotetramers [2], the determination of a single protein by SDS–PAGE suggested that this native PAL is a homotetramer composed of four 38 kD subunits.

PALs have generally been described as tetrameric enzymes with M_r between 280 000 and 330 000 except for an enzyme with M_r of 226 000, isolated from the procaryote *Streptomyces*, an actinomycete [1, 2]. Additionally, a PAL protein with a low M_r of 175 000, obtained from sunflower hypocotyls, was considered to be a trimeric form of the enzyme [10]. The quaternary structures have been reported to be either homogeneous or heterogeneous (differently sized subunits between 70 and 90 kD) [2]. Differences in the reported sizes of PALs may be due to post-translational events such as degradation or expression of a truncated polypeptide subunit [11]. The components with small subunit size (49.5 and 48 kD) on SDS–PAGE have been considered unstable and as degradable factors of intact enzyme or possible contaminants [2, 5]. In the basil methyl cinnamate chemotype, the native and subunit sizes (152–153 and 38 kD, individually) of PAL significantly differ from those previously reported in plants. Western analysis of PAL crude enzyme preparation (Fig. 1, lane 7) revealed identical migration pattern compared with pure PAL (Fig. 1, lanes 4 and 5), suggesting that this new PAL with 38 kD subunits is present in basil plants.

The *O. basilicum* PAL had a pI of 4.6, as determined by comparing the migration of the purified PAL with those of protein standards of known pI across the IEF gel in a vertical polyacrylamide migration system.

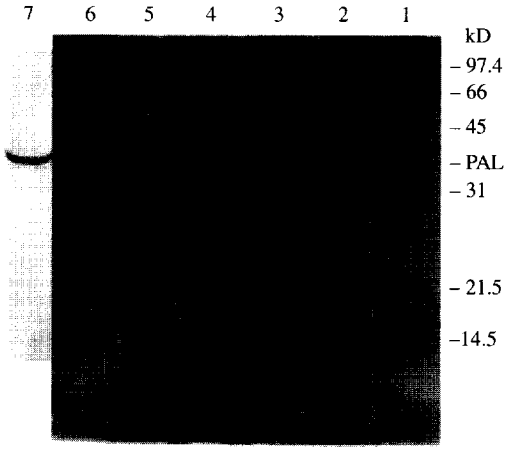

Fig. 1. SDS–PAGE (12% gel) documentation of the purification of *Ocimum basilicum* PAL. The position of molecular mass markers and the PAL (38 kD) from *O. basilicum* L. are shown on the right. Gels were stained with silver reagent. Lanes 1 and 6: molecular mass standards; lane 2: 1 μg DEAE-Sephacel eluate (1st separation); lane 3: 0.5 μg DEAE-Sephacel eluate (2nd separation); lane 4: 0.2 μg preparative PAGE eluate; lane 5: 0.1 μg preparative PAGE eluate; lane 7: western blot of 10 μg crude protein.

Confirmation of the identity of PAL

Although UV spectroscopic assay at A_{290} has shown that the purified protein with 38 kD subunits should possess PAL activity, to prove the catalytic function of this protein with lower M_r, the substrate labelled by stable isotope, L-phenylalanine-d_8, was incubated with purified protein at 40° for 2 hr. The enzyme conversion products were extracted by diethyl ether. The organic residues were silylated by bis(trimethylsilyl)trifluoroacetamide–trimethylchlorosilane (99:1, v/v) in pyridine at 80° for 15 min. GC-mass spectral analysis was employed to elucidate the structure of the silylated product with *d*-labelling, trimethylsilyl (TMSi)-*trans*-cinnamic acid-d_7. In the high-mass region of the EI mass spectrum of the silylated product, a molecular ion (m/z 227) can be observed. Additionally, all expected ions characteristic of TMSi-*trans*-cinnamic acid-d_7 were found at the four positions (m/z 212, 138, 110 and 82).

Kinetic properties

The PAL conformed to Michaelis–Menten kinetics, exhibiting a linear relationship for L-phenylalanine over the range 0.04–1.5 mM. The K_m and V_{max} values for L-phenylalanine were 329 μM and 11.43 μmol min^{-1}

mg^{-1} protein, as determined by single-reciprocal analysis (Hanes–Woolf plot). The effect of 2-aminoindan-2-phosphonic acid (AIP), *trans*-cinnamic acid (CA) and *trans*-methyl cinnamate (MC) on the reaction catalysed by PAL was evaluated by kinetic analysis. The Hanes–Woolf plots (Fig. 2) of initial velocities versus variable substrate concentrations (0.04–1.5 mM) clearly indicated that the inhibition of PAL activity by AIP, *trans*-

