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PURIFICATION AND CHARACTERIZATION OF PHENYLALANINE AMMONIA-LYASE FROM *USTILAGO MAYDIS*

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Key Word Index—*Ustilago maydis*; Basidiomycetes; corn smut fungus; phenylalanine ammonia-lyase; enzyme purification; subunit structure; polyclonal antibodies; Western blot.

Abstract—Phenylalanine ammonia-lyase (PAL; EC. 4.3.1.5) has been purified to homogeneity from liquid-cultured cells of the phytopathogenic fungus *Ustilago maydis* by use of heat treatment, protamine and ammonium sulphate precipitation, ion-exchange and gel filtration chromatography, and preparative PAGE. Its native molecular mass was estimated as 320±20 kDa and its subunit molecular mass as 80 kDa. No isoforms of the enzyme were detected, and there was no evidence of glycosylation of the protein. *Ustilago* PAL was most active at pH 8.8–9.2 and 30° and had a K_m for L-phenylalanine of 1.05 mM. The enzyme did not deaminate L-tyrosine. The synthetic inhibitor 2-aminoindan-2-phosphonic acid (AIP) strongly inhibited the enzyme, as did sulphhydryl reagents and carbonyl reagents, whereas *t*-cinnamate was only moderately inhibitory. *Ustilago* PAL activity had no requirement for metal ion cofactors, but was inhibited by heavy metal ions (Ag^+, Cu^{2+}, and Hg^{2+}). Polyclonal antibodies raised against the purified enzyme readily recognized *U. maydis* PAL in solution and on Western blots, but only weakly cross-reacted with higher plant PAL. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) catalyses the nonoxidative deamination of L-phenylalanine to form *trans*-cinnamic acid and a free ammonium ion. The enzyme occurs in plants [1, 2, 3], fungi [4, 5, 6, 7], and Actinomycetes [8], but not in animals. PAL has been used in experimental treatments and diagnosis of phenylketonurea, a human syndrome characterized by high levels of circulating phenylalanine [9], and the enzyme has industrial application in the production of L-phenylalanine from *trans*-cinnamic acid, i.e. the reverse of the normal *in vivo* reaction [10].

In plants, PAL is the entrypoint enzyme into phenylpropanoid metabolism and it regulates the biosynthesis of a wide range of phenylpropanoid secondary compounds, including lignin, flavonoids, furanocoumarin and isoflavonoid phytoalexins, and wound protectant hydroxycinnamic acid esters [11].

Ustilago maydis is the causal agent of corn smut. The fungus reproduces primarily in embryonic and protein-rich tissues of its host (*Zea mays*), and produces galls on any above-ground part of the host plant [12]. The presence of PAL in the related species, *U. hordei*, was reported earlier by Subba Rao *et al.* [13], but nothing is known about the role of PAL in the *Ustilago* life cycle or pathogenesis. In a yeast, *Rhodosporidium toruloides*, phenylalanine can act as the sole source of carbon, nitrogen, and energy [14]. Because PAL catalyses the initial reaction in the catabolism of the amino acid in this organism, the enzyme plays a key role in regulating phenylalanine-dependent metabolism. However, the biological function of PAL in *R. toruloides*, *U. maydis* and other fungi during normal (i.e. non-phenylalanine-dependent) growth and development is unclear. It is now possible to conduct gene replacement and gene disruption experiments in *U. maydis* [15]; these techniques provide a unique opportunity to address the role of PAL. In a preliminary study, we established that, like *U. hordei*, cultured cells of *U. maydis* grown in synthetic medium produce substantial amounts of PAL, and the present study reports the purification and characterization of the *U. maydis* enzyme.

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RESULTS

Purification of PAL

The enzyme was most readily extracted from cultured *U. maydis* cells by grinding the cell mass with alumina powder. The degree of purification and the yield of PAL activity at each purification step are shown in Table 1. The elution profiles from the DEAE

Table 1. Purification of phenylalanine ammonia-lyase from *Ustilago maydis* cells

