Effect of Light on Enzymes of Phenylpropanoid Metabolism and Hispidin Biosynthesis in *Polyporus hispidus*

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The effects of light on growth, pigmentation and the activities of enzymes involved in the deamination of phenylalanine and tyrosine and in the biosynthesis of hispidin were examined in *Polyporus hispidus*. Evidence is presented for the stimulation of phenylalanine ammonia-lyase activity by light. Tyrosine ammonia-lyase activity and aminotransferase activities for phenylalanine and tyrosine were higher in the dark. Tracer studies showed that conversion of cinnamate into *p*-coumarate is enhanced by light. *p*-Coumaric acid hydroxylase, catalysing the conversion of *p*-coumarate into caffeate, could be detected only in cultures exposed to light. These results suggest that the cinnamate pathway for the metabolism of phenylalanine, leading to hispidin synthesis, is regulated by light in *P. hispidus*.

In contrast with other fungi, some Basidiomycetes have the ability to synthesize cinnamic acid and *p*-coumaric acid from phenylalanine and tyrosine respectively (see Towers, 1969). The further metabolism of cinnamic acid may lead to ring cleavage and degradation to CO₂, as has been demonstrated in *Schizophyllum commune* and *Ustilago hordei* (Moore & Towers, 1967; Moore *et al.*, 1968), or to the elaboration of more-complicated molecules. For example, species of *Polyporus* and *Gymnopilus* are able to synthesize styrylpyrones (Bu'Lock & Smith, 1961; Edwards *et al.*, 1961; Ueno *et al.*, 1964; Hatfield & Brady, 1971) and recent tracer studies (Hatfield, 1973; Perrin & Towers, 1973) indicate that hispidin [6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone] is biosynthesized from phenylalanine through a cinnamyl derivative that is combined with either acetate or malonate. Preliminary investigations in our laboratory showed that the yellow pigmentation, characteristic of hispidin production, did not develop in cultures of *Polyporus hispidus* maintained in the dark. However, 2-week-old dark-grown cultures developed colour within 24 h on exposure to fluorescent light. These observations have stimulated us to investigate the effect of light more closely and, in particular, to examine its effect on enzymes related to phenylalanine and tyrosine metabolism and hispidin biosynthesis in this fungus.

In plants the activities of a number of enzymes are effected by light and in recent years phenylalanine ammonia-lyase (EC 4.3.1.5) has received considerable attention because it is peculiarly sensitive in this respect (Zucker, 1972). In fungi, however, although there are many examples of the effects of light on morphogenesis (Carlille, 1965), there are only a few

known cases in which light has an effect on enzymes. For example, light has been shown to stimulate the succinate–oxoglutarate–isocitrate cycle as well as nucleic acid synthesis in *Blastocladiella*, an aquatic organism (Turian & Cantino, 1959; Cantino & Turian, 1961). In the present study we have found that light is involved in the regulation of the activities of certain enzymes concerned with aromatic amino acid metabolism in *P. hispidus*.

Materials and Methods

Chemicals

DL-[1-¹⁴C]Phenylalanine and L-[U-¹⁴C]tyrosine were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. [2-¹⁴C]Cinnamic acid was purchased from International Chemical and Nuclear Corp., Irvine, Calif., U.S.A. All other chemicals were the best grades available from commercial sources.

Methods

Ultraviolet spectra. Unicam SP.800 or Unicam SP.500 series 2 spectrophotometers were used for all spectroscopic measurements.

Radioactivity measurements. Radioactive spots cut out from chromatograms or suitable portions of radioactive samples from the reaction mixture were transferred to scintillation fluid [1,4-bis-(5-phenoxyoxazol-2-yl)benzene, 4 g; 2,5-diphenyloxazole, 50 mg; toluene, 1 litre] and the radioactivity was measured in a Nuclear-Chicago 720 series liquid-scintillation spectrometer.

Organism and conditions of growth. *P. hispidus*

[University of British Columbia (UBC) 513] was grown in Roux bottles on a medium containing (per litre of water): glucose, 15g; yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.) 0.5g; Bacto-soytone (Difco Laboratories), 1g; KH_2PO_4 , 0.5g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25g; NaCl , 0.05g; CaCl_2 , 0.11g. The pH of the medium was adjusted to 5.8 with 1M-NaOH. Bottles containing 100ml of the above medium were inoculated with a mycelial homogenate of the fungus grown on the same medium, each flask receiving the same amount of inoculum. Stock cultures of the fungus were maintained on agar slopes containing (per litre): malt extract, 7g; yeast extract, 0.75g; Bacto-soytone (Difco Laboratories), 1g; agar, 15g.

