Role of Phenylalanine Deaminase and Tyrase in the Lignification of Bamboo

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Received January 27, 1966

A pronounced increase of the activity of phenylalanine deaminase and tyrase during the lignification of bamboo shoots was observed. With progressing maturation from the basal part to the upper part of immature bamboos the pattern of the activity of the enzymes moved toward the upper part of the tissues, where the most active lignification was taking place, and the amount of cinnamic acid derivatives in the tissues was found to be in good accordance with the activity of the enzymes. L-Phenylalanine-G-¹⁴C and L-tyrosine-G-¹⁴C were both well incorporated into the lignin of bamboos. These results indicate that phenylalanine deaminase and tyrase are synthesized progressively just before the lignification of the bamboos and by the enzymes L-phenylalanine and L-tyrosine are deaminated to cinnamic acid derivatives and incorporated into lignin.

Since the finding of phenylalanine deaminase (phenylalanine ammonia-lyase) and tyrase (tyrosine ammonia-lyase) by Koukol and Conn,¹⁾ and Neish,²⁾ respectively, several papers on the possible role of the enzymes in phenol metabolism have been reported. Yoshida and Shimokoriyama³⁾ found that phenylalanine deaminase activity develops in parallel with the lignification in stems of a buckwheat plant. Minamikawa and Uritani^{4~6)} showed that both phenylalanine deaminase and tyrase increase markedly in sweet potato root in response to wounding or infection by a certain pathogenic fungus and the increase of the enzymes are closely related to the biosynthesis of polyphenols in the tissues.

Zucker⁷⁾ also indicated an inductive synthesis of phenylalanine deaminase in culture of the sliced potato tuber, and that the activity of the enzyme is proportional to the initial rate of chlorogenic acid synthesis in the tissue.

In the authors previous papers^{8~9)} it was established that phenylalanine deaminase is found in several tissues cultured in vitro and in the cambial tissues of young stems of many conifers and broadleaved trees, and that the enzyme should be responsible for the lignification of those tissues.

The present paper provides evidence that phenylalanine deaminase and tyrase are both synthesized progressively during the lignification of bamboo shoots and the enzymes participate in the lignification of the bamboo.

MATERIALS AND METHODS

Samples

Bamboo shoots (length, 0.14 to 1.20 m) and the

1) J. Koukol and E. E. Conn, *J. Biol. Chem.*, **236**, 2692 (1961).

2) A. C. Neish, *Phytochem.*, **1**, 1 (1961).

3) S. Yoshida and M. Shimokoriyama, *Bot. Mag. (Tokyo)*, **78**, 14 (1965).

4) T. Minamikawa and I. Uritani, *Arch. Biochem. Biophys.*, **108**, 573 (1964).

5) T. Minamikawa and I. Uritani, *J. Biochem.*, **57**, 678 (1965).

6) T. Minamikawa and I. Uritani, *This Journal*, **29**, 1021 (1965).

7) M. Zucker, *Plant physiol.*, **40**, 779 (1965).

8) T. Higuchi and F. Barnoud, *Symp. Intern. Chimie, Biochimie, Lignine, Cellulose, Hemicelluloses*, p. 255 (1964).

9) T. Higuchi and F. Barnoud, *J. Jap. Wood Res. Soc.*, **12**, 36 (1966).

stems of immature bamboo (length, 4.0 to 8.8 m) of Mōso (*Phyllostachys pubescens*) and Madake (*Phyllostachys reticulata*) were collected from the Experimental Farm of Gifu University. They were cut into several portions in proportion to the length and to the lignification degree of the samples. The sheath was peeled off and 20 g of the tissue in each portion was cut into small pieces.

Extraction of Phenylalanine Deaminase and Tyrase

Following the procedure of Koukol and Conn,¹⁾ the enzymes were extracted from the acetone powder which was prepared from 20 g of small pieces of the tissues of bamboo shoots or immature bamboo stems.