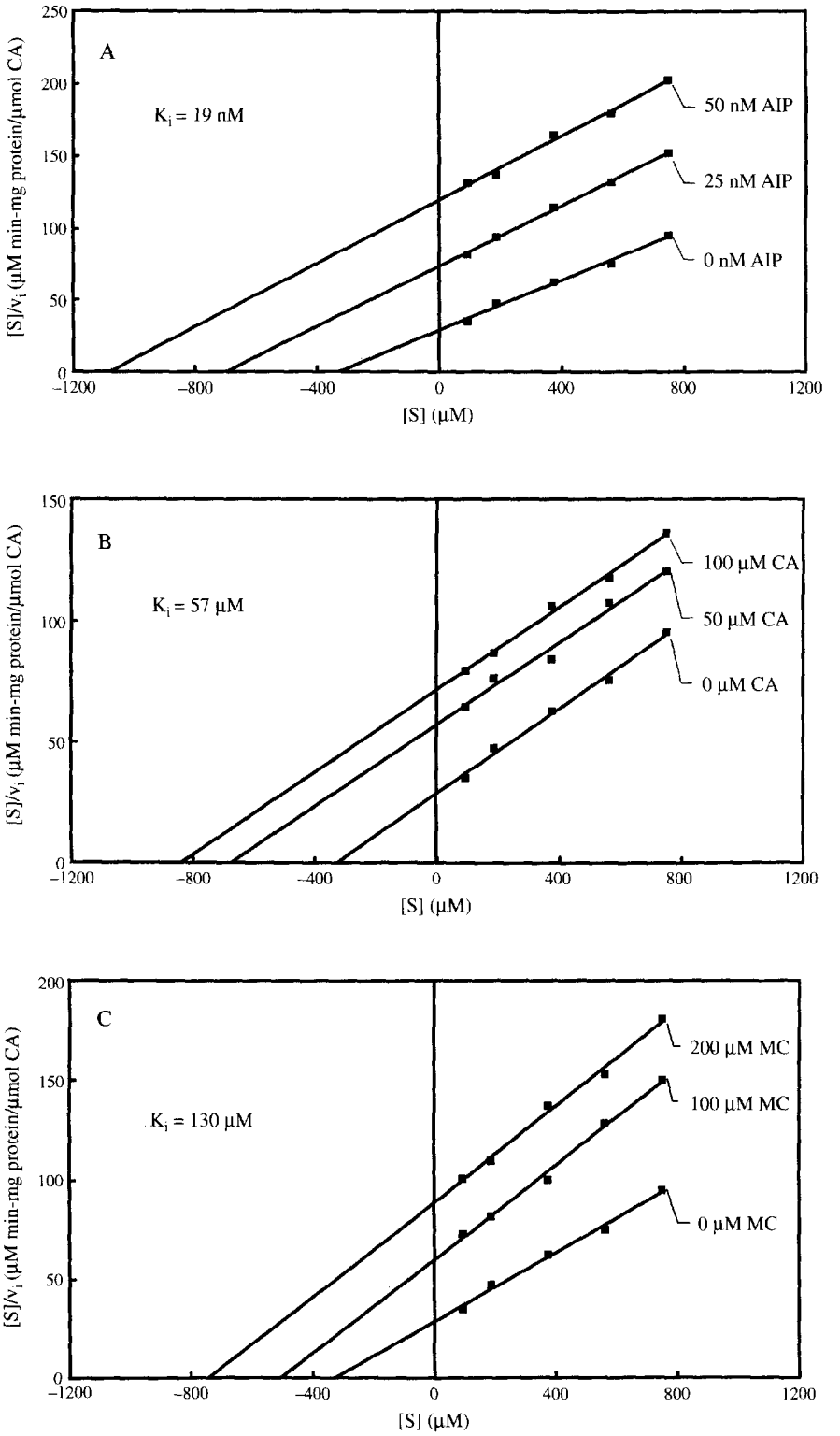

Fig. 2. Hanes–Woolf plots of the effects of 2-aminoindan-2-phosphonic acid (AIP, A), *trans*-cinnamic acid (CA, B), and *trans*-methyl cinnamate (MC, C) on the activity of PAL purified from *O. basilicum leaves*. [S] = phenylalanine.

cinnamic acid and *trans*-methyl cinnamate was also linearly competitive in each case [12]. AIP, the most potent *in vivo* inhibitor of PAL, inhibited the enzyme at a very low K_i/K_m ratio (0.000054) compared with the previously reported results for buckwheat PAL [13]. *trans*-Methyl cinnamate, an end product of phenylpropanoid metabolism in *O. basilicum*, inhibited PAL activity less (K_i 130 μM) than the direct reaction product, *trans*-cinnamic acid (K_i 57 μM). These results provide additional evidence that the potential regulation of PAL activity appears to be complex and not only related to feedback inhibition [2].

