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METABOLISM OF AROMATIC ACIDS BY *POLYPORUS HISPIDUS*

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Key Word Index—*Polyporus hispidus*; (Polyporaceae); phenylalanine metabolism; hydroxycinnamic acids; ammonia lyases; aromatic hydroxylases.

Abstract—When grown on glucose as principal carbon source the culture medium of *Polyporus hispidus* was found to contain phenolic acids, including *p*-coumaric and caffeic acids. ^{14}C-Studies indicated that phenylalanine is converted to cinnamic acid as well as to phenylpyruvic acid and tyrosine in cultures. Cell-free preparations of mycelium contained phenylalanine and tyrosine ammonia-lyse activities and were capable of effecting the hydroxylation of cinnamic, *p*-coumaric and benzoic acids.

INTRODUCTION

THE SPOROPHORES of *Polyporus hispidus*, a bracket fungus parasitic primarily on a variety of deciduous trees including *Fraxinus* and *Quercus*[1] contain hispidin (6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone).[2,3] Because styrylpyrones appear to be synthesized from cinnamic acid derivatives, and because *Polyporus hispidus* causes a white rot characteristic of strong lignin decomposition, we believe that an examination of aromatic acid metabolism in this organism bears investigating.

The metabolism of aromatic amino acids by Basidiomycetes has been examined a number of times,[4–7] and this subject has been included in a recent review.[8] Many Basidiomycetes have been shown to possess phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activity,[9,10] and the products are ultimately oxidized to carbon dioxide. However, some Basidiomycetes produce a variety of cinnamic acid derivatives,[11] possibly as end-products of metabolism, and as an extension of these studies, an examination of *P. hispidus* was undertaken. A preliminary report of our findings has already been presented.[12]

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RESULTS

DL-Phenylalanine-3-^{14}C was incubated with 2- and 3-week-old cultures of *Polyporus hispidus* for 24 hr. If the cultures were incubated on a 0·5% L-phenylalanine replacement solution for 48 hr prior to the radioactive feeding, the hydroxycinnamic acids detectable on chromatograms of extracts of the culture medium showed no incorporation of label. Radioactivity was present, however, in phenylpyruvic, phenyllactic and *p*-hydroxybenzoic acids as well as cinnamic and benzoic acids. The latter two acids were incompletely resolved on chromatograms. Smaller amounts of aromatic acids were detected in 3-week-old cultures than in 2-week-old cultures, and in the former, radioactivity was also incorporated into *p*-hydroxyphenylacetic, protocatechuic and homoprotocatechuic acids. When a cold phenylalanine replacement solution was not employed prior to administration of tracer, radioautography showed good incorporation of label into *p*-coumaric and caffeiç acids from radioactive phenylalanine. The degradative products of phenylalanine that were detected with replacement solution studies were also found to be radioactive. Although it was probable that *p*-coumaric acid was arising from the hydroxylation of cinnamate, the possibility that it could be formed via tyrosine was also examined. Recovery of the tyrosine from the pool of free amino acids in the medium after feeding 2-week-old cultures DL-phenylalanine-3-^{14}C for 24 hr showed that radioactivity was present in trace amounts in this compound.

TABLE 1. METABOLIC PRODUCTS OF VARIOUS AROMATIC ACIDS DETECTED IN THE CULTURE MEDIUM OF *Polyporus hispidus*

Compound fed	Growth stage (days)	Radioactivity (dpm) in: *p*-Coumaric acid	Caffeic acid	*p*-Hydroxybenzoic acid	*p*-Hydroxyphenyl acetic acid	Other labelled compounds
Phenylalanine-3-^{14}C	11	1000 (8%)	0	2800 (22%)	0	Phenyllactic and phenylpyruvic acids (20%)
	14	2700 (9%)	200 (1%)	1700 (7%)	1000 (5%)	Protocatechuic acid trace at 14 and 20 days
	20	2600 (9%)	1600 (6%)	1300 (5%)	2600 (9%)	Homoprotocatechuic acid 4·5% at 20 days
Tyrosine-3-^{14}C	14	1600 (24%)	500 (7%)	1000 (15%)	1200 (18%)	Homoprotocatechuic and *p*-hydroxyphenylpyruvic acids (20%)
Cinnamic acid-2-^{14}C	10	24 500 (67%)	1300 (4%)	0	0	Protocatechuic acid
	14	45 700 (82%)	1400 (3%)	0	0	14 days trace
	18	4400 (15%)	12 700 (42%)	0	0	18 days 5%
P-Coumaric acid-2-^{14}C	14	48 800 (80%)	3700 (6%)	0	0	Protocatechuic acid 5700 (10%)
Phenylacetic acid-2-^{14}C	21	0	0	7000 (1%)	510 000 (73%)	Protocatechuic acid 5000 (0·7%)
Benzoic acid-ring^{14}C (U)	14	0	0	21 600 (30%)	0	Protocatechuic acid 2100 (3%)

