Identification of Essential Amino Acids in Phenylalanine Ammonia-Lyase by Site-Directed Mutagenesis†

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Received March 25, 1997; Revised Manuscript Received June 16, 1997⊗

ABSTRACT: The postulated precursor of the prosthetic dehydroalanine of phenylalanine ammonia-lyase (PAL), serine 202, was changed to cysteine by site-directed mutagenesis. After cloning and heterologous expression in *Escherichia coli*, the gene product was assayed for PAL activity. Mutant S202C showed full catalytic activity, and its kinetic constants and the amount of thiol groups were identical to those of wild-type PAL. It must be concluded that in a posttranslational modification both water and hydrogen sulfide can be eliminated from the amino acid in position 202 to form dehydroalanine. In an attempt to identify further amino acids essential either for the posttranslational modification or for catalysis, arginine 174, glutamine 425, and lysine 499 were changed to isoleucine. Analysis of the heterologously expressed mutated gene products revealed that only the R174I mutant showed a significantly lower V_{max} value (1/450) identifying this arginine as important. This finding was supported by treatment of wild-type PAL and mutant R174I with phenylglyoxal and 2,3-butandione. Both react specifically with the guanidino group of arginine. They irreversibly inhibited wild-type PAL but had no influence of the V_{max} value of mutant R174I. Preincubation with L-phenylalanine protected wild-type PAL from inhibition by phenylglyoxal indicating that arginine 174 is close to the active site. Incubation with KCN irreversibly abolished the remaining activity of mutant R174I leading to the conclusion that arginine 174 is important in catalysis.

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5)[1] is an important enzyme in higher plants and catalyzes the elimination of ammonia from L-phenylalanine leading to *trans*-cinnamic acid. This reaction represents a branching point between primary metabolism and the formation of a various aromatic compounds, such as lignin, flavonoids, and coumarins (*1*, *2*).

It has been known for many years that PAL contains an electrophilic group that is essential for catalysis. The electrophilic property of the prosthetic dehydroalanine has been demonstrated by addition of various nucleophiles such as nitromethane (*3*−*5*), cyanide (*6*), and sodium borohydride (*4*, *7*). All of these reagents caused inactivation and, using radiolabel, incorporation of the radioisotope into the expected product. From these results it has been concluded that PAL contains a dehydroalanine residue at the active site (*7*), and cysteine or serine were discussed as possible precursors (*8*). By comparison of the amino acid sequences of PALs and HALs (histidine ammonia-lyases) and by the use of site-directed mutagenesis, we located serine 202 as the putative precursor of dehydroalanine (*9*). The behavior of various dehydroalanine-less mutants of PAL with 4-nitrophenylalanine as substrate and other kinetic experiments with wild-type PAL led us to postulate a novel mechanism for the PAL reaction in which a transient Friedel−Crafts-like acylation of the phenyl ring is the crucial step (*10*).

Here we report the exchange of serine 202 for cysteine by site-directed mutagenesis and its effect on the catalytic activity of PAL indicating that serine 202 is the precursor of the active site dehydroalanine. Furthermore we describe our successful search for further amino acids which play an essential role in the mechanism of action of PAL.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

Escherichia coli TG1 cells were used for the isolation of single-stranded DNA from M13 phages to carry out site-directed mutagenesis following the protocol of Amersham. Cells were grown and infected as described in the laboratory manual (*11*). *Escherichia coli* BL21(DE3) cells served both for site-directed mutagenesis following the protocol of Deng and Nickloff (*12*) and for the expression of wild-type phenylalanine ammonia-lyase and its mutants (*9*). For overexpression cells were grown in 1 L of Luria−Bertani medium (LB) supplemented with ampicillin (85 μg/mL) at 37 °C. At an OD_{600} of 1.0, 400 μmol of isopropyl thio-β-D-galactoside (IPTG) was added. Cells were harvested 4 h after induction.

The phage M13BM21 was from Boehringer Mannheim.

The expression vector pT7.7 was generously provided by Dr. Stanley Tabor (*13*).

The PAL1 gene from a cDNA library from elicitor-treated parsley (*Petroselinum crispum* L.) cells and antibodies against PAL were generous gifts of Prof. Dr. K. Hahlbrock (Max-Planck-Institut für Züchtungsforschung, Cologne, Germany) and Prof. Dr. N. Amrhein (ETH Zürich), respectively.