For enzyme extraction large numbers of cultures were maintained at 25°C in Psychrotherm incubators provided with 'daylight' fluorescent tubes. The light-grown mycelium was derived from bottles exposed to light for $\frac{1}{2}$ h/day, the dark-grown mycelium being totally unexposed to light. The mycelium was harvested by filtering through cheesecloth, washed with water and blotted between several layers of paper towels.

Preparation of cell-free extracts. Cell-free extracts of the fungus were obtained by grinding the mycelium with an equal weight of aluminium oxide (AnalaR, BDH Chemicals, Poole, Dorset, U.K.) in a chilled porcelain mortar and extracting twice with 0.1M-sodium phosphate buffer, pH 7.5 (2ml/g of mycelium). The resultant slurry was centrifuged at 15000g for 20min and the precipitate discarded. The supernatant was treated with solid $(\text{NH}_4)_2\text{SO}_4$ to 80% saturation. The precipitate formed in 45min was dissolved in a small volume of 0.1M-sodium phosphate buffer, pH 7.5. The preparation was de-salted by passage through a column of Sephadex G-25 (coarse grade) and the eluate was used as enzyme preparation.

Assay for phenylalanine and tyrosine ammonia-lyases. The reaction mixture containing 0.5ml of 0.05M-Tris-HCl buffer (pH 8.8), 0.5ml of the enzyme preparation, 5 μ mol of unlabelled substrate (L-phenylalanine or L-tyrosine) and 0.2 μ Ci of DL-[1- 14 C]phenylalanine (2.5mCi/mmol) or 0.25 μ Ci of [U- 14 C]tyrosine (50 μ Ci/ μ mol) in a final volume of 3ml was incubated at 30°C. After 1h the reaction was acidified with 0.2ml of 6M-HCl and extracted with diethyl ether. The radioactivity in the ethereal layer was determined and the amount of *p*-coumaric acid or cinnamic acid formed was determined as described by Camm & Towers (1969).

Assay for phenylalanine and tyrosine amino transferases. A modification of the assay described by Diamondstone (1966) was used. This is a fixed-time assay based on the alkali-catalysed oxidation of *p*-hydroxyphenylpyruvate to *p*-hydroxybenzaldehyde and oxalate. Our reaction mixture contained 5 μ mol of phenylalanine or tyrosine, 5 μ mol of α -oxoglutar-

ate and 0.2 μ mol of pyridoxal phosphate in a final volume of 0.9ml of 0.1M-sodium phosphate buffer, pH 7.6. The reaction was stopped by addition of 0.1ml of 10M-KOH with continuous agitation. Reaction mixtures were left at room temperature for 30min and then the extinction was read against a blank at 331 nm and the amount of product formed was calculated from a molar extinction coefficient of 19900M $^{-1}$ ·cm $^{-1}$. A unit of enzyme activity is the amount that catalyses the formation of 1 μ mol of *p*-hydroxyphenylpyruvate/min at 30°C.

Assay for cinnamic acid 4-hydroxylase. The enzyme was assayed in the microsomal fractions prepared as follows. The supernatant fraction obtained after centrifuging the mycelial homogenate at 15000g (see under 'Preparation of cell-free extracts') was further centrifuged at 105000g for 70min and the pellet, suspended in 0.1M-sodium phosphate buffer, pH 7.5, was used as microsomal preparation.

The assay system was similar to that described by Russell & Conn (1967). Microsomal preparation (1ml) was incubated with: [2- 14 C]cinnamic acid, 2 $\times$ 10 5 d.p.m. in 0.01 μ mol; *trans*-cinnamic acid, 1 μ mol; FAD, 1 μ mol; NADP $^+$, 2.5 μ mol; glucose 6-phosphate, 5.0 μ mol; glucose 6-phosphate dehydrogenase, 0.4 unit; in a total volume of 3.0ml at pH 7.5, including 1.3ml of 0.1M-sodium phosphate buffer. The reaction mixture was incubated at 30°C with shaking for 90min. After the reaction had been stopped by addition of 0.2ml of 6M-HCl, the solution was extracted with 3 $\times$ 6ml of diethyl ether. The ethereal extracts were combined, evaporated to dryness and the residue was redissolved in ethyl acetate and applied to strips of Whatman no. 1 chromatography paper together with unlabelled *p*-coumaric acid as a marker. The chromatogram was developed in benzene-acetic acid-water (10:7:3, by vol.) as solvent system and the *p*-coumaric acid was detected with long-wave u.v. light, under NH_3 vapour. The amount of radioactivity incorporated into *p*-coumaric acid was measured and was expressed as nmol of *p*-coumaric acid formed/h per mg of protein.