The tissue was ground in a high speed homogenizer with 200 ml of cold acetone (-18°C), and the homogenate was filtered by suction. The residue was washed with cold acetone twice and spread to dry on a filter paper in a draft chamber. The dry fibrous powder was extracted for 1 hour with cold 0.1 M borate buffer (pH 8.8) in a refrigerator. The extract was squeezed through cheese cloth and clarified by centrifugation. Solid ammonium sulfate was added into the extract little by little with stirring to 70% saturation at 3°C . The precipitated enzyme was collected by centrifugation and dissolved in a small amount of the same buffer and dialyzed against 0.05 M borate buffer (pH 8.8). The dialyzed enzyme solution contained both phenylalanine deaminase and tyrase.

Standard Assay of the Enzymes

Two ml of 0.1% L-phenylalanine or L-tyrosine in 0.1 M borate buffer (pH 8.8), 1 ml of enzyme solution and 2 ml of distilled water were added in a test tube, and the reaction mixture was shaken for 3 hours at 30°C in a Taiyo incubator type M 1. The reaction was stopped by adding 0.4 ml of 3 N hydrochloric acid and the reaction mixture was extracted twice with each 5 ml of ether, and ether was evaporated under air stream. The residue was dissolved in a suitable amount of 0.05 N sodium hydroxide, and the optical density of the solutions at $268\text{ m}\mu$ (for *trans*-cinnamic acid) or $333\text{ m}\mu$ (for *trans*-*p*-coumaric acid) was measured. The amount of *trans*-cinnamic acid or *trans*-*p*-coumaric acid formed was calculated from the molar extinction coefficient of the respective acids. No formation of the acids occurred when boiled enzymes were used as well as the enzymes or substrates were omitted from the reactions. The unit of the activity was defined as the amount of

enzyme catalyzing the formation of $1\text{ }\mu\text{g}$ of *trans*-cinnamic or *p*-coumaric acid per hour under standard assay condition.

Determination and Isolation of Phenolic Acids

Respective portions of a immature bamboo stem of Mōso were homogenized in hot ethanol, the homogenate was filtered, and the residue was washed with hot 80% ethanol. The filtrate and washings were combined, concentrated almost to dryness in a rotary evaporator, and the residue was dissolved in a suitable amount of hot water and filtered. The water-soluble fraction thus obtained was acidified and continuously extracted for 10 hours with ether. The ether-soluble fraction was separated into acid- and acid-free fractions by extraction of the ether solution with 5% sodium bicarbonate, and after acidification of the bicarbonate solution the acid fraction was re-extracted with ether. The ethereal acid solution was divided into two parts of the same volume and one part was used for the determination, and the other part was used for the identification and isolation of phenolic acids. For determination, ether was evaporated and the residue was dissolved in a suitable amount of 0.05 N sodium hydroxide and the amount of *trans*-cinnamic and *p*-coumaric acids were determined spectrophotometrically as described above. For identification the ether extract was subjected to paper chromatography in 1% acetic acid (v/v) and in the organic layer of toluene-acetic acid-water (4 : 1 : 5, v/v), respectively. The spots of phenolic acids were detected with the aid of a diazotized *p*-nitroaniline spray or ultraviolet light. The corresponding spots of *p*-coumaric and ferulic acids on the chromatogram irrigated with toluene-acetic acid-water were extracted with hot acetone,¹⁰⁾ and the acids thus obtained were further purified successively by paper chromatography in the organic layer of *n*-butanol-0.8 M ammonium carbonate-1.5 N ammonium hydroxide (2 : 1 : 1, v/v). The purified acids were dissolved in a suitable amount of 0.05 N sodium hydroxide and their ultraviolet spectra were measured by a Hitachi-Perkin Elmer 139 UV-VIS spectrophotometer.

Lignin Determination

The cell wall residue of acetone powder which the enzymes had been extracted was extracted with ethanol-benzene (1 : 1, v/v) for 10 hours and hot water for 4 hours, successively and lignin was determined by Klason method.

10) T. Higuchi and S. A. Brown, *Can. J. Biochem. Physiol.*, **41**, 621 (1963).

