Phenylalanine Ammonia-lyase of Bamboo Shoots

Ru-Yin CHEN, Tsung-Chain CHANG and Ming-Sai LIU

Food Industry Research and Development Institute,
P.O. Box 246, Hsinchu 30099, Taiwan,
Republic of China

Received January 11, 1988

Phenylalanine ammonia-lyase (PAL) was purified from bamboo shoots (*Bambusa oldhami* Munro) through steps of DEAE-Sephacel column chromatography, Sepharose CL-6B gel filtration, and a second DEAE-Sephacel column chromatography. Polyacrylamide gel electrophoresis showed that the purified enzyme (M_r 360,000) still contained two minor bands with much slower mobilities ($M_r \geq 660,000$) than the major one. The minor band could be the aggregated forms of native PAL, based on the fact that only a single band (M_r 86,000) was observed upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis or isoelectric focusing, and all bands shared common antigenic determinants as revealed by immunoblotting done with an antiserum raised against the major band. There was a sharp rise of PAL activity of bamboo shoots after harvest, and this was only partly due to the *de novo* synthesis of this enzyme as measured by Laurell's rocket immunoelectrophoresis.

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), which catalyzes the elimination of ammonium ions from L-phenylalanine to give *trans*-cinnamic acid, was first found by Koukol and Conn¹⁾ in barley seedlings in 1961. It is generally thought that PAL acts as a switching enzyme in diverting phenylalanine from the general pool of amino acids to the biosynthesis of phenylpropanoid compounds.^{2,3)} The activity of the enzyme increases significantly in a short time in response to various stimuli, such as wounding,^{4,5)} light,⁶⁾ and ethylene,⁷⁾ and after the increase in PAL activity, a decrease follows.^{4,8)} The rate of incorporation of [¹⁴C]leucine into PAL indicates that the increase of enzyme activity is partly due to *de novo* synthesis of the enzyme in sweet potatoes.⁴⁾ Recent reports showed the presence of inactivating systems for PAL in sunflower leaves,⁹⁾ apples,¹⁰⁾ potatoes,⁸⁾ and sweet potatoes.¹¹⁾ These systems might be responsible for the subsequent decrease in the enzyme activity *in vivo*.

In bamboo shoots, PAL activity increases greatly in response to the stimulus of harvest cutting, and then declines sharply.¹²⁾ This

change of PAL activity is similar to injured sweet potatoes,⁴⁾ potatoes,⁵⁾ and asparagus.¹³⁾ In an attempt to characterize the PAL of bamboo shoots, and to study whether the post-harvest increase of PAL activity is due to enzyme synthesis, the enzyme was purified from the plant and the antiserum raised against it was used as a probe to measure PAL in harvested bamboo shoots, either immediately after cutting or at different storage times.

MATERIALS AND METHODS

Purification of PAL from bamboo shoots. All purification was done at 0~4°C. Approximately 300g of bamboo shoots (*Bambusa oldhami* Munro), which were stored at 28~30°C for one day, was cut into small pieces and homogenized in 400 ml of 50 mM imidazole-HCl buffer (pH 6.4) containing 2 mM dithiothreitol (DTT), 0.1 mM EDTA, and 10 g/dl sucrose (buffer A). After centrifugation (27,000 $\times$ g, 15 min), solid ammonium sulfate was added to the supernatant, and the precipitate formed between 25 and 50% saturation of the salt was collected. The precipitate was dissolved in 25 ml of buffer A, dialyzed against the same buffer, and put on a DEAE-Sephacel (Pharmacia) column (2.6 $\times$ 24 cm) previously equilibrated with buffer A. After washing with 400 ml of the same buffer, the column was eluted with a linear gradient of 0 to