Assay for *p*-coumaric acid hydroxylase. The *p*-coumaric acid hydroxylase activity was measured by incubating a reaction mixture (1ml) containing citrate-phosphate buffer (Na_2HPO_4 , 40 μ mol; citric acid, 30 μ mol), pH 7.4, *p*-coumaric acid (1 μ mol), ascorbic acid (1 μ mol) and 0.3ml of enzyme at 30°C. The reaction was terminated by addition of 1ml of 0.5M-HCl at 5, 10 and 30min and the caffeic acid formed was measured by the method described by Arnou (1937) for 3,4-dihydroxyphenylalanine (dopa). One unit of hydroxylation activity is defined as the quantity that catalyses the production of 1 μ mol of caffeic acid/min under the conditions of the assay.

Experiments in vivo. Studies were carried out *in vivo* by incubating the cultures for 4h after adding 2 μ Ci of [2- 14 C]cinnamic acid to the growth medium. The

reaction was stopped by addition of 1 ml of 5M-HCl, and the mycelium and the growth medium were macerated by using a Vir-Tis tissue grinder. The homogenate was then extracted three times with 150ml of diethyl ether. The ethereal layer was evaporated to dryness after removal of traces of water with anhydrous Na_2SO_4 . The residue was dissolved in 1 ml of ethyl acetate and was subjected to two-directional chromatography on Whatman no. 3 chromatography paper, with benzene-acetic acid-water (10:7:3, by vol.) in the first direction and formic acid-water (9:1, v/v) in the second direction as solvents. The spots corresponding to *p*-coumaric acid and cinnamic acid were cut out from chromatograms and placed in scintillation vials together with 15ml of scintillation fluid for radioactivity determinations.

Mycelial dry-weight determinations. The growth medium was removed by filtering through a cheesecloth and the mycelium was dried to constant weight in an oven at 90°C. All weighings were performed at room temperature and humidity and were expressed as dry weight in grams per bottle.

Measurement of pigment production. The pigment formed in the mycelium was extracted in methanol by the method of Perrin & Towers (1973). The E_{370} of the extract was taken as a measure of the extent of pigmentation.

Protein. The method of Lowry *et al.* (1951) was used for the determination of protein. Bovine serum albumin (fraction V, Sigma Chemical Co., St. Louis, Mo., U.S.A.) was the standard.

Results

Growth and pigmentation

Mycelial dry weight of the fungus was higher when exposed to light than in the dark, the difference being more significant during the initial stages of growth (Fig. 1*a*). As depicted in Fig. 1*b*, there was no formation of pigment in the dark-grown mycelium even after 18 days of growth. When exposed to light pigment was detected in the mycelium after 10 days of growth, reaching a maximum value in 16 days (0.57 µg/mg of tissue).

Effect of light on phenylalanine and tyrosine ammonia-lyases

Fig. 2 depicts the formation and activities of phenylalanine ammonia-lyase in the fungus. The results show that non-oxidative deamination of phenylalanine to cinnamic acid was considerably higher in the light-grown mycelium. Maximum activities of phenylalanine ammonia-lyase were observed after 10 days of growth. Unlike phenylalanine ammonia-lyase, tyrosine ammonia-lyase activity was more pronounced in the cell-free extracts when the fungus was grown in the dark, exhibiting maximal activity at 14 days of growth (Fig. 3). In the light-grown mycelium activities of tyrosine ammonia-lyase were negligible.

Hydroxylation of cinnamic acid

Microsomal fractions were used for the assay of cinnamic acid 4-hydroxylase, since previous studies

Fig. 1. Effect of light on growth and pigmentation in *P. hispidus*

(a) Mycelial dry weight recorded at different days of growth in darkness (----) or with ½ h of illumination/day (—). (b) Pigmentation at different days in dark-grown (----) and light-grown (—) mycelium. Experimental details are given in the text.