† The work was supported by Deutsche Forschungsgemeinschaft and Fond der Chemischen Industrie.

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⊗ Abstract published in *Advance ACS Abstracts,* August 15, 1997.

[1] Abbreviations: PAL, phenylalanine ammonia-lyase; HAL, histidine ammonia-lyase; IPTG, isopropyl thiogalactoside.

S0006-2960(97)00699-5 CCC: $14.00

pT7.7PAL was produced as described by Schuster and Rétey (*9*).

Site-Directed Mutagenesis

Site-directed mutagenesis was performed following two different protocols. PALMutS202C was constructed as described by Schuster and Rétey (*9*) using the Amersham mutagenesis kit (Sculptor) (*14*). The oligonucleotide sequence used in this mutagenesis reaction was the following: 5′-CACTGCTTGTGGTGATC-3′.

Site-directed mutagenesis starting with double-stranded DNA was performed as described by Deng and Nickloff (*12*) following the protocol of stratagene Chameleon kit. The oligonucleotide sequences used in this mutagenesis reaction were as follows:

R174I: 5′-GGAATCATATTCGAAATTCTCG-3′

Q425I: 5′-GTTTGCTATATTATTTTCTGAAC-3′

K499I: 5′-CTCTTCAAGGATAAACATCAG-3′

selection primer:

5′-GTTAATAGTTTGATCAACGTTGTTG-3′

In brief, two primers are annealed on double-stranded plasmid DNA at the same time. One of the primers switches a codon and the other destroys a unique restriction site in the plasmid. Selection for mutants is performed by using the endonuclease which will not hydrolyze the mutated DNA. This mutagenesis results in 70−90% yield of mutant clones in 3 days.

Transformation. Either *E. coli* TG1 or *E. coli* BL21(DE3) cells were grown in 0.5 L of LB medium to an OD_{600} of 0.90−0.95 (*15*). The cells were collected by centrifugation at 4500*g*, and the cell pellet was resuspended in 0.5 L of ice-cold 10% glycerol solution. The cells were made competent in five steps by reduction of the suspension volume of 10% glycerol to a final volume of 1 mL. All steps were carried out at 4 °C. Aliquots of the concentrated competent cells (140 μL) were stored at −70 °C for two months without loss of competence. Transformation was performed by electroporation using a gene pulser from Bio-Rad. A 70 μL amount of competent cell suspension was transformed with 20 ng of vector DNA in a 0.4 mm cuvette at 2.5 kV and 0.2 kΩ (6.25 kV/cm) for 4.4 ms. Recombinant phages were selected by blue/white screening (*11*). Recombinant bacteria were detected either by blue/white screening or by restriction analysis.

Purification. Transformed *E. coli* BL21 cells were grown in 1 L of LB medium containing ampicillin (85 μg/mL) to an OD_{600} of 1.0, and then 400 μmol of IPTG was added. Cells were harvested 4 h after induction by centrifugation at 4500*g*. The cell pellet was resuspended in 10 mL of 10 mM potassium phosphate buffer, pH 6.6, containing 40 units of Benzonase (Merck, Darmstadt); 5 mM benzamidine, and 0.5 mM phenylmethanesulfonyl fluoride. Sonication (Branson, model 450, 70% power setting, 10 min ice bath) was followed by centrifugation at 30000g for 30 min. The clear supernatant was applied to an EMD-DEAE-650(M) column (gravity flow), using 10 mM potassium phosphate buffer, pH 6.6, as buffer A and 400 mM potassium phosphate buffer as buffer B. PAL eluted at 40% B. The PAL-containing fraction was dialyzed against 50 mM potassium phosphate buffer, pH 7.0. The filtered solution (0.45 μm filter) was then applied to a HILoad 16/10 Q-Sepharose high-performance column at a flow rate of 3 mL/min. 50 mM potassium phosphate buffer, pH 7.0, as start buffer and 400 mM potassium phosphate buffer, pH 7.2, as eluent was used. PAL was eluted at a concentration of approximately 120 mM potassium phosphate. PAL active fractions were pooled and concentrated (semipermeable cell with a molecular mass limit of 50 kDa, Centricon 30), to a final volume of 2 mL. The 2 mL aliquot was applied on a TSK 3000 SW gelfiltration column at a flow rate of 1.5 mL/min using 50 mM potassium phosphate buffer, pH 7.5, supplemented with 0.1 M KCl. The active fractions were pooled, concentrated to 0.5 mL (Centricon 30, Amicon), and mixed with 0.5 mL of 100% glycerol. The enzyme was stored at −20 °C.