Purification step	Total activity (pkat)	Protein (mg)	Specific activity (pkat mg^{-1} protein)	Purification (-fold)	Recovery (%)
Crude extract	17 670	3442	5	1	100
Protamine sulphate	14 102	2174	6.5	1.3	80
30–60% $(NH_4)_2SO_4$	5552	328	17	3.4	31
DEAE Cellulose	5132	50	103	20.6	29
Bio-Gel A-0.5 m	3331	2.1	1586	318	19
Preparative PAGE	530	0.090	5889	1178	3

cellulose and Bio-Gel gel filtration columns showed one major PAL activity peak in each case (Fig. 1). Following chromatographic fractionation, the most active fractions were electrophoresed under nondenaturing conditions and the resulting gels were sliced and assayed. PAL was detected as a strong trailing band of activity (Fig. 2), and electrophoresis in gels of different acrylamide concentrations (5–8%) showed that the mobility of the zone containing the highest PAL activity corresponded to a mass of 320±20 kDa. Isoelectric focusing of the chromatographically purified PAL yielded one major protein band with a pI of 6.3 (Fig. 3.A). No PAL isoforms were detected during either electrophoresis or chromatography, and the purified enzyme gave a negative reaction in the glycoprotein detection assay.

When the native PAGE gel slice corresponding to the zone of greatest enzyme activity was electro-eluted and subjected to SDS–PAGE, a single band of protein with a mass of 55 kDa was detected by Coomassie Blue staining. Silver staining, however, revealed three further minor bands (84, 73, and 30 kDa) (Fig. 3.B).

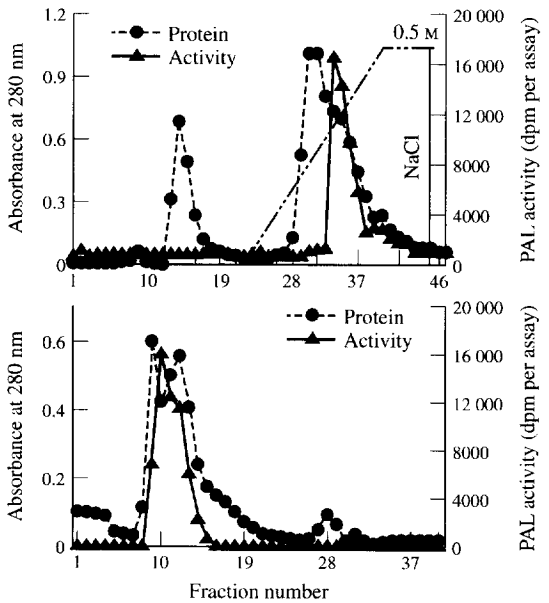

Fig. 1. Elution profiles of *Ustilago maydis* PAL and protein after DEAE cellulose chromatography (upper) and Bio-Gel A-0.5 m filtration (lower).

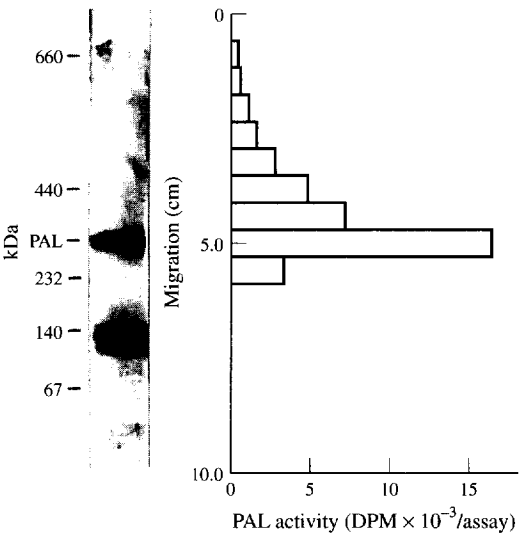

Fig. 2. Native PAGE analysis of purified PAL from *Ustilago maydis*. After preparative native PAGE (6% gel), one lane was silver stained (left) and another was sliced into 0.5 cm sections and assayed for PAL activity (histogram, right).

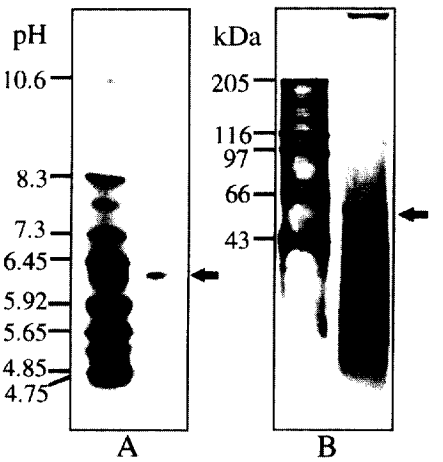

Fig. 3. Isoelectric focusing PAGE (A) and SDS-PAGE (B) analysis of *Ustilago maydis* PAL enzyme. Marker proteins (left lane) and enzyme (right lane) eluted from the native PAGE gel slice revealing highest PAL activity (see Fig. 2) were electrophoresed side by side and then silver stained. Arrow indicates the location of PAL enzyme.