0.4 M NaCl in buffer A (500 ml in each reservoir), with a flow rate of 20 ml/hr and 8 ml/fraction. The peak with PAL activity was pooled and concentrated by ultrafiltration. The concentrate was put onto a Sepharose CL-6B (Pharmacia) column (2.6 × 95 cm) and eluted with buffer A (flow rate 8 ml/hr). The fractions with enzyme activity were pooled, concentrated by ultrafiltration, and dialyzed against 50 mM Tris-HCl buffer (pH 7.2), containing 1 mM DTT, 0.1 mM EDTA, and 10 g/dl sucrose (buffer B). The dialysate was chromatographed on a second DEAE-Sephacel column (1.6 × 10 cm) equilibrated with buffer B, and eluted with a 0 to 0.4 M linear NaCl gradient in buffer B (150 ml in each reservoir), with a flow rate of 9.6 ml/hr and 4 ml/fraction.

Enzyme activity assay. One unit (U) of PAL is defined as the amount of enzyme catalyzing the formation of 1 μ mol of cinnamic acid per min at 40°C at the initial rate. The cinnamic acid formation was measured by the increase of OD₂₉₀.¹⁴ With the formation of each μ g of cinnamic acid in 3 ml of reaction mixture, the OD₂₉₀ will increase by 0.022. The reaction mixture (2.9 ml) contained 48 μ mol L-phenylalanine, 100 μ mol borate (Na⁺) buffer (pH 8.8), and 3–250 mU enzyme. After incubation at 40°C for 30 min, the reaction was stopped by adding 0.1 ml of 3 N HCl to the reaction mixture, and the OD₂₉₀ was measured.

Polyacrylamide gel electrophoresis. Gradient polyacrylamide gel electrophoresis (gradient PAGE) was done using Tris-glycine buffer.⁵ Polyacrylamide gradient gel (6–20%) was used for the molecular weight measurement.^{16,17} Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli.¹⁸ After electrophoresis, gels were stained with Coomassie brilliant blue and dried by the sandwich method.¹⁹ All gels used were 0.8 mm in thickness except that the gels for preparative purpose were 1.5 mm in thickness.

Preparation and testing of antibody. The purified PAL (0.165 mg) was emulsified with complete Freund's adjuvant and injected subcutaneously into several sites on the back of an adult rabbit. The animal was boosted at 2-week intervals in the same manner, except that incomplete Freund's adjuvant was used and the protein dosage was reduced to about 0.1 mg each time. Ten days after the second boosting, blood was collected from the ear vein. The immunoglobulin G (IgG) fraction was obtained from the serum by the method of DEAE-Sephacel column chromatography as described by Linn *et al.*²⁰ Ouchterlony double diffusion test,²¹ immunoelectrophoresis,²² and Laurell's rocket immunoelectrophoresis²³ were done on microscope slides coated with 1.5% agarose in barbital buffer (40 mM, pH 8.2). After double diffusion or electrophoresis, the plates were washed with phosphate-buffered saline (PBS, 0.15 M, pH 7.2), air-dried, and stained with Coomassie brilliant blue.

Isoelectric focusing (IEF). Analytical IEF was done by the method of Hudson and Hay.²³ Casting of thin-layer polyacrylamide gel (0.5 mm thickness) containing 1% Ampholine (LKB, pH range: 3.5 to 10.0), and all steps after IEF, namely fixing, staining, and preservation, were according to the LKB instruction pamphlet 1818-P.²⁴

Immunoblotting. Immunoblotting of PAL was done as previously described.²⁵ Anti-PAL IgG and horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Cappel Lab., Malven, PA, U.S.A.) were used as primary and secondary antibody, respectively. The bands were visualized by incubating the nitrocellulose papers in a DAB solution (100 ml PBS containing 5 mg of diaminobenzidine and 20 μ l of 30% H₂O₂).

The changes of PAL activity and content in the harvested bamboo shoots. Immediately after harvest, each bamboo shoot was longitudinally cut into halves. One half was stored at ambient temperature (about 28°C), and the other at 4°C. During the storage, the basal tissue of each half was removed for analysis of PAL activity and PAL content. About 5 g of the tissue was homogenized with 10 ml of buffer A, and centrifuged at 27,000 × *g* for 10 min. The supernatants were used for the measurement of PAL content by rocket immunoelectrophoresis,²³ and for the activity assay.