Nitrobenzene Oxidation

The extractive-free cell wall residue prepared previously was subjected into alkaline nitrobenzene oxidation. Cell wall residue (1 g), 8% sodium hydroxide solution (10 ml) and nitrobenzene (1 ml) were added in a small stainless tube vessel and oxidized at 160°C for 3 hours. The reaction mixtures were filtered and washed with ether. The washed alkaline solution was acidified to pH 3 with hydrochloric acid and the lignin degradation products were extracted with ether. Ether was evaporated and the residues were dissolved in 1 ml of acetone and analyzed by gas-liquid chromatography using a column of Apiezone N on Fluoropak.¹¹⁾

Administration of Labeled L-Phenylalanine and L-Tyrosine

L-Phenylalanine-G-¹⁴C and L-tyrosine-G-¹⁴C were purchased from the Radiochemical Center Amersham, Buckinghamshire, England. The labeled compounds (5 μ C) were administered as aqueous solutions through the cut ends of the young branches about 40 cm in length of Mōso and Madake and the plants were allowed to metabolize 24 hours in the light.

Ethanolysis of the Fed Plants

The fed plants were cut into about 1 cm pieces and extracted with boiled 80% ethanol. The extracted plant materials were dried and pulverized. The powder thus obtained was extracted with ethanol-benzene (1:1, v/v) and hot water, successively. The extracted powder was subjected into ethanolysis. Cell wall residue (10 g) and 150 ml of ethanol containing 3% hydrogen chloride were added in an Erlenmeyer flask and refluxed for 48 hours. The reaction mixtures were filtered and concentrated into a small volume (about 15 ml) and the concentrated ethanol solution was added dropwise into 100 ml of water to precipitate lignin. The lignin was filtered and dissolved in a small amount of ethanol and treated again in the same way. The filtrates in both cases were combined, concentrated and extracted with ether for 48 hours. The ethanolysis oil thus extracted was oxidized to diketones with ferric chloride in ethanol, and the ferric chloride was removed as ferric hydroxide with ammonia. The diketones produced were re-extracted with ether, and after evaporation of ether, the mixtures of diketones were dissolved in hot water and they were converted into their nickel glyoxime salts by refluxing with hydroxylamine

hydrochloride and nickel chloride in the presence of sodium acetate. The glyoxime salts obtained were hydrolysed with sulfuric acid and the free diketones were extracted with ether. Vanilloyl acetyl and syringoyl acetyl in the extract were isolated and purified by paper chromatography in the organic layer of ligroin-chloroform-methanol-water (7:2:1:5, v/v) as a solvent and the extracted diketones from the chromatogram were further purified by a small column (6 mm $\times$ 60 mm) of silicic acid (Mallinckrodt Chemical Works) using isopropyl ether saturated with water.¹²⁾

Measurement of Radioactivity

The radioactivity of the compounds was measured by a gas flow counter Tracerlab.

RESULTS AND DISCUSSION

Distributions of Phenylalanine Deaminase and Tyrase in Bamboo Shoots

The ultraviolet absorption spectrum and

FIG. 1. Effect of Enzyme Concentration on the Rate of Reaction.

- Enzymes from the basal part of bamboo shoot (length, 0.36 m) of *P. pubescens*.
- × Enzymes from the upper part of the same bamboo shoot.
- Cinnamic acid
- p-Coumaric acid

11) J. M. Pepper, M. Manolopoulou, and R. Burton, *Can. J. Chem.*, **40**, 1976 (1962).

12) T. Higuchi and I. Kawamura, *Holzforsch.*, **20**, 16 (1966).

R_F value of the products by phenylalanine deaminase and tyrase were compared with those of authentic *trans*-cinnamic and *trans*-*p*-coumaric acids, respectively, and these were completely identical with those of the respective acids.^{2,9)}

Fig. 1 shows that the formation of cinnamic and *p*-coumaric acids completely depend on the amount of enzyme solution used.

Typical examples of the activity of phenylalanine deaminase and tyrase of different portions of the bamboo shoots of Mōso (length, 0.90 m) and Madake (length, 1.0 m) are shown in Figs. 2 and 3, respectively. In both bamboo shoots the activity of the enzymes was proportional to the maturation degree of the plants and the activity was highest in the basal portion, rapidly decreasing toward the upper portion of the shoots.

The activity of tyrase was about one third of that of phenylalanine deaminase and the pattern of the enzyme activity was similar to

FIG. 2. Activity of Phenyl Alanine Deaminase and Tyrase of Different Portions of Bamboo Shoot (length, 0.90 m) of *P. pubescens*.

x Phenyl alanine deaminase.
● Tyrase.

