Phytochemistry, 1970, Vol. 9, pp. 1 to 4. Pergamon Press. Printed in England.

METABOLISM OF *p*-COUMARIC ACID DURING LIGNIFICATION OF A BAMBOO

MIKIO SHIMADA, TŌRU YAMAZAKI and TAKAYOSHI HIGUCHI

Division of Lignin Chemistry, Wood Research Institute,
Kyoto University, Uji, Kyoto, Japan

(*Received* 25 *March* 1969)

Abstract—Occurrence of cinnamic acid-4-hydroxylase in the shoot of bamboos, asparagus, gingko and in seedlings of Japanese cedar was found by using their sliced tissues. Cell-free extraction of the hydroxylase was tentatively achieved with asparagus shoot. *p*-Coumaric acid increased drastically in content from the top toward the lower parts of bamboo shoots, whereas ferulic acid did not increase so markedly as compared with *p*-coumaric acid. The activity of cinnamic acid hydroxylase also showed a tendency to increase along with the growth of a bamboo.

INTRODUCTION

IT HAS been well established that *p*-coumaric acid can serve as a precursor for lignins and flavonoids in higher plants.[1,2] Tracer and enzymic experiments suggested that *p*-coumaric acid should be derived from phenylalanine via cinnamic acid.[3] Subsequently Nair and Vining,[4] using spinach extracts, demonstrated that cinnamic acid was hydroxylated *in vitro* at the 4-position to form *p*-coumaric acid. Russell and Conn[5] confirmed recently that cinnamic acid-4-hydroxylase was located in a microsomal fraction of a pea seedling homogenate. Zenk,[6] using sliced plant tissue, showed that the reaction pattern of this hydroxylation of cinnamic acid belongs to one of the "NIH shift" reactions, which had been proposed by Guroff *et al.*[7]

The present paper deals with the occurrence of cinnamic acid hydroxylase in higher plants and describes the extraction of the enzyme from asparagus. Accumulation of free *p*-coumaric acid in growing bamboo shoot and changes in activity of cinnamic acid-4-hydroxylase are discussed in relation to the lignification of bamboos.

RESULTS

The occurrence of cinnamic acid hydroxylase was surveyed in shoots of bamboo, asparagus, ginkgo and poplar and in seedlings of Japanese cedar.

Sliced tissues of a young bamboo shoot (*Phyllostachys pubescens*) were shown to convert supplied cinnamic acid-2-^{14}C to *p*-coumaric acid, the latter being readily detected by scanning chromatograms of ethanolic extracts. After paper chromatography, the *p*-coumaric acid

[1] S. A. BROWN and A. C. NEISH, *Can. J. Biochem. Physiol.* **33**, 948 (1955).
[2] A. C. NEISH, in *Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), p. 295, Academic Press, London and New York (1964).
[3] D. R. MCCALLA and A. C. NEISH, *Can. J. Biochem. Physiol.* **37**, 537 (1959).
[4] P. M. NAIR and L. C. VINING, *Phytochem.* **4**, 161 (1965).
[5] D. W. RUSSELL and E. E. CONN, *Archs Biochem. Biophys.* **122**, 257 (1967).
[6] M. H. ZENK, *Z. Pflanzen Physiol.* **57**, 477 (1967).
[7] G. GUROFF, J. W. DALY, D. JERINA, J. RENSON, B. WITKOP and S. UDENFRIEND, *Science* **157**, 1524 (1967).

formed could be detected under an u.v. lamp or by spraying with diazotized sulfanilic acid. The location of the coloured spot on the chromatogram corresponded completely to the area of radioactivity. The same results were obtained with other plant materials except tissue from poplar tree.

Attempts were made to extract the hydroxylases from the plant tissues but no active enzymes were obtained except from asparagus. Cell-free extracts from asparagus shoot were found to convert cinnamic acid-2-^{14}C to *p*-coumaric acid-2-^{14}C in the presence of tetrahydrofolate and $NADPH_2$. However, purification of the enzyme and detailed examination of its cofactor requirements have not yet been successful.

The possible occurrence of phenylalanine hydroxylase[8] was also examined using labeled phenylalanine with sliced tissue of shoots of bamboos and asparagus, but no tyrosine was detected by autoradiography.

The pool size of free cinnamic acids in growing bamboos was surveyed in relation to turnover numbers of the enzymes concerned with their metabolism. The shoots only contained *p*-coumaric and ferulic acids; no cinnamic, caffeic, 5-hydroxyferulic or sinapic acids could be detected. Tables 1 and 2 show the variation in content of free *p*-coumaric and ferulic acids in bamboos with growth. Although the amount of ferulic acid did not increase markedly, that of *p*-coumaric acid increased greatly from the apical part toward the lower part of the shoot.