SDS/PAGE, Western Blot, and Protein Assay. SDS/PAGE using a 10% polyacrylamide gel was performed according to Laemmli (*16*) to monitor the purification of PAL. Staining of the gel was carried out with Coomassie Brillant Blue R 250. Western blotting was performed using the standard laboratory protocol adapted according to Symingteon et al. (*17*). Protein determinations were performed by measurement of A_{260} and A_{280} according to Warburg and Christian (*18*).

Enzyme Assay. PAL activity was determined spectrophotometrically of the reaction product cinnamate (*19*). Standard conditions for the measurement of PAL activity were 0.1 M Tris-HCl buffer, pH 8.8, 20 mM L-phenylalanine, 30 °C. The extinction coefficient (ϵ_{290}) of cinnamic acid is 10^4 L cm^{-1} mol^{-1}.

Reaction of Phenylglyoxal and 2,3-Butandione with Wild-Type PAL and Mutant R174I (20). 3.8 mg of phenylglyoxal or 150 mg of 2,3-butandione was dissolved in three buffers (5 mL) with different pH values (potassium phosphate buffer, pH 7.5/sodium carbonate buffer, pH 8.8 and 10.5). A 1 mg amount of PAL was added to each sample. Every 10 min a 200 μL aliquot of each reaction mixture was used for the enzyme assay.

Quantification of Thiol Groups in Wild-Type PAL and Mutant S202C. Determination of the amount of thiol groups was carried out as described in detail by Langer et al. (*21*). The molar extinction coefficient of the thiobenzoate anion (ϵ_{412}) at 412 nm is 13 600 L mol^{-1} cm^{-1}; the molar mass of the PAL homotetramer is 320 000 Da.

Reaction of KCN with Mutant R174I. A 0.4 mg amount of protein of mutant R174I was added to 1 mL of 0.1 M Tris-buffer, pH 8.8, containing 10 mM KCN. Every 20 min a 200 μL aliquot was used for the enzyme assay.

RESULTS

Expression and Purification of Recombinant Wild-Type (wt) PAL

Expression of wild-type PAL has been described in detail. Overexpression was performed as described by Schuster and Rétey (*9*) using the pT7.7 expression system. Purification of recombinant PAL proteins was carried out by modification of the procedure as described by Schuster and Rétey (*10*). Table 1 summarizes the individual islolation steps for wild-type PAL isolated from *E. coli* BL21(DE3). Depending on the oxygen available in the culture medium, the total amount of PAL protein varies from 1 to 6 mg per liter of *E. coli*

Table 1: Summary of the Individual Purification Steps for Recombinant wt PAL Isolated from *E. coli* BL21(DE3)

purification step	total protein (mg)	total activity (units)	specific activity (units/mg)	yield (%)
crude extract	260	23	0.09	100
EMD-DEAE-650(M)	110	22	0.2	96
Q-Sepharose	38	19	0.5	83
TSK-3000SW	6	18	3.0	78

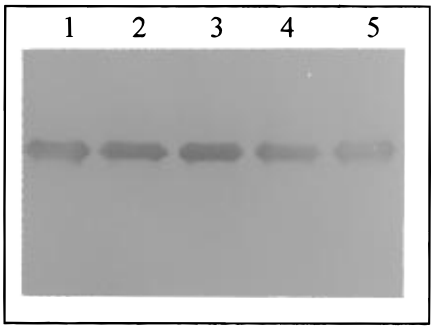

FIGURE 1: Western blot of SDS/PAGE gel showing overexpressed wild-type PAL (lane 1), mutant S202C (lane 2), R174I (lane 3), Q425I (lane 4), and K499I (lane 5) demonstrating that all proteins are indistinguishable by their molecular masses.

Table 2: Summary of the Yields of Recombinant wt PAL and the Different Mutants Isolated from 1 L of *E. coli* BL21(DE3) Culture

	protein				
	wt PAL	S202C	R174I	Q425I	K499I
total protein (mg)	0.72	0.007	0.24	0.25	0.72

culture. Specific activities between 1 and 3 units/mg can be achieved depending on the rate of expression.