FIG. 3. Activity of Phenyl Alanine Deaminase and Tyrase of Different Portions of Bamboo Shoot (length, 1.0 m) of *P. reticulata*.

x Phenyl alanine deaminase.
● Tyrase.

that of phenylalanine deaminase in both species of bamboo shoot.

The upper part of the bamboo shoots gave weak positive reactions for phloroglucinol-hydrochloric acid and Mäule reagent in only spiral vessels of the apex and then the reactions extended around the other types of vessels and sieve tubes in lower portions. In the middle and basal portions the reactions became stronger in the vessels and sieve tubes but the reactions were still negative in the surrounding parenchyma tissues.

Distributions of Phenylalanine Deaminase, Tyrase, Cinnamic acids and Lignin in Immature Bamboo

In further experiments young immature bamboo stems of Mōso (length, 4.0 m) were cut into 7 portions and the activity of the enzymes in the respective portions were compared. The results are shown in Fig. 4. The activity of phenylalanine deaminase and tyrase increased progressively from the top of the stem to lower portions, but at the basal portion, where the tissue was quite hard, the

FIG. 4. Activity of Phenyl Alanine Deaminase and Tyrase of Different Portions of Immature Bamboo Stem (length, 4.0 m) of *P. pubescens*.

× Phenyl alanine deaminase.
● Tyrase.

FIG. 6. Activity of Phenyl Alanine Deaminase and Tyrase of Different Portion of Immature Bamboo Stem (length, 6.6 m) of *P. reticulata*.

× Phenyl alanine deaminase.
● Tyrase.

FIG. 5. Activity of Enzymes and Amount of Cinnamic Acids and Lignin of Different Portions of Immature Bamboo Stem (length, 8.8 m) of *P. pubescens*.

—×— Phenyl alanine deaminase.
—●— Tyrase.
---x--- Cinnamic acid.
---●--- p-Coumaric acid.
—▲— Lignin.

vessels gave strong lignin reactions and the parenchyma tissues also gave weak reactions, the enzyme activity decreased.

Figs. 5 and 6 show the movement of the localisation of the activity of phenylalanine deaminase and tyrase as well as the pattern of the amount of cinnamic acids and lignin depending on the progressing maturation from the basal part to the upper part of immature bamboo stems.

In the youngest shoot the activity increased from the top to the basal portion progressively, whereas the enzyme activity of the more matured bamboo stem (length, 4.0 m) increased progressively from the top to lower portions but at the basal portion where the lignification began in the parenchyma tissue the activity decreased. In the most matured bamboo (length, 8.8 m, branches are beginning to develop and lower two third of the stem is quite hard and lignified) the activity increased from the top to the adjacent portions but the activity decreased at the part of one third of

the stem length where the tissue is quite hard and parenchyma is lignified. In other words, the pattern of the enzyme activity moves toward the upper part of the tissue, where the lignification is taking place most actively, during maturation of bamboo.

The pattern of the amount of cinnamic acids in the respective portions was found to be in good accordance with that of the activity of the enzymes, and *p*-coumaric and ferulic acids were identified by paper chromatography and by ultraviolet absorption spectrum.¹⁰⁾ The amount of lignin gradually increased with maturation of the bamboo and the increase of lignin was likely parallel with that of the enzyme activity.

These results are consistent with a previous work¹³⁾ which showed the synthesis of phenylalanine deaminase and tyrase during germination of corn. There was no enzyme in the corn seed but in germination both enzyme were formed rapidly and followed by the formation of ether-soluble phenolic compounds, which were mainly consist of cinnamic acids, and lignin successively.