EXPERIMENTAL

Materials. All chemicals and biochemicals were of analyt. grade and purchased from Sigma unless stated otherwise. Experiments were performed with 50-day-old basil plants grown in the Horticulture Department glasshouses at 22±2°. The newest three fully expanded leaves of each plant were cut and immediately frozen in deep liquid N_2 and stored at −70° until used.

Determination of PAL activity and protein concentration. PAL activity was detected using an assay modified from a previously reported method [14]. The reaction mixt. contained 50 mM Tris–HCl (pH 8.8), 2 mM 2-mercaptoethanol, 12 mM L-phenylalanine and enzyme in a total vol. of 2.0 ml. The incubations were allowed to proceed at 40° for 60 min in activity-direction assay and for 70–280 min in kinetic activity assay. The reaction was stopped by addition of 0.2 ml 6 N HCl. The reaction mixt. was then extracted with 2.0 ml toluene by vortexing for 10 sec, and the mixt. centrifuged at 750 *g* for 5 min to separate the phases. The A_{290} of the *trans*-cinnamic acid recovered in the toluene phase was measured against a toluene blank. Enzyme activity is expressed in nkat (1 kat represents the conversion of 1 mol substrate to product sec^{-1}). Protein concn was determined by dye-binding microassay, using BSA as standard [15].

Extraction and purification of PAL. All purification steps were carried out in a cold room at 4°. Frozen leaves (*ca* 3 kg) were homogenized in a Waring blender containing Dowex 1 (Cl^-, dry mesh: 50–100, 2.5% w/v), PVPP (1.5 w/v) and 50 mM Tris–HCl (pH 8.8) containing 2 mM 2-mercaptoethanol (buffer A, 1:4 w/v). The slurry was filtered through 4 layers of cheesecloth (pre-moistened with buffer A). The filtrate was centrifuged at 20 000 *g* for 30 min (×2). $(NH_4)_2SO_4$ fractionation was carried out on the supernatant, and the frs pptg between 25 and 55% of satn were taken for further purification. The ppt was resuspended in a minimum vol. of 20 mM Tris–HCl (pH 7.5) containing 10% ethylene glycol and 2 mM 2-mercaptoethanol (buffer B) and dialysed against two changes of buffer B. Insoluble material was removed by centrifugation at 14 000 *g* for 20 min. The supernatant was diluted to a protein concn of <15 mg ml^{-1} and loaded on to a 2.0 × 20 cm column of DEAE-Sephacel (100 mg ml^{-1} DEAE-Sephacel gel material). The column was washed with buffer B until protein content of effluent returned to baseline level, determined by the dye-binding assay. Linear gradient elution was then carried out with 300 ml each of buffer B and 400 mM Tris–HCl (pH 7.5) containing 10% ethylene glycol and 2 mM 2-mercaptoethanol (buffer C), and 20-ml frs were collected. Frs containing higher PAL activity were pooled and concd to 30 ml using centrifugal ultrafiltration devices. The concd material was then loaded on to a small DEAE column (1.0 × 20 cm) and eluted with 200 ml each of buffer B and C again, and 5-ml frs were collected. Frs containing the highest PAL activity were pooled and concd to 10 ml as described for the previous step. The concd proteins were further purified using prep. PAGE (2.5 mm thick, 16 × 16 cm and 6% cross-linked with bisacrylamide) [16]. Electrophoresis was performed in a commercial slab gel apparatus. Proteins were localized by cutting two longitudinal strips from the sides of the slab gel and a narrow longitudinal strip from the centre. These strips were stained with Coomassie blue R-250 while keeping the rest of the gel on a glass plate covered with cling film in a refrigerator. The stained side strips were carefully lined up along the edges of the unstained gel (but without any physical contact) and used as guides to cut out the corresponding band from the unstained gel. The proteins on the gel slice were then electroeluted using a previously reported method [17], using a current of 30 mA for 8 hr.