RESULTS

Purification of PAL

Through steps of ammonium sulfate fractionation (25–50% saturation), DEAE-Sephacel column chromatography, Sepharose CL-6B gel filtration, and second DEAE-Sephacel column chromatography, PAL was purified to apparent homogeneity. The chromatographic profiles were presented in Fig. 1. A single band (*M_r* 86,000) was observed when the purified enzyme was analyzed by SDS-PAGE (Fig. 2). However, gradient PAGE showed, in addition to a major band (*M_r* 360,000), two minor bands with much slower mobility (*M_r* ≥ 660,000) (Fig. 3, Lanes 2 and 3). The minor bands were considered to be aggregated forms of the major band, as a similar gradient PAGE pattern was observed when the major band was electrophoretically eluted from polyacrylamide gel and reelectrophoresed (Fig. 3, Lane 3). Furthermore, the major and minor bands had common antigenic determinant(s) as revealed by immunoblotting (Fig. 3, Lanes 5 and 6).

FIG. 1. Elution Patterns of the Different Chromatographic Steps of PAL Purification from Bamboo Shoots.
 A. First DEAE-Sephacel column chromatogram of 25~50% ammonium sulfate precipitate of crude extract from bamboo shoot.
 B. Sepharose CL-6B column chromatogram.
 C. Second DEAE-Sephacel column chromatogram.
 OD₂₈₀ (●); PAL activity (×); NaCl gradient (-----).

using the antiserum produced from the electrophoretically eluted PAL as an antigen. Minor bands in Lanes 5 and 6 (Fig. 3) might be due to trace impurities present in the purified PAL for immunization, therefore minor bands were visualized in the immunoblot because of amplification effect of immunoblotting. Non-specific faint bands in Lanes 8 and 9 (Fig. 3) was perhaps due to a low anti-PAL activity present in the preimmune serum, because PAL is normally present in higher plants and perhaps in the rabbit feed.

Since Ouchterlony double diffusion and im-

muno-electrophoresis showed single precipitin bands (Fig. 4), the high molecular weight aggregates of PAL (Fig. 3, Lanes 2 and 3) seems to depend on the buffer systems used.

Isoelectric point

The isoelectric point of PAL was pH 4.7 in a thin-layer (0.5 mm) polyacrylamide gel (pH range: 3.5~10.0).

Postharvest changes of PAL activity and PAL content

During storage at 28°C, the PAL activity of

FIG. 2. SDS-PAGE (Acrylamide; 7.5%) of Purified PAL.

Lane 1, low molecular weight calibration markers for electrophoresis; Lane 2, high molecular weight calibration markers for electrophoresis; Lanes 3 and 4, purified PAL (3.9 and 5.3 μ g, respectively).

bamboo shoots increased several-fold one day after harvest, then decreased on the second day (Fig. 5). The increase in the enzyme activity was accompanied by a concurrent increase in the PAL content, as revealed by rocket immunoelectrophoresis. So it is concluded that the increase of PAL activity in harvested bamboo shoots is to some extent due to the *de novo* synthesis of this enzyme. However, the increases in the enzyme activity and enzyme content were not parallel. In this study, during the first day, the PAL activity was found to increase more than ten-fold, however only about 50% increase in the enzyme content was observed (Fig. 5). Both enzyme activity and enzyme content also increased during storage at 4°C for two days. Again, the disproportion in the increases of enzyme activity and enzyme content was evident (Fig. 5). Because the increase in PAL content was not proportional to the increase in enzyme activity, it is possible that activation rather than *de novo* synthesis is mainly responsible for the rise of PAL activity.

DISCUSSION

During the purification of PAL from bam-

FIG. 3. Gradient PAGE (Acrylamide; 6 to 20%) of Purified PAL and Immunoblotting.