Analysis of Wild-Type PAL and the Mutants S202C, R174I, Q425I, and K499I

The PAL mutant S202C was constructed as described by Schuster and Rétey (*9*). Mutants R174I, Q425I, and K499I were constructued using the method of Deng and Nickoloff (*12*). As revealed by Western blot analysis of the SDS/PAGE the overexpressed wild-type and mutant PAL proteins are indistinguishable by their molecular masses (Figure 1). After purification the total amount of the expressed proteins are compared. The yields of wild-type PAL protein and mutants are shown in Table 2. Wild-type PAL and the mutants R174I, Q425I, and K499I are expressed in comparable amounts: 0.72 mg of wt PAL; 0.72 mg of mutant K499I; 0.24 mg of mutant R174I, and 0.25 mg of mutant Q425I could be isolated from 1 L of *E. coli* culture, whereas isolation of mutant S202C yielded 100 times less protein.

The kinetic measurements were carried out using the spectrophotometric enzyme assay. The substrate concentration varied between 0.05 and 5 mM and the kinetic parameters were determined using the double-reciprocal plot. The kinetic behavior of the different PAL proteins are compared in Table 3. The K_m and V_{max} values from wild-type PAL and the mutants S202C and K499I are nearly identical and comparable to those of native PAL isolated from parsley (*19*). A slightly lower V_{max} value was determined for the mutant Q425I whereas significant differences can be seen between wild-type PAL and the mutant R174I. Mutant R174I reacts 450 times more slowly with L-phenylalanine as substrate than does wild-type PAL. This implies an important role of R174 in the formation of fully active enzyme.

Table 3: Comparison of the Kinetic Konstants of wt PAL and the Mutants S202C, R174I, Q425I, and K499I with L-Phe as Substrate

protein	K_m (mM)	V_{max} (units/mg)	$V_{maxwtPAL}/V_{maxmutPAL}$
wtPAL	0.17	0.74	
S202C	0.17	0.70	1.1
R174I	0.03	1.6×10^{-3}	450
Q425I	–	0.012	62
K499I	0.10	0.79	0.9

Table 4: Titration of the Thiol Groups of Wild-Type PAL and Mutant S202C per Monomer

PAL	SH groups
recombinant wtPAL	9.0
mutant S202C	8.9

Quantification of Free Thiol Groups in Wild-Type PAL and Mutant S202C

The kinetic constants indicate that mutant S202C is posttranslationally converted into wild-type PAL. To test this presumption the amount of cysteines in mutant S202C were determined and compared to that in wild-type PAL. The deduced sequence of PAL from parsley (*22*) reveals nine cysteines per subunit. To quantify the free thiol groups in the mutant S202C Ellman's reagent (DNTB) was used. The titration experiments are summarized in Table 4. Both wild-type PAL and mutant S202C show nine SH groups per subunit. This result suggests that mutant S202C is indistinguishable from wild-type PAL by the number of SH groups.

Reaction of Phenylglyoxal, 2,3-Butandione, and KCN with Wild-Type PAL and Mutant R174I

To investigate the role of arginine in the catalytic activity PAL was chemically modified. Phenylglyoxal and 2,3-butandione are known to react specifically with the guanidino group of arginine residues (*20*). Two molecules of phenylglyoxal and three molecules of 2,3-butandione are involved in forming a complex with arginine.

Wild-type PAL was therefore treated with phenylglyoxal and 2,3-butandione at different pH values. The results are shown in Figure 2. Both phenylglyoxal and 2,3-butandione act as irreversible inhibitors of PAL. 2,3-Butandione reacts faster with wtPAL than phenylglyoxal. The time for 50% inactivation is less than 15 min for phenylglyoxal and less than 5 min for 2,3-butandione, respectively. The rate of inactivation was decreased significantly when the pH of the reaction was lowered. After 6 h of incubation with phenylglyoxal at a pH value of 7.5, less than 10% of the original activity of PAL remained; 20 min of incubation with 2,3-butandione had the same effect. At a pH value of 10.5 the time for 90% inactivation was reduced to 15 min for phenylglyoxal and 7 min for 2,3-butandione. In contrast to wtPAL incubation with phenylglyoxal did not influence the residual activity of mutant R174I.

