Decarboxylative Conversion of Hydroxycinnamic Acids to Hydroxystyrenes by *Polyporus circinata*

By H. G. BAYNE, B. J. FINKLE AND R. E. LUNDIN

*Western Regional Research Center, Agricultural Research Service,
U.S. Department of Agriculture, Berkeley, California 94710, U.S.A.*

(Received 25 November 1975; revised 9 March 1976)

INTRODUCTION

Higher plants synthesize a large number of aromatic acids that often occur as aromatic alcohol derivatives (phenolic acids) or as esters. Many of these compounds are resistant to biodegradation. They are, however, metabolized by various micro-organisms, including members of the family Polyporaceae which can decompose the lignin of woody plants.

We examined the ability of the white rot fungus *Polyporus circinata* to utilize 3,4-dihydroxycinnamate as a sole carbon source. In the course of our investigations, it became apparent that an early degradative step was a non-oxidative decarboxylation to hydroxystyrene. The bacterium *Aerobacter aerogenes* is known to degrade hydroxycinnamic acids in a similar manner, but such a reaction has not been reported previously for mycelial fungi or higher organisms.

METHODS

Polyporus circinata NRRL2903 was obtained from the Northern Regional Research Center, Peoria, Illinois, U.S.A. The culture was grown and maintained on Sabouraud Maltose Broth containing 2 % (w/v) yeast extract. For large scale cell production, several 48 h cultures grown at 28 °C on a rotary shaker were inoculated into six Fernbach flasks each containing 350 ml broth. They were incubated for an additional 48 h on a reciprocal shaker (100 strokes/min) washed with 6 l sterile water, and then introduced into two flasks, each containing 500 ml salts-substrate medium. This medium contained (per l): NaCl, 1 g; NH₄Cl, 1 g; MgSO₄·7H₂O, 0.05 g; MnSO₄, 0.001 g; FeCl₃·6H₂O, 0.01 g; CaCl₂, 0.005 g; 1.0 M-*cis,cis,cis,cis*-1,2,3,4-cyclopentane tetracarboxylic acid (CIS) buffer pH 5.0, 200 ml; 0.2 % (w/v) 3,4-dihydroxycinnamic acid in 0.05 M-potassium phosphate pH 6.3, 200 ml. The cells were incubated for 0.75 to 1.5 h, washed, weighed and frozen until used. The yield was usually about 300 g wet weight of cells from six flasks. Thawed cells were used in all experiments reported here.

For the decarboxylation reaction, a suspension of 160 g cells in 0.02 M-CIS buffer pH 5.2 (160 ml) was inoculated into 0.2 % (w/v) 4-hydroxycinnamic acid or 3,4-dihydroxycinnamic acid (480 ml), 1.0 M-CIS buffer pH 5.0 (160 ml) and salts medium (160 ml), then incubated at 28 °C on a reciprocal shaker. At intervals samples were removed, filtered and the filtrates diluted 1:100 in 0.05 M-potassium phosphate buffer pH 6.3 for spectrophotometric analysis. The mixture remaining after completion of a reaction was filtered through Whatman no. 5 paper and frozen; and the acidified filtrate was extracted with ether, concentrated, and stored at -30 °C.

Cell-free extracts were prepared from a 25 % (w/v) cell suspension in 0.02 M-CIS buffer pH 5.2 employing a Manton-Gaulin APV Industrial Homogenizer. Centrifugation was at

27000g for 40 min. The crude supernatant obtained was fractionated by precipitation in 20, 40, 60 or 80 % saturated ammonium sulphate solution. Ammonium sulphate fractionation was followed by dialysis for 18 h in 0.02 M-CIS buffer pH 5.2.

RESULTS

Incubation of whole cells of *P. circinata* with 4-hydroxycinnamic acid for 5 h produced a compound having an absorption peak at 257 nm. Paper chromatography demonstrated the presence of a reaction product showing a dark blue fluorescence. Its R_F values relative to 4-hydroxycinnamic acid in four solvents, and also its extinction coefficient at 258 nm, conformed to those previously reported for 4-hydroxystyrene (Finkle *et al.*, 1962). The product was extracted and recrystallized according to the procedures of Dale & Hennis (1958), and showed the characteristic reactions of 4-hydroxystyrene: m.p., 70 to 71 °C; grey-green colour with 1 % $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$; cornflower blue colour with concentrated HCl; yellow-orange colour with concentrated H_2SO_4 , later turning red. Identification of the crystalline product was confirmed by nuclear magnetic resonance spectrometry. It had a proton magnetic resonance spectrum (100 MHz, dry $[\text{}^2\text{H}_6]\text{acetone}$) as follows: $\delta = 8.34$ (s, 1 H, aromatic OH); 6.66 (dd, 1 H, $J = 17, 10$ Hz,); 5.03 (dd, 1 H, $J = 10, 1$ Hz,); 5.58 (dd, 1 H, $J = 17, 1$ Hz,); 7.30 ($\frac{1}{2}$ AA'BB', 2 H, *meta* to OH); 6.81 ($\frac{1}{2}$ AA'BB', 2 H, *ortho* to OH). Except for a shift in the position of the hydroxyl resonance that is often observed, all shielding values agree with those for 4-hydroxystyrene reported by Finkle *et al.* (1962).

