The Role of Laccase in Carpophore Initiation in *Coprinus congregatus*

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The mycelium of the basidiomycete *Coprinus congregatus* is not uniformly competent to differentiate. Differentiation occurs only in localized areas of young mycelia. Studies on laccase activities from whole mycelium extracts suggest a correlation between overall laccase levels and the development of mushroom primordia. However, studies of higher resolution on the localized areas of developmental competence, under a variety of light, nutrient and temperature regimes, indicate that laccase is probably not involved in the actual development of the primordia. If laccase is involved in *C. congregatus* differentiation, it is only at the initial, light-requiring, step.

INTRODUCTION

Studies on differentiation in the basidiomycete *Coprinus congregatus* showed that only certain parts of mycelia grown on solid substrates were capable of being induced by obligatory light exposure. Only such induced areas or zones could differentiate into mushroom primordia (Ross, 1982). Because of the strict localization of zones of primordia, it was suggested that the use of whole mycelia to study differentiation in higher fungi was probably inappropriate. Yet virtually all previous studies on the induction of basidiomycete development have used whole mycelia as the source of extracts from which to fractionate the light receptor system and the developmentally active components, all with very little success to date. This is particularly true of work on phenol oxidases, which are commonly produced by fungi and have been implicated in a variety of fungal developmental systems. Clutterbuck (1972) reported that laccase appeared during conidiation in *Aspergillus nidulans*, and Law & Timberlake (1980) showed that this laccase was a developmentally regulated *de novo* gene product. Leonard (1971), Leonard & Phillips (1973) and Phillips & Leonard (1976*a*) have shown an involvement of laccase in basidiocarp formation in *Schizophyllum commune*. Wood & Goodenough (1977) and Wood (1980*a,b*) have reported a similar association in the cultivated mushroom, *Agaricus bisporus*. Several workers have reported laccase activity in developing fruiting bodies (see Leatham & Stahmann, 1981), and there are reports of the presence of laccase and tyrosinase in many mature basidiocarps (Matsubura & Iwasaki, 1972). Most mycelium-produced laccases are extracellular and can readily be found in the substrate (Wood, 1980*a*; Leonard, 1971; Froehner & Eriksson, 1974; Bocks, 1967; Leatham & Stahmann, 1981); there have been occasional reports of intracellular activity (Molitoris & Esser, 1971; Phillips & Leonard, 1976*a*). All the workers on basidiomycetes examined laccase activity as a function of mycelial age and developmental state, using whole mycelia or large amounts of substrate as the source of the laccase.

Since the differentiation of primordia in *C. congregatus* was strictly localized and preliminary work had found a phenol oxidase with laccase substrate specificity in mycelial extracts, this study was initiated to determine if laccase activities were spatially localized, and if their presence correlated with some developmental stage in *C. congregatus*. Since the phenol oxidase produced by *C. congregatus* is not secreted into the medium, this fungus is particularly useful for this type of study.

Fig. 1. Method of sampling localized growth zones from day 3 (zone III) through to day 9 (a). Note ring of primordia in zone III of untouched control culture (b) and in the same zone of sampled culture.

Table 1. *Representative laccase activities and extractable protein from L/D grown cultures*

Intracellular laccase was extracted at the times indicated from each growth zone.

Day after inoculation	Growth zone...	III	IV	V	VI	VII
Relative activity (ΔA_{590} per unit time)*						
3		0.41 $\pm$ 0.05				
4		0.46 $\pm$ 0.05	0.50 $\pm$ 0.02			
5		0.31 $\pm$ 0.02	0.45 $\pm$ 0.02	0.49 $\pm$ 0.01		
6		0.20 $\pm$ 0.03	0.39 $\pm$ 0.04	0.47 $\pm$ 0.01	0.48 $\pm$ 0.06	
7		0.12 $\pm$ 0.01	0.28 $\pm$ 0.04	0.41 $\pm$ 0.02	0.46 $\pm$ 0.03	0.45 $\pm$ 0.01
Protein [μ g (ml extract) ⁻¹]						
3		444 $\pm$ 20				
4		488 $\pm$ 45	470 $\pm$ 45			
5		617 $\pm$ 63	560 $\pm$ 52	464 $\pm$ 48		
6		573 $\pm$ 25	550 $\pm$ 8	610 $\pm$ 45	460 $\pm$ 31	
7		550 $\pm$ 8	580 $\pm$ 28	593 $\pm$ 38	593 $\pm$ 25	470 $\pm$ 65
Specific activity [units (mg protein) ⁻¹ †]						
3		150 $\pm$ 18				
4		160 $\pm$ 19	174 $\pm$ 7			
5		87 $\pm$ 3	148 $\pm$ 6	182 $\pm$ 4		
6		61 $\pm$ 8	116 $\pm$ 10	131 $\pm$ 2	178 $\pm$ 20	
7		38 $\pm$ 1	78 $\pm$ 11	105 $\pm$ 5	128 $\pm$ 8	167 $\pm$ 3

* Mean value of five replicate samples $\pm$ S.D. Activity measured as over a set period of time (see Methods).