Phenylalanine deaminase and tyrase are both key enzymes in the synthesis of phenolic compounds because these enzymes irreversibly divert the phenylalanine and tyrosine in the protein metabolism into the synthesis of phenolic compounds. In young meristematic tissues the protein metabolism is active but in the cesation of cell division cell wall synthesis takes place with cell growth. In the cell wall synthesis polysaccharides formation precedes and after the completion of the synthesis lignification begins at the corner of primary cell wall.¹⁴⁾ During this stage phenylalanine deaminase and tyrase should be formed increasingly and the phenylalanine and tyrosine which have been incorporated into protein begins to deaminate to cinnamic acids as precursor of phenolic compounds

TABLE I. MOLECULAR RATIO OF AROMATIC ALDEHYDES FROM LIGNIN IN NITROBENZENE OXIDATION OF AN IMMATURE BAMBOO STEM OF *P. pubescens*

The part sample taken (Distance from top) (m)	Molecular ratio of aldehydes		
	Vanillin	Syring- aldehyde	<i>p</i> -Hydroxy- benzaldehyde
2	1	0.51	1.40
3	1	0.62	1.10
4	1	0.67	0.95
7	1	0.73	0.90
8	1	0.77	0.91

including lignin.

In Table I the ratio of aromatic aldehydes in the alkaline nitrobenzene oxidation of the respective portions of the immature bamboo stem (length, 8.8m) of Mōso is given. The results are consistent with an earlier work¹⁵⁾ which showed that *p*-hydroxybenzaldehyde is predominant and the amount of vanillin and syringaldehyde is quite low in the upper portion, whereas in the lower portions the amount of vanillin and syringaldehyde increases in comparison to the amount of *p*-hydroxybenzaldehyde and that in the most matured tissue syringaldehyde is predominant.

In a separate experiment,¹⁶⁾ the present author found that a considerable part of *p*-hydroxybenzaldehyde formed in the alkaline nitrobenzene oxidation of grasses is originated from the *p*-coumaric acid connected with lignin by an ester linkage. Considering that the activity of tyrase is very strong in grasses as compared to that in dicotyledones the *p*-coumaric acid connected with lignin should be formed by the catalysis of tyrase.

Incorporation of L-Phenylalanine-G-¹⁴C and L-Tyrosine-G-¹⁴C into Lignin

The results of the incorporation of L-phenylalanine-G-¹⁴C and L-tyrosine-G-¹⁴C into the lignins of Mōso and Madake are shown in Table II. The incorporation of both com-

13) F. Lefevre, *Thèse Doctorat, 3^e cycle. Université de Grenoble, France* (1964).

14) A.B. Wardrop and D.E. Bland, *Proc. 4th Intern. Congr. Biochem.*, II, 92 (1958).

15) T. Higuchi, I. Kawamura, I. Morimoto and T. Kanda, *J. Jap. Forest. Soc.*, **36**, 101 (1954).

16) T. Higuchi and I. Kawamura, Unpublished work.

pounds into vanilloyl acetyl and syringoyl acetyl obtained in the ethanolysis of the fed

TABLE II. INCORPORATION OF L-PHENYLALANINE-G-¹⁴C AND L-TYROSINE-G-¹⁴C INTO LIGNINS OF BAMBOO STEMS

Species of plant	Compound	Dose, $\mu\text{M/g}$ fresh wt.	Specific activity of diketones from lignin, $\mu\text{C/mm}$		Dilution	
			VA*	SA**	VA	SA
<i>P. pubescens</i>	{P ^a	3.5	1.10	1.30	319	270
	{T ^b	3.6	0.80	0.93	381	328
<i>P. reticulata</i>	{P	3.5	1.25	1.21	280	290
	{T	3.6	0.95	1.16	321	263

* VA; vanilloyl acetyl. ** SA; syringoyl acetyl.

^a P; L-phenylalanine-G-¹⁴C (351 $\mu\text{C/mm}$), ^b T; L-tyrosine-G-¹⁴C (305 $\mu\text{C/mm}$).

plants was quite good, and as expected the conversion of L-tyrosine to the diketones was about as good as L-phenylalanine as a precursor of bamboo lignin.¹⁷⁾

Thus the results of present experiment conclusively show that phenylalanine deaminase and tyrase are both synthesized progressively in parallel with the lignification of bamboo and by the enzymes L-phenylalanine and L-tyrosine are deaminated to cinnamic acids and incorporated into lignin.

Acknowledgement. The technical assistance of Mr. K. Kojima is gratefully acknowledged.

17) S. A. Brown and A. C. Neish, *Can. J. Biochem. Physiol.*, **34**, 769 (1956).