TABLE 1. VARIATIONS IN THE CONTENT OF FREE *p*-COUMARIC AND FERULIC ACIDS IN BAMBOO (*Phyllostachys pubescens*) WITH GROWTH

Distance from the top (cm)	*p*-Coumaric acid (μg/10 g fresh weight)	Ferulic acid (μg/10 g fresh weight)
0– 20	2·6	0·2
80– 90	2·9	1·1
140–150	10·6	3·1
190–200	55·6	3·8
260–270	248·8	4·5
340–350	398·7	1·7

TABLE 2. VARIATIONS IN CONTENT OF FREE *p*-COUMARIC AND FERULIC ACIDS WITH GROWTH OF A BAMBOO (*Phyllostachys reticulata*)

Distance from the top (cm)	*p*-Coumaric acid (μg/10 g fresh weight)	Ferulic acid (μg/10 g fresh weight)
0– 10	2·7	2·0
40– 50	5·0	2·1
90–100	55·5	3·9
120–130	152·8	5·2
170–180	116·8	5·5
210–220	161·6	7·3

[8] P. M. NAIR and L. C. VINING, *Phytochem.* **4**, 401 (1964).

Since the preparation of a cell-free extract of cinnamic acid hydroxylase from bamboo proved to be difficult, sliced tissue was employed for the assay of enzyme activity. The changes in the activity of cinnamic acid hydroxylase with the growth of a bamboo shoot were then examined. The net amount of *p*-coumaric acid formed by slices from the apical part of the shoot was low and increased markedly toward the lower parts.

DISCUSSION

Previous papers have dealt with the changes in activities of the enzymes participating in lignin biosynthesis of bamboo.[9–14] However, there is still some uncertainty about the regulatory role of the metabolism of the hydroxycinnamic acids during the lignification process. It seems probable that the activity of cinnamic acid-4-hydroxylase may be quite important as it is the first hydroxylation step occurring on aromatic ring, and the distribution of the enzyme is probably widespread. Distribution of phenylalanine hydroxylase, on the other hand, seems to be more restricted in the plant kingdom, although Nair and Vining[15, 16] detected it in spinach extracts.[8]

El-Basyouni *et al.*[15, 16] suggested that the actual pool of hydroxycinnamic acids involved in the biosynthesis of lignin occurred in the form of esters combined with some enzyme proteins. However, free *p*-coumaric acid and to a lesser extent ferulic acid was found to accumulate in growing bamboo shoots whereas cinnamic acid, caffeic, 5-hydroxyferulic and sinapic acids could not be detected using conventional methods. Although a comprehensive interpretation cannot be made at this point, differences in the accumulation patterns of cinnamic acids are presumably related to the turnover number of each enzyme participating the successive transformation reactions, the unextractable chemical forms or to the instability of the cinnamic acids. *p*-Coumaric and ferulic acids, for example, are far more stable than 5-hydroxyferulic and sinapic acids. The accumulation of *p*-coumaric acid may possibly be due to the abundant accumulation of tyrosine and to an increased amount of tyrosine ammonia-lyase as well as to cinnamic acid hydroxylase during growth. As previously reported, however, *p*-coumaric acid seems to be formed more readily from phenylalanine via cinnamic acid than from tyrosine.[14]

In any case, a large amount of free *p*-coumaric acid may perform a role as a pool from which the other various hydroxycinnamic acids and their esters are formed.[17] A considerable amount of *p*-coumaric acid is found esterified in grass lignins including bamboo milled wood lignin[18] whereas the amount of ferulic acid in grass lignin is very much smaller.

Previous papers reported the occurrence of phenylalanine- and tyrosine ammonia-lyases[9] and *O*-methyltransferase[13] in growing bamboo and the increase in the amount of these enzymes paralleling the progress of lignification.

The fact that the cinnamic acid hydroxylase also tends to increase in activity is quite in accordance with the results previously obtained. It is presumed therefore that cinnamic

[9] T. HIGUCHI, *Agric. Biol. Chem.* **30**, 667 (1966).
[10] T. HIGUCHI and M. SHIMADA, *Plant Cell Physiol.* **8**, 61 (1967).
[11] T. HIGUCHI and M. SHIMADA, *Plant Cell Physiol.* **8**, 71 (1967).
[12] T. HIGUCHI and M. SHIMADA, *Agric. Biol. Chem.* **31**, 1179 (1967).
[13] T. HIGUCHI, M. SHIMADA and H. OHASHI, *Agric. Biol. Chem.* **31**, 1459 (1967).
[14] T. HIGUCHI and M. SHIMADA, *Phytochem.* **8**, 1185 (1969).
[15] SAID, Z. EL-BASYOUNI, A. C. NEISH and G. H. N. TOWERS, *Phytochem.* **3**, 627 (1964).
[16] SAID, Z. EL-BASYOUNI and A. C. NEISH, *Phytochem.* **5**, 683 (1966).
[17] E. C. BATE-SMITH, in *Wood, Extractives* (edited by W. E. HILLIS), p. 136, Academic Press, New York (1962).
[18] T. HIGUCHI, Y. ITO, M. SHIMADA and I. KAWAMURA, *Phytochem.* **6**, 1551 (1967).

acid hydroxylase and other related enzymes might perform an important role in the production of precursors of lignin in higher plants. However, further systematic investigations of the overall biochemical reactions involved in lignin biosynthesis are needed to elucidate the biochemical control of lignification.