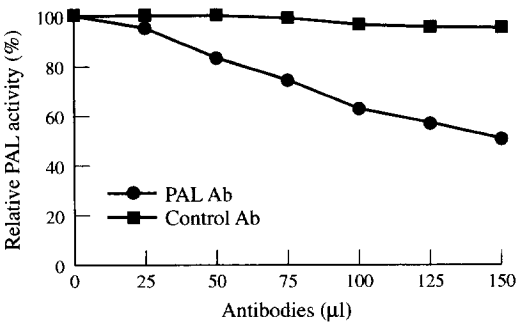

Fig. 4. Inhibition of *Ustilago maydis* PAL activity by *U. maydis* PAL antibodies. The purified γ-globulin fractions from *U. maydis* PAL antiserum and from pre-immune serum (control antibody) were incubated with PAL enzyme for 1 hr at 25° and assayed for PAL activity. Activity is expressed as a percentage of a control not related with antibodies.

Antibody production and characterization

Polyclonal antibodies against *U. maydis* PAL protein eluted from preparative nondenaturing PAGE gels produced a precipitin arc in immuno-double diffusion assays, with reaction to proteins from the crude extracts and from PAL-active fractions derived from ion-exchange and gel filtration separations (data not shown). The antibody titre was 12 000-fold, as measured by dot immuno-blot assay.

To test the effect of the *U. maydis* PAL antibodies on PAL enzyme activity, the *U. maydis* PAL enzyme was co-incubated with the γ-globulin fraction of the *U. maydis* PAL antiserum. The PAL activity decreased linearly with increasing amounts of antibody, whereas the preimmune antibodies had no effect (Fig. 4). Immobilization of the anti-PAL γ-globulin fraction to form an immunoaffinity matrix, and chromatography of partially purified *U. maydis* PAL preparations in this matrix, allowed recovery of low levels of PAL activity. The active fractions migrated as a single protein on nondenaturing PAGE and displayed the same mobility as conventionally purified PAL (data not shown).

Western blotting

On western blots, *U. maydis* PAL polyclonal antiserum detected a 320 kDa band in crude cell extracts, and in gel filtration-purified *U. maydis* PAL preparations, and also an equivalent band in recombinant poplar PAL preparations (Fig. 5). When poplar PAL polyclonal antiserum [16] was used as a probe, a weak band at 320 kDa was detected in purified *U. maydis* PAL (Fig. 5), but not in crude *U. maydis* cell extracts (data not shown). Activity assay of gel slices excised from the zone corresponding to the 320 kDa protein band detected on Western blots confirmed that the highest PAL activity was associated with this protein (data not shown). SDS–PAGE fractionation and western blotting (Fig. 6) of *U. maydis* PAL preparations at different points in the purification revealed two strong

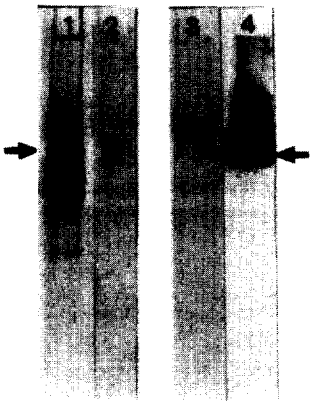

Fig. 5. Western blot analysis of reciprocal cross-reactivity between recombinant poplar PAL, *Ustilago maydis* PAL and their respective antisera. *Ustilago maydis* PAL (1 or 10 µg) from Bio-Gel filtration and purified recombinant poplar PAL (1 or 10 µg) [16] were electrophoresed separately in 7.5% native polyacrylamide gels and electro-blotted onto PVDF membrane. The blots were probed with either *U. maydis* PAL antiserum (1 µg sample of *U. maydis* PAL and 10 µg sample of poplar PAL), or poplar PAL antiserum (10 µg sample of *U. maydis* PAL and 1 µg sample of poplar PAL). After being probed with alkaline phosphatase-conjugated secondary antibodies, both the blots were developed with NBT/BCIP. Lane: 1 µg *U. maydis* PAL/*U. maydis* PAL antiserum $1:10^4$. Lane 2: 10 µg poplar PAL/*U. maydis* PAL antiserum $1:10^3$. Lane 3: 10 µg *U. maydis* PAL/poplar PAL antiserum $1:10^3$. Lane 4: 1 µg poplar PAL/poplar PAL antiserum $1:10^4$. Arrow indicates the location of PAL enzyme.