Percentages refer to the portion of radioactivity detected on the chromatogram that was present in that compound.

DL-Phenylalanine-3-^{14}C, DL-tyrosine-3-^{14}C, cinnamate-2-^{14}C, *p*-coumarate-2-^{14}C, phenylacetate-2-^{14}C and benzoate-ring^{14}C (U) were fed to cultures of various ages and the radioactivity in the metabolites was detected by radioautography and the activities determined with scintillation counting (Table 1). When labelled benzoic acid was fed to the cultures, 5% of the administered radioactivity was recovered in carbon dioxide after 6 hr.

Cell-free preparations demonstrating enzyme activities related to the metabolism of aromatic acids were obtained. These preparations contained phenylalanine and tyrosine ammonia-lyase activities and were capable of effecting the hydroxylation of cinnamic, *p*-coumaric and benzoic acids. The maximum activity of PAL (E.C. 4.1.1.5) occurred near

the end of the logarithmic phase of growth. Benzoic and cinnamic acid 4-hydroxylase activities were found in the same enzyme preparations. Benzoic acid was hydroxylated much more readily (12% conversion) than was cinnamic acid (2% conversion).

DISCUSSION

Two pathways of phenylalanine degradation, similar to those found in *Schizophyllum commune*,[4] appear to be operative in *Polyporus hispidus*. Radioactive cinnamic and benzoic acids were always detected when cultures were incubated with DL-phenylalanine-3-^{14}C. Although phenyllactate and phenylpyruvate were not completely separated by PC, the presence of label in both compounds after phenylalanine feedings was readily determined by radioautography. Most phenolic compounds were easily distinguished on chromatograms, and the incorporation of radioactivity into *p*-hydroxyphenylacetic, *p*-hydroxybenzoic and protocatechuic acids from radioactive phenylalanine was observed. Thus the degradative pathway via cinnamate, benzoate and protocatechuate, and a pathway via phenylpyruvate and phenylacetate appear to be present. However, in *P. hispidus* we have found no evidence for the *ortho*-hydroxylation of phenylacetic acid, nor for the formation of *o*-hydroxyphenylacetate from phenylpyruvate.

Although *Penicillium chrysogenum*[13] and other micro-organisms[8] will readily hydroxylate administered phenylacetic acid in the *para* position, it has not been suggested that this represents a natural degradative route. On the other hand, formation of *o*-hydroxyphenylacetic acid directly from phenylpyruvate, and subsequent conversion to homogentisate has been postulated as a natural sequence in the degradation of phenylalanine in a number of fungi.[8]

In feeding experiments with *P. hispidus*, radioactive *o*-hydroxyphenylacetic acid was never detected, while *p*-hydroxyphenylacetate became radioactive after incubation with phenylalanine, tyrosine and phenylacetic acid. Phenylacetic acid-2-^{14}C was further metabolized to *p*-hydroxybenzoate and protocatechuate suggesting that this might be a natural degradative sequence in this organism. When 3-week-old cultures were incubated with labelled tyrosine, radioactive homoprotocatechuic acid was also detected. While this compound could serve as a suitable substrate for ring fission as has been shown in *Tilletiopsis*,[14] further degradation to protocatechuic acid prior to ring cleavage could also occur.