Lanes 1 to 3, Coomassie brilliant blue stain; Lanes 4 to 9, after electrophoresis, proteins were blotted onto nitrocellulose paper, and then probed with PAL antibody (0.45 μ g/ml) (Lanes 4 to 6), or preimmune rabbit serum (Lanes 7 to 9); Lanes 1, 4 and 7, high molecular weight calibration markers for electrophoresis (Pharmacia); Lanes 2, 5 and 8, PAL purified from the 2nd DEAE-Sephacel column chromatography (each contained 3.7 μ g enzyme); Lanes 3, 6, 9, PAL electrophoretically eluted from the main band of Lane 2 (each contained 3.8 μ g enzyme).

FIG. 4. Ouchterlony Double Diffusion (A) and Immunoelectrophoresis (B) of Purified PAL.

(A): The central well 1. contained 0.9 µg of purified PAL and the surrounding wells 2., 3., 4., 5. and 6. contained 16, 8, 4, 2, and 1 µg of anti-PAL IgG respectively, and well 7. contained 0.1 mg of preimmune rabbit serum protein.

B: Well 8. contained 0.9 µg of purified PAL. After electrophoresis, anti-PAL antibody were added to the upper (0.92 mg/ml) and lower (0.69 mg/ml) troughs.

FIG. 5. The Changes of PAL Content and Activity of Bamboo Shoots during Storage at 28°C or 4°C.

Six bamboo shoots were longitudinally cut into halves. Six of them were stored at ambient temperature (28°C), and the other six halves stored at 4°C. At the indicated times, the basal parts of each halves were removed, sliced into small pieces, and assayed for PAL activity and content. Vertical bars denote S.E. of the mean for six separate measurements. PAL content (—); PAL activity (---); bamboo shoots stored at 28°C (○) or 4°C (●).

boo shoots, the enzyme gradually lost its activity. The reason for the instability is not known. Havir²⁷⁾ showed that the use of a DEAE-Sephadex column could separate some saccharides from PAL, and this apparently affects the enzyme activity. Kishore²⁸⁾ suggested the use of polyhydric alcohols and polyethylene glycol (M_r 400) to increase the PAL

stability.

The native molecular weight and subunit molecular weight of bamboo shoot PAL were 360,000 and 86,000, respectively. Obviously the bamboo PAL was a tetramer. These are very close to the previous reports for parsley (M_r 330,000, subunit M_r 83,000),²⁹⁾ sweet potato (M_r 285,000 ~ 320,000, subunit M_r 80,000),³⁰⁾ tulip anthers (M_r 320,000, subunit M_r 83,000),³¹⁾ and carrot (M_r 340,000, subunit M_r 84,000).³²⁾ Schopfer³³⁾ showed that the enzyme from mustard is unstable in a dilute Tris buffer and aggregates to a form of molecular weight greater than 600,000. Therefore, the minor bands of Lanes 2 and 3 in Fig. 3 might be the aggregates of native PAL. This is supported by the results of SDS-PAGE (Fig. 2), isoelectric focusing (in both cases single band was observed) and immunoblotting (Fig. 3).

Although there were increases in both PAL content and activity, no evident increase in phenylpropanoid compounds was found in the basal part of stored bamboo shoots.²⁶⁾ On the contrary, even though large amounts of phenylpropanoid compounds were found in the tip part of stored shoots, no significant increase in PAL activity was observed.²⁶⁾ Therefore, the relationship between PAL activity (or content)

and phenylpropanoids remains unknown.

The changes of PAL activity of postharvest bamboo shoots was very similar to that of the enzyme in cut-injured sweet potatoes,⁴⁾ potatoes,⁵⁾ and asparagus.¹³⁾ The increase in PAL activity in harvested bamboo shoots is partly due to the *de novo* synthesis of this enzyme as shown in Fig. 5. The *de novo* synthesis of PAL was also demonstrated in sweet potatoes.⁴⁾ However, the large disproportion of the increases in the enzyme activity and enzyme content could not be explained now. This suggests that activation of the enzyme may contribute greatly to the increase in the enzyme activity.