To localize R174 in the three-dimensional structure PAL was treated with phenylalanine before inactivation with phenylglyoxal. Figure 3 shows that in the presence of the natural substrate inactivation is slower. This indicates that

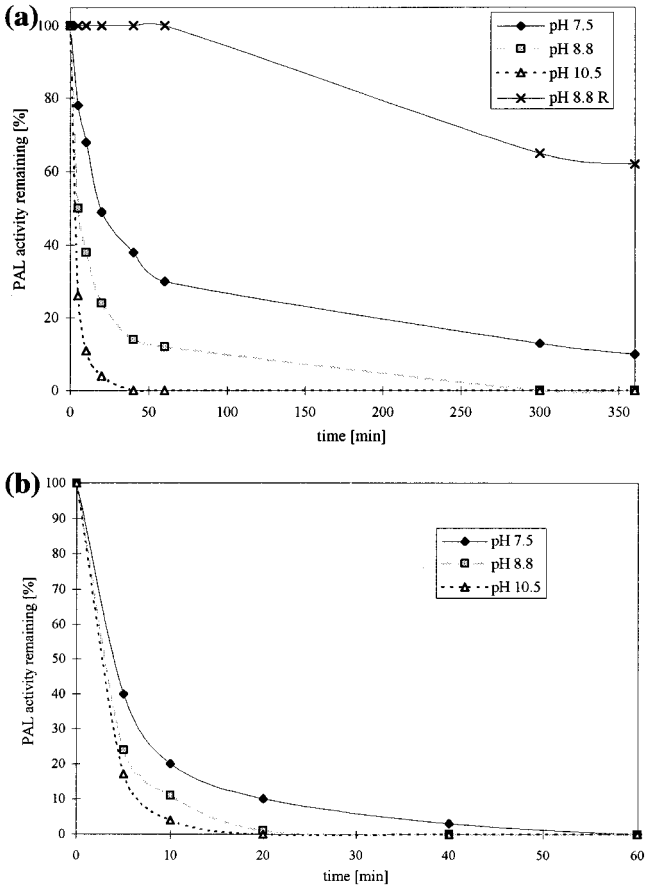

FIGURE 2: Rates of inactivation of wtPAL at different pH values and of mutant R174I at a pH value of 8.8 (pH 8.8 R) by reaction with phenylglyoxal (Figure 2a) and inactivation of wtPAL by reaction with 2,3-butandione (Figure 2b).

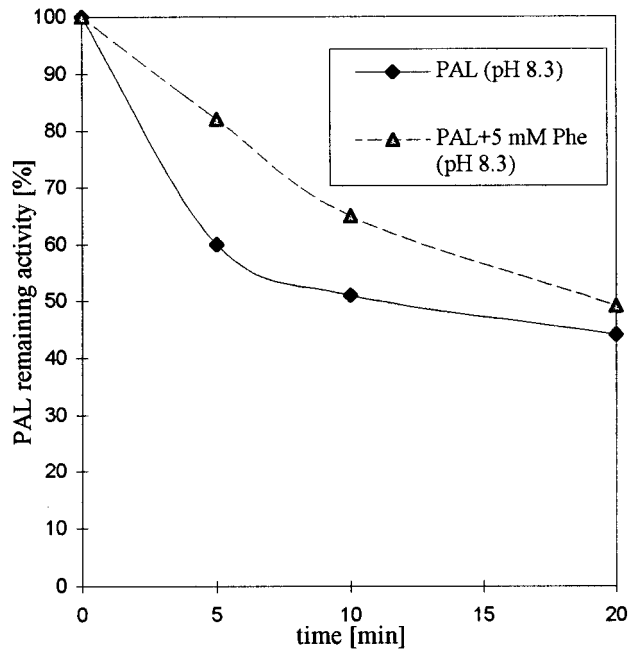

FIGURE 3: Rates of inactivation of PAL at a pH value of 8.3 by reaction with phenylglyoxal in the presence and absence of L-phenylalanine.

R174 is located close to the active center because phenylalanine protects it from inactivation by phenylglyoxal.