Tyrosine appeared to be degraded in a similar manner to phenylalanine, i.e. *via* the ammonia-lyase and *via* oxidative deamination or transamination. Although *p*-hydroxyphenylpyruvic acid was not detected, it is a likely intermediate in the formation of *p*-hydroxyphenylacetic acid, which showed good incorporation of label from tyrosine-3-^{14}C. Radioactive *p*-hydroxymandelic acid was also detected in trace amounts of chromatograms of medium extracts after tyrosine and phenylalanine feedings. *p*-Hydroxymandelic acid might be an intermediate in the formation of *p*-hydroxybenzoic and protocatechuic acids.

In addition to being degraded to the corresponding benzoic acid derivative, cinnamic acid and *p*-coumaric acid were readily hydroxylated in cultures of the fungus, In fungi, this activity is apparently restricted to a few Basidiomycetes.[11] The potential of these hydroxycinnamic acids as styrylpyrone precursors or as subunits for a sporophore-toughening polymer in this organism is very important. While no direct evidence of the composition of molecules responsible for the woodiness of various Basidiomycetes has been found, it has been suggested that cinnamic acid derivatives might be partly responsible, as they are in the

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lignin of higher plants.[11,15] The importance of these compounds in styrylpyrone synthesis will be discussed in a subsequent paper.

Maximum PAL activity was recorded for 14-day-old cultures which compares favourably with the observed conversion of phenylalanine to cinnamic acid *in vivo* (Table 1). However, *P. hispidus* differs from *Rhodotorula glutinus* and *Sporobolomyces roseus* in the time of maximum PAL activity. While the other organisms showed maximum PAL activity as the cultures entered the stationary phase of growth,[16,17] maximum PAL activity in *P. hispidus* occurs near the end of the logarithmic phase of growth.

Cultures at this growth stage were examined for other enzymes associated with aromatic acid metabolism. TAL activity was determined in a crude enzyme preparation with a 14-hr incubation period. Cinnamic acid 4-hydroxylase was prepared using the methods employed for higher plants.[18–20] As in higher plants,[19,21] a microsomal preparation gave the best activity obtained.

Cell-free preparations which effect the hydroxylation of *p*-coumaric acid were first isolated from spinach,[22] but have since been obtained from fungi[23] and *Streptomyces*.[24] In our preparations, as in those from higher plants, ascorbate functions effectively as a reducing agent. While so far only crude enzyme preparations have been obtained from cultures of *P. hispidus*, a more thorough examination of these enzymes is being undertaken in this laboratory.

EXPERIMENTAL

Culturing. The fungus (UBC culture collection No. 513) was cultured in Roux bottles containing 110 ml of medium and inoculated by pipetting 10 ml of culture homogenate into the bottle. The medium contain glucose (15 g), yeast extract (0·5 g), Soytone (Difco Laboratories) (1 g), KH_2PO_4 (0·5 g), $MgSO_4.7\,H_2O$ (0·25 g), NaCl (0·05 g) and $CaCl_2{\cdot}2\,H_2O$ (0·02 g)/l. of dis. H_2O. The homogenate was prepared by blending a 2-week-old agar culture with 250 ml of sterile dis. H_2O. The organism was maintained on MYP agar containing malt extract, 7 g; yeast extract, 0·75 g; Soytone (Difco Laboratories), 1 g and agar 15 g/l. of dis. H_2O. Agar cultures were prepared by pipetting 1 ml of the homogenate into a standard 10 cm Petri dish containing approximately 25 ml of agar.

Preparation and administration of radioactive compounds. DL-Phenylalanine-3-^{14}C (3·9 μCi/μM) and phenylacetic acid-2-^{14}C (2·9 μCi/μM) were obtained from New England Nuclear Corporation. Cinnamic Acid-2-^{14}C (20 μCi/μM) and DL-tyrosine-3-^{14}C (6·9 μCi/μM) were obtained from International Chemical and Nuclear Corporation and benzoic acid-ring ^{14}C (45 μCi/μM) was obtained from the Radiochemical Centre, Amersham, England. *p*-Coumaric acid-2-^{14}C (0·18 μCi/μM) and caffeic acid-2-^{14}C (0·16 μCi/μM) were prepared by condensation of the appropriate benzaldehyde with malonic acid-2-^{14}C in pyridine with a trace of piperidine.[25] 2 μCi of each compound was administered on the appropriate day directly into the culture medium of each Roux bottle.