protein bands (80 kDa and 52 kDa) and one weak band (160 kDa), with the 52 kDa protein predominating as the purification proceeded.

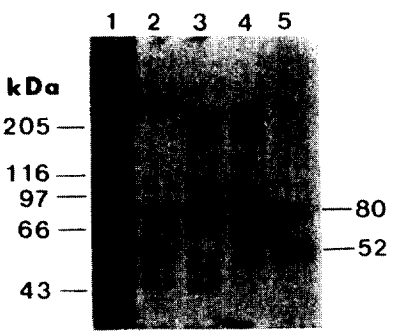

Fig. 6. Western blot analysis of *Ustilago maydis* PAL subunit sizes at different stages of enzyme purification. Samples of PAL were taken from different purification stages, separated on 7.5% SDS polyacrylamide gels, and electro-blotted onto PVDF membrane. The molecular weight marker lane in the blot was stained with Coomassie Blue. Other lanes in the blot were probed with *U. maydis* PAL antibodies and alkaline phosphatase-conjugated secondary antibodies, followed by colour development with NBT/BCIP. Lane 1: molecular weight markers. Lane 2: crude extract. Lane 3: 30–60% ammonium sulphate precipitate. Lane 4: Bio-Gel A-0.5 m eluate. Lane 5: electro-eluate from a single band on a preparative native PAGE gel.

Stability

When PAL enzyme in 0.01 M Na-phosphate buffer (pH 7.0) was heated for 10 min at 50°C, cooled to room temperature and assayed at 30°, no significant activity was lost. Heating at 60° or 70°, however, resulted in activity losses of 30% and 100%, respectively. When stored at 4°, the enzyme retained 95% activity for a week, and 60% activity after one month.

pH optimum

The optimal pH for PAL activity was pH 8.8–9.2. The enzyme displayed 50% of maximum activity at pH 6.8.

Catalytic properties

Ustilago maydis PAL partially purified by DEAE cellulose ion-exchange chromatography displayed normal Michaelis–Menten kinetics. The apparent K_m value for L-phenylalanine was 1.05 mM, and the V_{max} was 51 pkat mg^{-1}. The enzyme displayed no detectable activity against L-tyrosine using either spectrophotometric or radiometric assay methods.

Inhibitors and activators

Ustilago maydis PAL was readily inactivated by carbonyl reagents such as NaCN and $NaBH_4$ (Table 2), as well as by the sulphhydryl reagent, *p*-chloromercuribenzoate. The enzyme was moderately sensitive to its reaction product, *t*-cinnamic acid ($K_i = 0.41$ mM), and to the product of cinnamate hydroxylation, *p*-coumaric acid, but the synthetic substrate analogue, 2-aminoindan-2-phosphonic acid (AIP) [17], was a very effective inhibitor ($K_i = 0.33$ μM).

None of the metal ions tested produced any enhancement of PAL activity, but heavy metal ions generally inhibited the enzyme (Table 3). Treatment with EDTA was not inhibitory, but the copper chelator, 8-hydroxyquinoline (12.5 mM), produced a moderate level of inhibition. PAL inhibition produced by Hg^{2+} could be partially reversed by treating the inhibited enzyme with β-mercaptoethanol, but 8-hydroxyquinoline was unable to reverse the inhibition produced by Cu^{+} or Cu^{2+} ions. Although PAL requires a low level of a thiol protectant for long-term stability, high concentrations (>25 mM) of β-mercaptoethanol also inhibited the enzyme.