EXPERIMENTAL

Bamboos (*Phyllostachys pubescens* and *Phyllostachys reticulata*) used for this experiment were taken from the bamboo grove of the experimental farm of Gifu University. Asparagus (*Asparagus officinalis*) was grown in the field near Gifu University.

Cinnamic acid-2-^{14}C (77·0 μc/mM) was synthesized from malonic acid-2-^{14}C and benzaldehyde according to the method of Neish.[19] Tetrahydrofolic acid was prepared by hydrogenation of folic acid in the presence of PtO_2 and H_2 according to the method of Blakley.[20] G-6-P dehydrogenase was purchased from Sigma Chemical Company.

Determination of Free p-*Coumaric and Ferulic Acids in Bamboo Shoots*

Young bamboos, 2·5–3·6 m in height, were cut and 10 g of fresh tissue taken from six different parts of the shoot, and homogenized with the Ultraturax homogenizer with about 50 ml of hot 80% EtOH. The homogenate was filtered through a celite-bedded glass filter and washed with ethanol. The concentrate was dissolved in 40 ml of hot H_2O, again filtered and the filter washed with a small amount of saturated $NaHCO_3$. The filtrate and washings were extracted with Et_2O and, after acidifying the aqueous solution with 10% HCl, it was extracted continuously for 16 hr. The ether extract, after washing with water, was dried (Na_2SO_4) and evaporated to dryness. The concentrate was dissolved in a suitable amount of 50% EtOH and chromatographed in toluene–acetic acid–water (4:1:5, v/v) until the ferulic acid had moved to about 80% of the chromatogram. The spots corresponding to *p*-coumaric acid and ferulic acid were marked under an u.v. lamp, cut out and eluted with 95% EtOH and the eluate evaporated to dryness. The concentrate was rechromatographed quantitatively in the same way. After rechromatography, *p*-coumaric and ferulic acids were eluted with 5 ml of 95% ethanol at 50° for 20 min and the absorbance determined at 310 nm and 323 nm for *p*-coumaric and ferulic acids, respectively.

Examination of Cinnamic Acid Hydroxylase Activity in Plants

Shoots of bamboo and asparagus, young branches of gingko (*Gingko biloba*) and the poplar (*Populus nigra*) and seedlings of Japanese cedar (*Cryptomeria japonica*) were used.

Excised fresh tissue (5 g) was cut into small pieces (*ca.* 2 mm cubes) and cinnamic acid-2-^{14}C (0·5 mg, 0·26 μc) in 1·0 ml H_2O was infiltrated. The tissue was allowed to stand at room temperature for 4 hr and then homogenized in 50 ml 95% EtOH. The extract was chromatographed using chloroform–acetic acid–water (2:1:1, organic layer). Radioactive *p*-coumaric acid was detected on the chromatogram with a chromatogram scanner.

Pattern of Hydroxylating Activity at Different Stages of Bamboo Growth

From each of five different parts of the bamboo shoot (*P. pubescens*) 2 m in height, 5 g of tissue was cut out, sliced and incubated with 0·5 mg of the labeled cinnamic acid at 25° for 5 hr. Net synthesis of *p*-coumaric acid was determined above.

Hydroxylase Assay with Cell-Free Extracts

Asparagus shoots (20 g) cooled to 10° were homogenized with 10 ml of 1 M phosphate buffer (pH 7·5) containing 2·0 g of Polyclar AT in a mortar at 0–5°. The homogenate was squeezed through gauze. After centrifuging the filtrate at 10000 × *g* for 10 min, the supernatant solution obtained was used as a crude enzyme preparation.

Reaction mixture contained 0·1 ml of cinnamic acid-2-^{14}C (0·3 μmole, 0·03 μc), 0·2 ml of tetrahydrofolic acid (1·0 μmole), 0·1 ml of NADP (5·0 μmoles), 0·1 ml of G-6-P (10·0 μmoles), G-6-P dehydrogenase (1·6 units) and 2·0 ml of the enzyme solution. The reaction mixture was incubated at 30° for 2 hr with shaking. The reaction was stopped by the addition of 0·5 ml of 10% HCl and *p*-coumaric acid was extracted with ether, and the ether fraction submitted to paper chromatography using toluene–acetic acid–water (4:1:5, upper layer). *p*-Coumaric acid was detected under an u.v. lamp and by spraying with diazotized sulfanilic acid reagent, in parallel with radioautography.

Acknowledgement—The authors are indebted to Dr. W. D. Loomis for kindly supplying us with Polyclar AT.

[19] A. C. NEISH, *Can. J. Biochem. Physiol.* **87**, 1431 (1959).
[20] R. L. BLAKLEY, *Biochem. J.* **65**, 331 (1957).