The incubation of whole cells with 3,4-dihydroxycinnamic acid for 5.5 h yielded a product having an absorption peak and extinction coefficient at 258 nm characteristic of 3,4-dihydroxystyrene (Finkle *et al.*, 1962). Precipitates from cell-free extracts 80 % saturated with ammonium sulphate were also able to degrade 3,4-dihydroxycinnamic acid, yielding a product with an absorption peak at about 258 nm. If the ammonium sulphate fraction was boiled for 10 min, degradation of 3,4-dihydroxycinnamic acid or 4-hydroxycinnamic acid no longer occurred. Protocatechuic and cinnamic acids, catechol, dihydroxyphenylalanine and dihydroxyphenylethylamine were inactive as substrates. Refractionation with ammonium sulphate demonstrated 3,4-dihydroxycinnamic decarboxylase activity in the 40 to 60 % saturated ammonium sulphate fraction (pH optimum, 6.1). At higher ammonium sulphate concentrations (70 to 80 % saturated), the fractions were devoid of decarboxylase activity but readily attacked chlorogenic acid, the quinic ester of 3,4-dihydroxycinnamic acid, to give a spectrum with peaks near 287 nm and 312 nm. These are characteristic of 3,4-dihydroxycinnamic acid, presumably formed by hydrolysis (Lewis & Thompson, 1961).

DISCUSSION

This paper describes the biological decarboxylation of hydroxycinnamic acids to hydroxystyrenes by a mould, or extracts of a mould. When placed in contact with the substrate, whole cells of *P. circinata* that had been grown in the absence of substrate, rapidly degraded 3,4-dihydroxycinnamic acid, strongly suggesting that the decarboxylase is constitutive. The reaction promoted by whole cells and cell-free extracts resembled that of the bacterium

Aerobacter aerogenes in decarboxylating hydroxycinnamic acids. Similar conversion reactions have been observed for other bacteria (Finkle *et al.*, 1962; Indahl & Scheline, 1968), for yeasts (Chen & Pepler, 1956; Manitto, Gramatica & Ranzi, 1975), and in the cooking and alcoholic fermentation of grain (Steinke & Paulson, 1964).

Fungal decarboxylation of *trans*-cinnamic acid to styrene was recorded for *Aspergillus niger* by Herzog & Ripke (1908) and for *Penicillium* sp. by Jaminet (1950). Decarboxylation of hydroxycinnamic acids by fungi other than yeasts has not previously been reported. This decarboxylation and complementary reactions such as oxidations, polymerizations and demethylations are assumed to take place in the formation of soil humic acids (Finkle, 1965) through the activity of associations of micro-organisms that decompose the lignin of woody material.

The authors acknowledge with appreciation the assistance of Mr Noel Hurd, University of California, Berkeley, for cooperative use of the Manton-Gaulin Homogenizer.

REFERENCES

- CHEN, S. L. & PEPLER, H. J. (1956). Conversion of cinnamaldehyde to styrene by a yeast mutant. *Journal of Biological Chemistry* **221**, 101–106.
- DALE, W. J. & HENNIS, H. E. (1958). Substituted styrenes. III. The synthesis and some chemical properties of vinylphenols. *Journal of the American Chemical Society* **80**, 3645–3649.
- FINKLE, B. J. (1965). Soil humic acid as a hydroxypolystyrene: a biochemical hypothesis. *Nature, London* **207**, 604–605.
- FINKLE, B. J., LEWIS, J. C., CORSE, J. W. & LUNDIN, R. E. (1962). Enzyme reactions with phenolic compounds: formation of hydroxystyrenes through the decarboxylation of 4-hydroxycinnamic acids by *Aerobacter*. *Journal of Biological Chemistry* **237**, 2926–2931.
- HERZOG, R. O. & RIPKE, O. (1908). Conversion of cinnamic acid into styrene by molds. *Zeitschrift für physiologische Chemie* **57**, 43–45.
- INDAHL, S. R. & SCHELIN, R. R. (1968). Decarboxylation of 4-hydroxycinnamic acids by *Bacillus* strains isolated from rat intestine. *Applied Microbiology* **16**, 667.
- JAMINET, F. (1950). Hydrocarbonic fermentation in a total syrup mixture. *Journal of Pharmacology, Belgium* **5**, 191–201.
- LEWIS, J. C. & THOMPSON, P. A. (1961). Microbiological production of chlorogenicase. *Applied Microbiology* **9**, 205–208.
- MANITTO, P., GRAMATICA, P. & RANZI, B. M. (1975). Stereochemistry of the decarboxylation of phenolic cinnamic acids by *Saccharomyces cerevisiae*. *Journal of the Chemical Society, Chemical Communications* 442–443.
- STEINKE, R. D. & PAULSON, M. C. (1964). The production of steam-volatile phenols during the cooking and alcoholic fermentation of grain. *Journal of Agriculture and Food Chemistry* **12**, 381–387.