Table 2. Effect of chemical modification reagents and substrate analogues on *Ustilago maydis* phenylalanine ammonia-lyase activity

Inhibitor	Concentration	Relative activity (%)
None	—	100%
NaCN	5 mM	0
$NaBH_4$	5 mM	13
Phenylhydrazine HCl	5 mM	91
Iodoacetate	5 mM	109
N-Ethylmaleimide	5 mM	32
p-Chloromercuribenzoate	1 mM	11
β-Mercaptoethanol	25 mM	40
AIP	0.1 μM	55
	10 μM	1
t-Cinnamate	1.0 mM	75
	10 mM	8
p-Coumarate	10 mM	97
	30 mM	0

Table 3. Effect of metal ions and chelators on *Ustilago maydis* phenylalanine ammonia-lyase activity

Chemicals	Concentration (mM)	Relative activity (%)
No addition	–	100
$AgNO_3$	5	3
$Cd(CH_3CO_2)_2$	10	38
$CoCl_2$	10	62
CuCl	6	6
$CuCl_2$	3	1
$HgCl_2$	1	0.5
$ZnCl_2$	10	54
8-Hydroxyquinoline	12.5	15

$CaCl_2$, $FeSO_4$, $MgCl_2$, $MnCl_2$, $Na_2Cr_2O_7$, NaF, and EDTA at 10 mM. Concentrations did not reduce activity below 90% of control.

DISCUSSION

Phenylalanine ammonia-lyase has been purified to varying degrees from numerous higher plant species but only from a limited number of fungi [18]. It is a relatively low abundance protein and obtaining homogeneous preparations usually requires extensive fractionation of initial cell extracts. PAL is also prone to degradation during purification, and this lability can generate confusion over the native and subunit structure of the enzyme.

The estimated molecular mass of native *U. maydis* PAL (320 kDa) is similar to most other known PAL enzymes, which typically range in mass from 300 to 340 kDa, although exceptions have been reported for PAL from *Streptomyces* (226 kDa) [8], strawberry (266 kDa) [19], and *Alternaria* (556 kDa) [20]. There is convincing evidence from studies of the heterologous expression of both fungal and plant PAL cDNAs that PAL is normally a homotetrameric protein consisting of four copies of the same gene product [21–22]. This implies that the subunit of the *U. maydis* enzyme should have a mass of 80 kDa, and a protein of this size is prominent on western blots of preparations obtained at various points during the purification procedure (Fig. 6). The western blots show, however, that the original 80 kDa subunit population is accompanied by a strongly immunoreactive 52 kDa fragment throughout the normal purification process. The most highly purified active *U. maydis* PAL preparations (Fig. 2) consist of holoenzyme that yields almost exclusively this smaller fragment upon denaturation (Fig. 3.B; Fig. 6).

It is interesting to note that the undenatured enzyme appears to retain its native M_r even when the results of SDS–PAGE and western blot analysis suggest that most of the subunits in the population have been cleaved to yield a discrete, substantially smaller, polypeptide fragment. This behaviour, which has also been observed in other systems [23–24], implies that the cleavage fragments remain firmly bound within the tetrameric structure of PAL. Heterotetrameric quaternary structures for PAL have been reported from *Rhizoctonia* [25] and sunflower [3], but partial degradation of the enzyme cannot be ruled out in either case.

The *U. maydis* PAL antiserum has a high affinity for both the native fungal enzyme and its denatured subunits. The *U. maydis* PAL antiserum was also able to recognize a higher plant PAL protein, although the reaction was far weaker than with the homologous protein. The reciprocal cross-reaction between poplar PAL antiserum and *U. maydis* PAL protein (Fig. 5), and between lucerne PAL antiserum and *U. maydis* PAL protein (S. H. Kim, J. W. Kronstad and B. E. Ellis, unpublished data), was similarly detectable but weak. While antisera have been raised to several plant and fungal PAL proteins (e.g. lucerne [26], bean [27], and *Mycosphaeria maculans* [28]), we are not aware of any other reports on the degree of cross-reactivity between plant and fungal PAL. The cross-reactivity observed here is consistent with the presence of some short stretches of highly conserved sequence within plant and fungal PAL genes [29], but the weakness of the cross-reaction also emphasizes the extent to which the structures of the *Ustilago* and higher plant PAL proteins may have diverged.

Many of the physico-chemical properties of *U. maydis* PAL are typical of this enzyme from other sources (e.g. M_r, pH optimum, sensitivity to carbonyl reagents, requirements for thiol protectants). Other properties are more unusual, including the temperature stability, a relatively high apparent K_m for phenylalanine, and a pI of 6.3. PAL proteins from other species have been reported to display apparent K_m values ranging from 11 μM to 450 μM L-phenylalanine, while the reported pI values range from 4.1 [8] to 5.75 [30].