Fig. 2. Effect of light on phenylalanine ammonia-lyase activity in *P. hispidus*

Phenylalanine ammonia-lyase activity was assayed in the cell-free extracts derived from dark-grown (----) and light-grown (—) mycelium at different days of growth. One unit of activity is the amount of enzyme that catalyses the formation of $1\mu\text{mol}$ of cinnamic acid from DL-phenylalanine in 1 h at 30°C . Experimental details are given in the text.

Fig. 3. Effect of light on tyrosine ammonia-lyase activity in *P. hispidus*

Tyrosine ammonia-lyase activity was assayed in the cell-free extracts derived from dark-grown (----) and light-grown (—) mycelium at different days of growth. One unit of activity is the amount of enzyme that catalyses the formation of $1\mu\text{mol}$ of *p*-coumaric acid from L-tyrosine in 1 h at 30°C . Experimental details are given in the text.

with *P. hispidus* had shown that this enzyme is localized in this fraction (C. P. Vance, A. M. D. Nambudiri & G. H. N. Towers, unpublished work). As shown in Table 1, cinnamic acid 4-hydroxylase activity in the microsomal fraction was maximal at 10 days of growth when the organism was exposed to light. In the present study, no attempts were made to determine the activities of cinnamic acid hydroxylase in the dark-grown mycelium. Feeding of $[2-^{14}\text{C}]$ -cinnamic acid *in vivo* indicated that the formation of

Table 1. Conversion of cinnamic acid into *p*-coumaric acid *in vitro* by microsomal preparations from *P. hispidus*

For details see the text.

Age of mycelium (days)	Specific activity (nmol of <i>p</i> -coumaric acid formed/h per mg of protein)
8	15.0
10	51.0
12	27.0
14	8.0
16	0.0
18	0.0

Fig. 4. Conversion of cinnamic acid into *p*-coumaric acid *in vivo* by *P. hispidus*

Dark-grown (----) and light-grown (—) cultures were incubated with $[2-^{14}\text{C}]$ cinnamic acid and the percentage incorporation of the label into *p*-coumaric acid was measured. Experimental details are given in the text.

p- $[2-^{14}\text{C}]$ coumaric acid was maximal at 12 days and the conversion was more (16.8%/h per g dry wt.) in the light-grown mycelium than in the dark-grown (5.3%/h per g dry wt.) (Fig. 4).

Hydroxylation of *p*-coumaric acid to caffeic acid

Cell-free preparations of *P. hispidus* did not exhibit any *p*-coumaric acid hydroxylase activity until the mycelium was 10 days old (Fig. 5). Preparations contained maximum specific activity when the cultures were 14 days old. Elaboration of this enzyme and the appearance of hispidin followed a similar pattern (Fig. 1b). Neither pigment nor *p*-coumaric acid hydroxylase activity was detected in mycelium grown in the dark.

Fig. 5. Effect of light on *p*-coumaric acid hydroxylase activity in *P. hispidus*

p-Coumaric acid hydroxylase activity was assayed in cell-free extracts derived from dark-grown (----) and light-grown (—) mycelium at different days of growth. Conditions of assay and other details are given in the text. One unit of enzyme activity is defined as the quantity that catalyses the production of 1 μ mol of caffeic acid/min.

Fig. 6. Effect of light on phenylalanine aminotransferase activity in *P. hispidus*

Phenylalanine aminotransferase activity was assayed in cell-free preparations derived from dark-grown (----) and light-grown (—) mycelium at different days of growth. Experimental details are given in the text. One unit of enzyme activity is the amount that catalyses the formation of 1 μ mol of phenylpyruvic acid/min.

Oxidative deamination of phenylalanine and tyrosine

Cell-free extracts of *P. hispidus* catalysed the conversion of phenylalanine and tyrosine into the corresponding oxo acids, i.e. phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid. As shown in Figs. 6 and 7, the activities of transaminases for phenylalanine and tyrosine were significantly higher in the dark-grown mycelium than in the light-grown. The activities of these enzymes decreased in the light-

Fig. 7. Effect of light on tyrosine aminotransferase activity in *P. hispidus*

Tyrosine aminotransferase was assayed in cell-free preparations derived from dark-grown (----) and light-grown (—) mycelium at different days of growth. Experimental details are given in the text. One unit of enzyme activity is the amount that catalyses the formation of 1 μ mol of *p*-hydroxyphenylpyruvic acid/min.

grown mycelium after 10 days and after 16 days no activity was detected in either dark-grown or light-grown organisms.