Analysis for aromatic acids. After 24 hr incubation with the radioisotopes, the culture medium was removed by filtration, acidified to pH 2 with 2 N HCl and extracted with 200 ml Et_2O. The Et_2O extract was analyzed by two-dimensional PC in benzene-HOAc-H_2O (10:7:3, upper phase) in the first dimension and 2% aq. HCO_2H in the second. Spots were visualized under long wave UV light and by spraying with diazotized *p*-nitroaniline reagent[26] or with 2% ethanolic $FeCl_3$. Radioactive compounds were detected by radioautography. Unsprayed duplicate chromatograms were examined quantitatively by cutting the spots from the

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chromatograms and counting them directly by scintillation employing a Nuclear-Chicago 720 series or Unilux II liquid scintillation counting. The scintillation fluid employed consisted of 4 g 2,5-diphenyloxazole (PPO) and 30 mg *p*-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in 1 l. of toluene.

Recovery of radioactive carbon dioxide. Filtered air was passed through the culture incubated with ring-labelled benzoic acid and subsequently bubbled slowly through a 1 M methanolic solution of hyamine hydroxide. At the end of the incubation period the solution was examined by scintillation counting.

Phenylalanine ammonia-lyase. Cultures were filtered, the mycelium blotted and then extracted by grinding with a weight of aluminium oxide equal to the wet wt of mycelium, and twice this wt of 0·5 M Tris-HCl buffer pH 8·6. The slurry was centrifuged at 18 000 *g* for 10 min and 5 ml of the supernatant was passed onto a column of Sephadex G25, for removal of the low MW molecules. 5 ml of eluate was collected and aliquots were added to 1 ml of 2·5 μmol phenylalanine in buffer solution and sufficient buffer to make a total of 3 ml. These solutions were assayed spectrophotometrically at 290 nm and at 30°. Relative enzyme activities are reported with respect to the dry wt of fungus.

Tyrosine ammonia-lyase. 8 ml of the enzyme preparation employed for the 14-day PAL assay were combined with 1 μCi tyrosine-3-^{14}C and 3 drops of 5% 2-mercaptoethanol in a test tube. The mixture was incubated at room temp. for 14 hr, acidified and extracted with Et_2O. The Et_2O extract was examined by PC and radioautography.

Benzoic and cinnamic acid-4-hydroxylases. The fungus was harvested and macerated as described for PAL, employing 0·1 M phosphate buffer, pH 7·5. The slurry was centrifuged at 27 000 *g* for 10 min and the supernatant at 40 000 *g* for a further 10 min. This supernatant was then centrifuged at 105 000 *g* for 90 min to obtain a microsomal pellet. The microsomal pellet was suspended in two 2-ml aliquots of 0·1 M phosphate buffer pH 7·5. To 1-pellet suspension was added cinnamic acid-2-^{14}C, 0·3 μCi; non-labelled cinnamic acid, 0·15 mg; 2-mercaptoethanol, 0·24 mg; and 3 ml of the NADPH generating system. To the second pellet suspension was added 2 μCi labelled benzoic acid instead of the cinnamic acid. To provide NADPH for the enzymes, a mixture of 1·6 mg NADP, 1·4 mg glucose-6-phosphate and 0·4 units glucose-6-phosphate dehydrogenase in 3 ml buffer was prepared and incubated at 30° for 10 min before addition to the assay mixture. The assay mixture was incubated at 30° with agitation for 90 min. Radioactive *p*-coumaric and *p*-hydroxybenzoic acids were detected by PC.

p-*Coumaric acid hydroxylase.* 14-day-old cultures were harvested and macerated as described for PAL, employing 0·1 M citrate-phosphate buffer, pH 7·0. The crude extract was centrifuged at 10 000 *g* for 20 min and passed onto a column of Sephadex G25. Twice the volume applied to the column was collected and employed as the crude enzyme preparation. 2 ml of crude enzyme, 3 μmol *p*-coumaric acid-2-^{14}C and 3 μmol ascorbate in a total of 4 ml buffer were mixed in a test tube and incubated at 30° for 0·5 hr. At the end of the reaction time, the mixture was treated with 0·5 ml 5 N HCl and extracted with a total of 15 ml Et_2O. The Et_2O extract was examined by PC and radioautography.

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