PAL preparations from some sources, including sweet potato [31], tobacco [32] and yeast [33], are strongly inhibited by the reaction product, *t*-cinnamate, but cinnamate has comparatively little effect on the activity of the *U. maydis* enzyme. In this, the *Ustilago* PAL resembles the enzyme for *Streptomyces* [8], lucerne [26], pine [30], and tomato [34].

Like most known PALs, the *Ustilago* enzyme is sensitive to heavy metal ions and displays no metal ion requirement for catalytic activity (Table 3). Again, there is considerable interspecific variability in these responses, since Hg^{2+} completely inhibited the *U. maydis* PAL enzyme at 0.5 mM, whereas sunflower PAL was less strongly inhibited, retaining some activity even in 2 mM Hg^{2+} [2]. Similarly, Cu^{2+} (1 mM) was a potent inactivator of the *U. maydis* PAL but had no effect on the enzyme from sweet potato [31]. The significance, if any, of these patterns may become apparent only when more is learned about the detailed structure of PAL proteins and their active site(s).

EXPERIMENTAL

Materials. DIG-Glycoprotein Detection kit and pI markers were purchased from Boehringer Mannheim. Molecular mass markers for native PAGE and HiTrap-NHS matrix were obtained from Pharmacia Biotechnology Products. DEAE cellulose and Freund's adjuvant were purchased from Sigma. Bio-Gel A-0.5m agarose beads, Ampholyte, alkaline phosphate-conjugated goat anti-rabbit antibody, SDS–PAGE molecular markers, and BCIP (5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt)/NBT (*p*-nitro blue tetrazolium chloride) were obtained from Bio-Rad. Westran PVDF (polyvinylidine difluoride) membrane and Centriprep Concentrator were purchased from Schleicher & Schuell and Amicon, respectively.

Fungal cultures. Ustilago maydis strain 518 was maintained on potato dextrose agar medium (PDA) and grown in Complete Liquid Medium as described in ref. [25]. For enzyme isolation, Complete Liquid Medium (50 ml) in 250 ml Erlenmeyer flasks was inoculated with sporidia of the fungus grown on PDA and incubated at 30° for 21 hr on a gyratory shaker (250 rpm).

Purification of U. maydis *phenylalanine ammonia-lyase.* All procedures were carried out at 4°, unless otherwise mentioned. The fungal cells were harvested by centrifugation (10 000 *g*, 10 min), washed twice with distilled H_2O, frozen in liquid N_2 and stored at 70° until needed. Cells (30 g) were mixed with 60 g alumina and homogenized for 30 min in a mortar and pestle. The homogenate was extracted with 120 ml 50 mM sodium phosphate buffer (pH 8.0) by stirring for 1 hr, and centrifuged (20 000 *g*, 30 min). The supernatant (115 ml) was heated at 50° for 10 min, cooled in ice and centrifuged (20 000 *g*, 30 min). Protamine sulphate (2%, pH 7.0) was slowly added to the supernatant (final concn 0.1%) and stirred for 30 min. After centrifugation (20 000 *g*, 30 min), the pellet was discarded, and the supernatant was fractionated between 30% and 60% saturation with $(NH_4)_2SO_4$. The pellet was dissolved in 10 ml buffer A (1 mM sodium phosphate, pH 7.0) and dialysed against 3 l buffer A (16 hr × 3 changes). The centrifuged dialysate was applied to a DEAE cellulose column (2 × 28 cm), washed with buffer A, and eluted with buffer A containing NaCl in a linear 0–0.5 M gradient. The highest PAL activity frs were pooled and brought to 70% $(NH_4)_2SO_4$ satn. After centrifugation, the pellet was dissolved in 4 ml buffer A, dialysed, centrifuged, and concd on a Amicon Centriprep concentrator. The concentrate (1 ml) was applied to a Bio-Gel A-0.5m column (1 × 110 cm) and eluted with 100 ml buffer A. The highest PAL activity fractions were combined, concd, and dialysed. A cooled 6% prep. vertical polyacrylamide slab gel was used for the final purifica-

tion step (see below for electrophoresis). Two 0.5 cm wide vertical strips were excised from the centre of each gel. One was silver stained and the other was divided into 0.5 cm wide segments. Each segment was minced separately in 300 μl K-borate buffer (pH 8.8), shaken for 30 min, ground with a mini-pestle, centrifuged, and assayed for PAL activity and protein.