Discussion

This is not the first report on the stimulation by light of vegetative growth in a fungus. Other fungi such as *Blastocladiella* (Cantino & Horenstein, 1956; Cantino, 1959) and *Thraustochytrium roseum* (Goldstein, 1963) have been shown to be similarly effected. In *Blastocladiella emersonii*, a stimulatory effect of light on the internal pools of soluble protein and soluble polysaccharide and dry weight/cell during ontogeny has been demonstrated (Goldstein & Cantino, 1962).

Light has also been shown to play a role in the synthesis of specific groups of compounds in various fungi. Blue light decreased melanogenesis and tyrosinase activity in a *Neurospora* mutant (Schaffer, 1953), whereas melanin formation was stimulated by light in *Aurobasidium pullulans* (Lingappa *et al.*, 1963) and *Cladosporium mansonii* (Sussman *et al.*, 1963). Similarly, carotenoid synthesis has been shown to be stimulated by light in *Pyronema confluens* (Carlille & Friend, 1956), *Phycomyces blakesleeanus* (Garton *et al.*, 1951), *Neurospora crassa* (Zolaker, 1954) and *Dacryopinax spathularia* (Vail & Lilly, 1968).

Our studies with *Polyporus* indicate a clear-cut effect of light on aromatic metabolism. There are two known pathways in *Polyporus* for the non-protein metabolism of phenylalanine and tyrosine, namely

Scheme 1. *Phenylalanine and tyrosine metabolism in P. hispidus*

Heavy arrows represent processes stimulated by light, i.e. (1) phenylalanine ammonia-lyase activity; (2) hydroxylation of [¹⁴C]cinnamate *in vivo*; (3) *p*-coumarate acid hydroxylase; (4) hispidin production.

non-oxidative deamination leading to the cinnamic acid and ultimately to hispidin, and oxidative deamination, catalysed by aminotransferases and leading to phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid (Scheme 1). Whether the degradation of the latter acid occurs via homogentisic acid, as has been shown for certain bacteria (see Kluyver & Van Zijp, 1951; Evans, 1963; Rogoff, 1961; Treccani, 1963), is not known in *Polyporus*.

Of the four enzyme reactions involved in the deamination of these two amino acids the only one stimulated by light is that with phenylalanine ammonia-lyase. Tyrosine ammonia-lyase and aminotransferase activities for phenylalanine and tyrosine are lowered by light. Also the maximum activity for phenylalanine ammonia-lyase is exhibited at a different growth time from that of tyrosine ammonia-lyase. These differences in behaviour between

phenylalanine and tyrosine ammonia-lyases suggest that either two separate lyases for phenylalanine and tyrosine are present in *Polyporus* or that light is affecting, in some way, a single protein with activities towards both substrates. Good evidence has been presented for *Sporobolomyces* (Parkhurst & Hodgins, 1971) and for maize (Reid *et al.*, 1972) that both activities may reside in one enzyme.

A second effect of light, as shown in the tracer study, is to enhance the hydroxylation of cinnamate to *p*-coumarate. Photo-induction of cinnamic acid 4-hydroxylase along with phenylalanine ammonia-lyase has been demonstrated in buckwheat seedlings (Amrhein & Zenk, 1968, 1970, 1971) and parsley tissue culture (Hahlbrock *et al.*, 1970).

A third effect of light is to trigger the synthesis or induce the activity of a *p*-coumarate-hydroxylating system. Hydroxylation of *p*-coumaric acid is known in plants as well as micro-organisms, and enzymes catalysing the reaction have been isolated from plants (Vaughn & Butt, 1969), fungi (Sato, 1969) and *Streptomyces* (Nambudiri *et al.*, 1972). Light-stimulation of the production of this enzyme, however, was hitherto unknown. *p*-Coumarate hydroxylase activity and pigment formation are totally lacking in dark-grown cultures of *P. hispidus*. Tyrosine ammonia-lyase activity, however, was higher in the dark, but the fate of the product, i.e. *p*-coumaric acid, under these conditions is unknown. The evidence in the present paper shows that the cinnamate pathway (Scheme 1) leading from phenylalanine to hispidin and perhaps to other similar compounds is regulated by light. Whether phenylalanine and tyrosine ammonia-lyase activities are located in one enzyme or in two remains to be determined. The significance of the alternate oxo-acid pathway has not been examined.

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