† ΔA_{590} measured during linear portion of reaction curve. One enzyme unit equals ΔA_{590} of 0.001 min⁻¹.

METHODS

Organism and culture. Stock cultures of *C. congregatus* were maintained as described previously (Ross, 1982). The dikaryon (cc8 $\times$ cc16) was used as previously. All cultures were grown on Emerson's YpSs agar (Difco) in 100 mm diameter plastic Petri dishes containing 30 ml agar at 25 °C, unless otherwise stated. Light and dark conditions were as described previously.

Sampling method. Samples were taken from the culture at 24 h intervals from 72 h onwards using a no. 3 cork borer to cut plugs of mycelium and agar. This size was chosen because the 5.5 mm diameter of the cork borer was just smaller than the average 24 h growth increment. Each growth zone was sampled each day from the end of day 3 to the end of day 10. Even though this caused a considerable amount of agar and mycelium to be removed from each plate over a period of several days, such trauma did not affect either the growth rate or the developmental potential of the remaining mycelium (Fig. 1). Each day's growth was considered a zone and given a Roman numeral. All samples taken on a particular day were given an Arabic number corresponding to that day. All experiments were done in quintuplicate and repeated at least twice. Samples from dark-grown cultures were taken by the light of a single dissecting microscope lamp covered with a Kodak Wratten no. 58 (green) filter. All samples were removed from the Petri dishes with forceps, placed individually into 6 $\times$ 50 mm test tubes, and immediately frozen and stored at -20 °C until assayed.

Homogenization method. One major problem with the localization studies was the difficulty of obtaining quantitatively repeatable homogenization of the small samples in a rapid manner. Several methods were tried, including a Virtis Homogenizer, a Brinkman Polytron, a Parr disruption bomb, and grinding in a mortar and pestle, but all suffered from an inability to repeat homogenizations quantitatively. This was mainly due to the difficulty of disrupting the thick walls of the hyphae and of transferring the homogenized material to centrifuge tubes. A method was developed that used a combination of a high-speed drill with a special bit that just fits in 6 mm diameter tubes (to be described elsewhere). This method is rapid, simple and quantitative, as shown by the standard deviations in Table 1.

Enzyme assay. Frozen mycelium samples were thawed in 200 μ l 0.1 M-sodium phosphate buffer, pH 6.6, on ice. After 30 s homogenization, the samples were centrifuged and 50 μ l of supernatant from each sample placed in individual tubes on ice, with the addition of 1.0 ml 1.0 mM-tolidine (3,3'-dimethylbenzidine) in 0.1 M-sodium acetate buffer, pH 4.7 (Phillips & Leonard, 1976*b*; Leslie & Leonard, 1979). The tubes were incubated at 25 °C for 1 h, then at 4 °C for 15 h. After the 4 °C incubation, samples were equilibrated at room temperature for 30 min and read at 590 nm on a Beckman DB-G spectrophotometer at 28 °C, against a blank of substrate and buffer only. A change in absorbance of 0.001 min⁻¹ was defined as one unit of enzyme activity (Phillips & Leonard, 1976*a*). The enzyme activity is given as relative activity in most graphs (absorbance change per total incubation time per sample). In some experiments, specific activity was determined [units (mg protein)⁻¹] and plotted with the relative activity (Table 1). Protein was determined by the Lowry method. The protocol permitted daily accumulation of many hundreds of samples and uniform analysis of all the samples of any one experiment. Because the mycelium samples were stored at -20 °C and removed at random for assaying, the five replicates of any one sampling were rarely processed together, but the readings from the replicates correlated very well (Table 1).

RESULTS

General enzyme characteristics

The *C. congregatus* enzyme has the substrate specificity of a laccase, since it will oxidize substrates such as gallic acid, *p*-phenylenediamine, *N,N*-dimethyl-*p*-phenylenediamine, but not 3-(3,4-dihydroxyphenyl)-L-alanine (DOPA) or tyrosine. The enzyme oxidizes *o*-tolidine and guaiacol (*o*-methoxyphenol) and has other substrate specificities that do not fit exactly the definition of a laccase (*p*-diphenol: oxygen oxidoreductase, EC 1.10.3.2), as discussed for other fungal laccases by Scháněl & Esser (1971). No laccase activity has been detected in the medium in either agar or liquid-grown cultures.