Molecular weight of native enzyme and subunits and pI. Non-denaturing PAGE and gel filtration were used to determine the M_r of the native protein. Migration of marker proteins (MW-ND-500 kit) and the purified PAL in non-denaturing gels of different acrylamide concns (4.5–10%) were used to calculate the M_r of the native protein (Sigma Technical Bulletin MKR-137) [18]. The protein material was loaded on to a 1.8 × 35 cm Sephacryl S-300 gel filtration column, eluted with buffer B, and 2 ml frs were collected. Thyroglobulin (M_r 669 000), *apoferritin* (M_r 443 000), β-amylase (M_r 200 000), BSA (dimer M_r 132 000; monomer M_r 66 000) and carbonic anhydrase (M_r 29 000) were used as reference proteins. SDS–PAGE was performed in 12% acrylamide gels [19]. Prior to electrophoresis, protein samples and SDS–PAGE marker proteins were boiled in 2 × Laemmli sample buffer for 3 min. Protein was visualized by Coomassie blue R-250 and silver staining. The pI of the purified native PAL was determined using rapid isoelectric focusing in a vertical 1.5 mm thick polyacrylamide mini-gel system (Mini Protein II, Bio-Rad) [20], polymerized from the following mixt. 7 ml double-distilled H_2O, 2 ml acrylamide–bisacrylamide (15:0.4), 2.4 ml 50% (v/v) glycerol, 0.6 ml ampholytes (pH range 3–10), 50 μl 10% (w/v) ammonium persulphate and 20 μl TEMED. Protein standards and purified PAL were mixed with an equal vol. of 60% (v/v) glycerol and 4% (v/v) ampholytes of the same pH range used to prepare the gel. The cathode soln was 25 mM NaOH and the anode soln 20 mM HOAc. Electrophoresis was performed at 200 V for 1.5 hr, then increased to 400 V for an additional 1.5 hr. The pH gradient was measured on the gel surface with

a flat-bottomed pH electrode and then the gel was stained with 0.25% (w/v) Coomassie blue R-250 in 45% (v/v) MeOH and 10% (v/v) glacial HOAc for 10 min with gentle shaking and then destained in 45% (v/v) MeOH and 10% (v/v) glacial HOAc. The pI of PAL was obtained from the curve generated from the standard protein (IEF MIX 3.6–9.3 marker) plotted against their migration distance (data not shown).

Western blot. Protein samples were transferred to a nitrocellulose membrane using Semi-Dry electroblotter with graphite plates. Membranes were incubated with polyclonal antibodies prepd from *ca* 100 μg pure protein in the Department of Biological Sciences (Dr David J. Asai), Purdue University, according to standard procedures. Immunocross-reactivity was detected following incubation with secondary antibodies linked to goat anti-rabbit alkaline phosphatase.

Analysis of compounds with stable isotopes. L-Phenylalanine-d_8 (12 mM) (Cambridge Isotope Laboratories, Andover, MA), 2 mM 2-mercaptoethanol and 10 μg purified protein obtained from prep. PAGE were mixed in 50 mM Tris–HCl (pH 8.8) in a total vol. of 5.0 ml. The reaction was allowed to proceed for 120 min at 40° and stopped by addition of 0.2 ml 6 N HCl. The reaction mixt. was then extracted with 5.0 ml Et_2O by vortexing for 10 sec, and the mixt. centrifuged at 750 *g* for 5 min to separate the phases. Et_2O was then removed under N_2. To the dry residue 100 μl bis(TMSi)- trifluoroacetamide– trimethylchlorosilane (99:1, v/v) combined with 100 μl pyridine were added and the mixt. was heated to 80° for 15 min [21]. Analysis of the silylated product was performed on a Finnigan 4000 GC/MS using EI ionization. GC conditions: direct injection of 1.0 μl sample diluted with pyridine; fused silica capillary column (30 m × 0.25 mm i.d.) with SPB™-5 bonded phase (5% diphenyl:95% dimethylsiloxane, 0.25 μm film thickness, He as carrier gas at 10.5 p.s.i. (72 395 Pa) and split vent of 40 ml min^{-1}; oven programme, 120° for 10 min, raised to 215° at 2° min^{-1}. The injector and detector were set at 260° and 300°, respectively. MS conditions: ionization voltage, 70 eV; emission current, 400 μA; scan rate, 1 scan sec^{-1}; mass range, 40–350 Da; ion source temp., 160°.

Kinetic properties. Experiments using the A_{290} assay were conducted to determine kinetic and inhibition parameters. Suitable blanks and controls in each case were used to determine the cinnamic acid formed due to enzyme reaction. Reaction rates in presence and absence of inhibitors (AIP, *trans*-methyl cinnamate, *trans*-cinnamic acid) were linear over the time course used for kinetic studies. Michaelis constants for substrate (L-phenylalanine) and inhibition constants for inhibitors were determined by non-linear regression using Microsoft Excel. Inhibition constants were determined with respect to substrate. The concn range for each inhibitor was chosen as the range resulting in 20–80% inhibition of enzyme activity. The lines generated for each inhibitor were the best fit of the data determined by non-linear regression using Microsoft Excel, which was also used to calculate slopes and intercepts for inhibition replots from primary Hanes–Woolf plots.

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