Now the question arises what is the function of arginine 174. Is this amino acid important in formation of dehydroalanine or in catalysis? In earlier experiments Hanson and Havir (*7*) showed, that KCN reacts with dehydroalanine leading to an irreversible inhibition of PAL. If arginine was necessary for the formation of dehydroalanine, the mutant R174I would not be able to form this prosthetic group and incubation with KCN would not influence the V_{max} value. If treatment with KCN had an effect at the activity of the mutant PAL, the arginine would be important in catalysis. Therefore mutant R174I was incubated with KCN. After 1 h incubation with KCN no catalytic activity remained (Figure 4). This result suggests that arginine 174 is important in catalysis and not in the formation of the dehydroalanine prosthetic group.

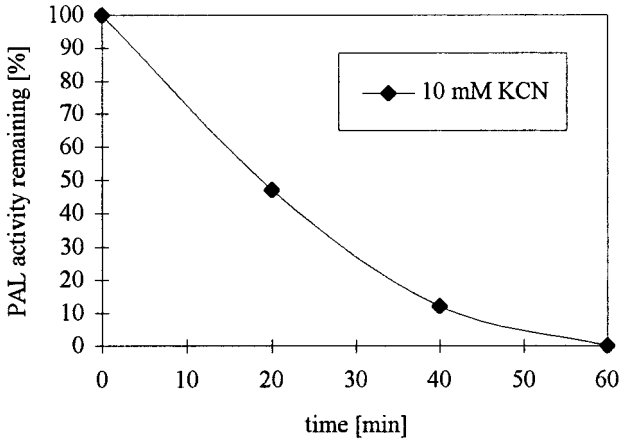

FIGURE 4: Rate of inactivation of mutant R174I at a pH value of 8.8 by reaction with KCN.

DISCUSSION

The heterologous expression of the PAL gene from parsley in *E. coli* afforded PAL in the fully active form. The lack of PAL in the wild-type *E. coli* suggests that the posttranslational modification of serine 202 to the prosthetic dehydroalanine occurs by autocatalysis (i.e., without intervention of components of the host cell) and with remarkable regiospecifity (*9*, *22*). The domain of PAL responsible for modification is still unknown. Equally unknown is the mechanism of the dehydration of serine 202. The processing of our S202C mutant to fully active PAL shows that instead of water also hydrogen sulfide can be eliminated, revealing a lack of specificity in the autocatalytic site for the leaving group. The posttranslational modification domain of HAL behaves similarly (*21*). The relatively low expression of the S202C mutant was not the result of degradation of the protein. It may lie in the unfavorable codon usage of *E. coli* for the mutated codon. Nevertheless, because of potential hairpin structures of alternative mutagenesis primers, we had to make this compromise resulting in a lower expression rate.

Basic groups of the enzyme must play a role in the biosynthesis of the prosthetic dehydroalanine and in the catalytic reaction. We therefore mutated arginine 174 and lysine 499, which are in very conserved environments in all PALs and HALs of known sequence to isoleucine. Since the posttranslational modification might be initiated by phosphorylation of serine 202, we also mutated glutamine 425 which is an environment reminiscent of the active site of serine/threonine protein kinases. While the mutants K499I and Q425I show no significant differences, mutation of arginine 174 is much more detrimental. This mutated protein

showed a significant lower V_{max} value compared to wild-type PAL. Now we had to investigate the role of arginine 174. Therefore we incubated mutant R174I with KCN, which led to the loss of the residual activity indicating that dehydroalanine is present.

Recently, Weber and Rétey (*23*) showed that in HAL an arginine residue is close to the active center. We therefore treated wild-type PAL with the arginine specific reagents, phenylglyoxal and 2,3-butandione. In both cases, time-dependent irreversible inhibition occurred which was faster with 2,3-butandione than with phenylglyoxal (Figure 2b). Higher pH values also favored the inhibition, while addition of phenylalanine partially protected PAL from inhibition with phenylglyoxal (Figure 3). Incubation with phenylglyoxal had no effect on the residual activity of mutant R174I (Figure 2a). These results indicate that arginine 174 is close to the active site in the 3D folded polypeptide chain of PAL and that arginine 174 is likely important in catalysis.

ACKNOWLEDGMENT

We thank Prof. Dr. K. Hahlbrock of MPI für Züchtungsforschung, Cologne, and Prof. Dr. N. Amrhein of ETH Zürich for the gift of the PAL gene from parsley and for PAL specific antibodies, respectively. B.L. thanks the Land Baden-Württemberg for a scholarship for graduate students.

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BI970699U