Whole mycelium studies

Because correlations between laccase activity and development had all been previously made with whole mycelia, an experiment was performed using large portions of the mycelium. A wedge, representing 25% of all growth zones of the existing mycelium, was cut from the agar and macerated in a Brinkman Polytron. Buffer and mycelium volumes were kept proportional to the amounts used for the smaller samples taken with the no. 3 cork borer. The curve shown in Fig. 2 is typical of 'whole mycelium' activity, showing an overall increase in laccase activity during growth and a sudden decrease coinciding with the appearance of primordia.

Localized studies: light and dark grown cultures

If cultures were sampled as shown in Fig. 1, with each sample representing a small portion of a particular day's growth, the results revealed a more complex correlation between laccase and development. In Fig. 3, the laccase activities in each zone were plotted each day. In the cultures grown under alternating light/dark (L/D) conditions (Fig. 3*a*), there was a considerable difference in the laccase activities across a culture on any one day (reading all points with the same Arabic number). As the mycelium grew from day 3 (one sample point) to day 7 (5 sample points) the highest activity was either at the edge of the mycelium or just behind it. In general, maximum activities obtained from days 4 to 7 were approximately the same. Laccase activities declined rapidly each day after a zone had grown. Activity in zone III declined very rapidly and the rates of decline of all the zones increased after day 7 (the day on which the mycelium reached the edge of the Petri dish). Primordia formed in zone III on day 8. In dark-grown (CD) cultures (Fig. 3*b*) the laccase activity was similar to that of L/D cultures (Fig. 3*a*) through to day 7.

Fig. 2. Laccase activity in extracts of segments of mycelia covering several zones at once.

Fig. 3. Laccase activity in small samples of each growth zone taken each day from day 3 (single sample point) to day 10. The Arabic numbers on the curves indicate the day taken. The Roman numerals on the abscissa indicate growth zone sampled. (a), Cultures grown under alternating light/dark (L/D) conditions; (b), cultures grown under constant dark (CD) conditions; (c), cultures grown in dark for 6 d, given 12 h light and returned to dark (D-L₆-D).

Unlike L/D cultures, CD cultures did not display the rapid decline of laccase activity in zones V, VI and VII, as seen in L/D cultures.

The curves in Fig. 3(c) show the laccase activities of cultures dark grown for 6 d and then exposed to a single 12 h light induction (D-L₆-D). At the time of light reception, laccase activities were highest in zone VI, and primordia formed in that zone on day 8, when laccase levels were still very high. Laccase activities had been equal up to the sixth day in the CD cultures and those dark grown to day 6. After light induction, the activities in the induced cultures began to decline faster than those in the CD cultures.

Fig. 4. Effect of medium volume on laccase activity (see text for details).

Localized studies: volume of media

There is a major difference in location of zones of primordia depending on the amount of media in the Petri dishes (Ross, 1982). Laccase activities were monitored in cultures grown in 15, 30 and 45 ml agar per plate (Fig. 4a–c). Laccase activities in the 15 ml cultures were highest in zone IV on day 5 and did not reach that level in any other zone on any other day. Laccase activity was greater in 30 and 45 ml cultures than in any 15 ml culture; each successive zone showed equally high levels of enzyme activity. The cultures were maintained under L/D conditions and primordia formed in zone III of both the 30 and 45 ml cultures, but in zone IV of the 15 ml cultures. Activities of laccase in cultures grown on 15 ml agar never attained those reached in cultures grown on 45 ml agar, and reached only the lowest activities observed in cultures grown on 30 ml agar. To see if this effect was a function of the amount of nutrients or of volume (depth) of medium, cultures were grown on 30 ml half-strength YpSs. The maximum activity and overall profile of laccase in the half-strength cultures resembled those of the 15 ml cultures (Fig. 4d). Primordia formed in zones IV and V of these plates under L/D conditions.

In previous experiments where cultures were grown in 15 ml agar (Ross, 1982), primordia formed only in zone III, but in the present experiment they formed only in zone IV. The difference has been attributed to the amount of media dispensed by the pump used: there may be 2–3 ml differences between the '15 ml' amounts in the two experiments. Such differences appear to be critical when using volumes of less than 30 ml.

Localized studies: temperature effects

Only cultures grown at 25 °C are able to be light induced and to develop primordia (Ross, 1982). Cultures grown at 30 °C cannot be induced and never develop primordia, while cultures grown at 20 °C can be induced, but cannot develop primordia at that temperature. Laccase activities in such cultures are shown in Fig. 5. Cultures at 25 °C displayed the normal pattern for cultures on 30 ml full-strength YpSs under L/D conditions. Cultures at 20 °C had considerably higher laccase activities, which remained high through to day 8. The 30 °C cultures did not

Fig. 5. Effect of temperature on laccase activity. All cultures were grown under alternating light/dark (L/D) conditions. Specific activity (vertical lines) has been plotted with relative activity (vertical hatched bars) to show correlation (see Methods).

attain the laccase activities of cultures grown at 20 and 25 °C, and the decline by day 8 was substantial.