REFERENCES

- 1) J. Koukol and E. E. Conn, *J. Biol. Chem.*, **236**, 2692 (1961).
- 2) E. L. Camm and G. H. N. Towers, *Phytochemistry*, **12**, 961 (1973).
- 3) K. Hahlbrock and H. Grisebach, *Ann. Rev. Plant Physiol.*, **30**, 105 (1979).
- 4) Y. Tanaka and I. Uritani, *Eur. J. Biochem.*, **73**, 255 (1977).
- 5) H. Hyodo, *J. Biochem.*, **79**, 277 (1976).
- 6) M. Zucker, *Ann. Rev. Plant Physiol.*, **23**, 133 (1972).
- 7) K. Matsushita and I. Uritani, *Plant Cell Physiol.*, **16**, 203 (1975).
- 8) M. Zucker, *Plant Physiol.*, **43**, 365 (1968).
- 9) L. L. Creasy, *Phytochemistry*, **15**, 673 (1976).
- 10) S. C. Tan, *J. Am. Soc. Hort. Sci.*, **104**, 581 (1979).
- 11) Y. Tanaka, K. Matsushita and I. Uritani, *Plant Cell Physiol.*, **18**, 1209 (1977).
- 12) M. S. Liu, R. Y. Chen and S. S. Tzeng, Research Report No. 348, Food Industry Research and Development Institute, Hsinchu, Taiwan, Republic of China, 1984.
- 13) L. D. Goldstein, P. H. Jennings and H. V. Marsh, Jr., *Plant Cell Physiol.*, **12**, 657 (1971).
- 14) E. A. Havir and K. R. Hanson, *Biochemistry*, **7**, 1897 (1968).
- 15) J. M. Clark, Jr. and R. L. Switzer, "Experimental Biochemistry," 2nd Ed., Freeman and Company, San Francisco, 1979, pp. 43 ~ 56.
- 16) J. Margolis and K. G. Kendrick, *Anal. Biochem.*, **25**, 347 (1968).
- 17) G. G. Slater, *Anal. Chem.*, **41**, 1039 (1969).
- 18) U. K. Laemmli, *Nature (London)*, **227**, 680 (1970).
- 19) R. H. Juang, Y. D. Chang, H. Y. Sung and J. C. Su, *Anal. Biochem.*, **141**, 348 (1984).
- 20) T. G. Linn, A. L. Greenleaf, R. G. Shorestein and R. Losick, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 1865 (1973).
- 21) O. Ouchterlony, *Acta Pathol. Microbiol. Scand.*, **26**, 507 (1949).
- 22) L. Hudson and F. C. Hay, "Practical Immunology," 2nd Ed., Blackwell Scientific Publications, London, 1980, pp. 122 ~ 131, 184 ~ 187.
- 23) C. B. Laurell, *Anal. Biochem.*, **15**, 45 (1966).
- 24) Instruction for High-Performance Analytical Electrophoresis in 0.5 mm Thin-Layer Polyacrylamide Gels, 1818-p, LKB, Bromma, Sweden.
- 25) T. C. Chang, S. C. Lee and J. C. Su, *Agric. Biol. Chem.*, **51**, 187 (1987).
- 26) R. Y. Chen, J. S. Yang and M. S. Liu, Research Report No. 294, Food Industry Research and Development Institute, Hsinchu, Taiwan, Republic of China (1983).
- 27) E. A. Havir, *Plant Sci. Lett.*, **16**, 297 (1979).
- 28) G. M. Kishore, European Pat. Appl. Ep 0136996 A2 (1985).
- 29) A. Zimmermann and K. Hahlbrock, *Arch. Biochem. Biophys.*, **166**, 54 (1975).
- 30) Y. Tanaka and I. Uritani, *J. Biochem.*, **81**, 963 (1977).
- 31) B. Kehrel and R. Wiermann, *Planta*, **163**, 183 (1985).
- 32) W. Noé and H. U. Seitz, *Planta*, **154**, 454 (1982).
- 33) P. Schopfer, *Planta*, **99**, 339 (1971).