Enzyme assay. PAL activity was measured radiometrically as described in ref. [36] using L-[U-^{14}C]phenylalanine as substrate. Protein concn was estimated by the dye-binding method [37], with bovine serum albumin as standard. All assays were done in triplicate. To test the effect of inhibitors and activators on *in vitro* activity of PAL, Bio-Gel purified PAL (Table 1) in PAL assay buffer was mixed with compounds to be tested. After 20 min incubation, the mixt. was filtered through a Sephadex G25 column and assayed with the standard reaction mixt. For the detection of tyrosine ammonia-lyase (TAL) activity, crude and Bio-gel purified PAL enzyme preparations were assayed by the spectrophotometric method, and by the radiometric method using tritium-labelled L-tyrosine as substrate.

Polyacrylamide gel electrophoresis. Vertical slab gels were used for all PAGE analyses. Polyacrylamide gels (5, 6, 7, and 8%) were prepared for native PAGE and run with the Laemmli discontinuous buffer system [38] in which SDS was omitted from the loading and running buffer. To determine native M_r of PAL protein, the mobility of PAL at each gel concentration was compared with that of thyroglobulin (660 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and BSA (67 kDa). Subunit M_r was determined by mobility in 7.5% SDS–PAGE gels, compared with that of myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), BSA (66 kDa) and ovalbumin (43 kDa). Separated protein bands were detected by Coomassie Brilliant Blue R-250 or silver staining. To detect PAL charge isoforms, the purified PAL was run in a native IEF–PAGE system [39] using pH 3-10 Ampholyte, and silver stained. For pI determination, the mobility of the PAL band was compared with that of cytochrome *c* (horse heart, pI 10.6), myoglobin met (whale sperm, pI 8.3), myoglobin met (horse, pI 7.3), myoglobin met (porcine, pI 6.45), trifluoracetylated myoglobin met (porcine, pI 5.92), azurin (*P. aeruginosa*, pI 5.65) and C-phycocyanin (*A. nidulans*, pI 4.75 and 4.85).

Antibody production and immuno-affinity chromatography. PAL protein excised from preparative PAGE gels was minced in 0.5 ml Freund's adjuvant and New Zealand White rabbits immunized with one subcutaneous and three intramuscular injections of 50 μg PAL at 10-day intervals. Specificity of the antiserum was assayed by double diffusion in agar and the titre was determined by dot immuno-blot assay. The γ-globulin fraction was isolated from *U. maydis* PAL antiserum by DEAE cellulose chromatography [40]. After incubation of a mixt. of purified *U. maydis* PAL and the γ-globulin fraction at 25°C for 1 hr, the solution was assayed directly for PAL activity. For affinity purification of the *U. maydis* PAL enzyme, the isolated γ-globulin was coupled to a HiTrap-NHS matrix according to the supplier's protocol. *U. maydis* PAL purified through the DEAE cellulose step was applied to the affinity column and eluted with 0.1 mM Na-citrate buffer, pH 3.0.

Western blot. PAL proteins sepd on a 7.5% native or SDS–PAGE gel were electrophoretically transferred to Westran polyvinylidene fluoride membrane (100 V, 1 hr, 4°). Tris-glycine buffer (25 mM Tris-HCl, 190 mM glycine, pH 8.3) was used as transfer buffer for native PAGE gels. MeOH (10%) was included in the transfer buffer for SDS–PAGE gels. After electroblotting, the M_r marker lane was cut off the membrane and stained with Coomassie Brilliant Blue R-250. The remaining blot was washed twice with TBS (100 mM Tris-HCl, 150 mM NaCl, pH 7.5), blocked with 3% BSA in TBS-Tween buffer (0.1% Tween-20 in TBS) for 1 hr and probed with *U. maydis* PAL polyclonal antibodies for 2 hr at room temp. After washing ×3 with TBS-Tween buffer, the blot was incubated (1 hr) with an alkaline phosphatase-conjugated goat anti-rabbit antibody. The blot was washed again and the secondary antibody detected with NBT/BCIP staining.

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