DISCUSSION

Fungal laccases appear to be developmentally regulated enzymes (Clutterbuck, 1972; Esser & Minuth, 1970; Law & Timberlake, 1980; Leonard, 1971; Leonard & Phillips, 1973; Wood, 1980*a*). It is important, therefore, to determine the exact stage or state of differentiation associated with changes in laccase activity. In the literature on basidiomycete fruiting, this association has been closely correlated with fruiting body formation. Leonard & Phillips (1973) state, 'The results indicate that phenoloxidase activity increases in colonies subsequent to the formation of aggregates of hyphae, continues to increase until mature, sporulating fruiting structures are produced, and then declines significantly.' Wood & Goodenough (1977) also reported similar changes in laccase activities correlated with basidiocarp formation, although Wood (1980*b*) disagreed with Phillips & Leonard (1976*a*) on possible causes for the laccase decline. The laccase activity profile in Fig. 2 does indeed conform to those previous concepts. Laccase activity, measured from large segments of mycelia, appears to correlate with the appearance of primordia.

Laccase activities, when examined in strictly localized areas of mycelia, do not fit such a simple picture of developmental correlation (Fig. 3). Cultures grown in L/D conditions form primordia in zone III, but such primordia do not form until at least day 8, 5 d after induction. At the time of induction, the laccase levels in zone III were high, but by the time of the first differentiation of the primordia, had fallen substantially. Yet, in dark-grown cultures illuminated on day 6, laccase levels were high in zone VI (Fig. 3) and also on day 8, when primordia did form in zone VI. Apparently the level of laccase activity may be quite variable without affecting differentiation.

There is a correlation in L/D cultures between laccase activities and edge contact. Laccase activity was highest at the periphery (the inducible zone) and remained high through to day 7, at which time all zones declined rapidly in laccase activity. Cultures grown under CD conditions presented a very similar laccase activity profile through to day 7, but remained high after EC. The act of light-induction at some previous time seems to trigger laccase decline, but only after

edge contact. In the absence of light induction (CD conditions), edge contact alone is not sufficient to cause a significant decline in laccase activity.

The implication from the results shown in Fig. 3 is that any involvement of laccase in fruiting is restricted to the initial light-requiring induction process, rather than the actual development of primordia. This conclusion is supported by the experiments conducted to determine the influence of media volume and temperature. In both, there is a correlation between high laccase activities and the competence to become induced. On 30 ml agar, cultures displayed equally high levels of laccase activity in successive growth zones III to VII. These levels correlate with the expanding zone of inducibility reported previously (Ross, 1982). On 15 ml agar, in contrast, zone IV had the highest level of laccase activity and subsequent growth zones did not attain that level. This correlates with the observation that primordia formed only in zone IV of these cultures, no matter when illuminated. In the temperature study, in which cultures grown at 30 °C could neither be induced nor differentiate, laccase levels were all lower than those of cultures grown at 20 and 25 °C, again correlating high laccase activities with the competence to be induced.

Wood & Goodenough (1977) noted the lack of proof that the laccase activity changes are directly correlated with the differentiation process and are not merely secondary events in the developmental processes. There does, however, seem to be a direct correlation between laccase, light and induction. The localization work reported earlier (Ross, 1982) implied that light induction could 'fix' the light receptor system in the inducible zone and thus prevent other, later growth zones from becoming induced. Laccase activity does not begin to decline in L/D grown cultures until after edge contact, several days after zone III has been 'fixed' as the induced zone, therefore it would appear that high laccase levels alone are not sufficient to permit induction, since none of the zones with high activities (IV–VII) can be induced once zone III has been induced.

Ross (1982) showed that the action of light stabilizes the induced zone and that it can occur at low temperatures and prior to edge contact, with no further differentiation taking place until after edge contact or a rise in temperature. This suggests that whatever is activated must have the capacity to remain in that state for some time without decay or translocation. The variable levels of laccase that can be present at the actual time of differentiation suggest that there is no critical laccase level responsible for such development. On the other hand, the constant correlation between high levels of laccase activity and light-inducibility could implicate laccase in the initial induction or triggering event of differentiation.

Investigations into the initiation and regulation of differentiation in basidiomycetes should focus on localized areas of the mycelium rather than on 'whole mycelium' observations.

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