Disordered ultrastructure in lignin-peroxidase-secreting hyphae of the white-rot fungus *Phanerochaete chrysosporium*

Laura Zacchi,1 Ian Morris2 and Patricia J. Harvey1

Author for correspondence: Patricia J. Harvey. Tel.: +44 181 331 9972. Fax: +44 181 331 8305.
e-mail: p.j.harvey@greenwich.ac.uk

INTRODUCTION

The white-rot fungus *Phanerochaete chrysosporium* is able to degrade and metabolize polymeric lignin as well as a broad range of recalcitrant organopollutants (for reviews see Gold & Alic, 1993; Reddy & D'Souza, 1994). As such, it represents a promising candidate for bioremediation purposes (Aust, 1990; Lamar et al., 1990; Holroyd & Caunt, 1997). Lignin peroxidase (LiP) is one of the most important of the haem proteins associated with the degradative enzyme pathway of the fungus. It oxidizes dimethoxylated aromatics to radical cations with redox potentials in excess of 1–4 V (Kersten et al., 1985; Bietti et al., 1998). Its value in bioremediation lies in the fact that, unlike the cytochrome-P450-type enzymes linked to xenobiotic degradation, LiP is extracellular and uses the oxidizing power of \( \text{H}_2\text{O}_2 \) to catalyse the one-electron oxidation of its substrates via redox active mediators. The most physiologically significant of the mediators investigated to date is the fungal secondary metabolite veratryl alcohol (Harvey et al., 1986; Hammel & Moen, 1991; Goodwin et al., 1995; Candeias & Harvey, 1995). Redox mediation during catalysis is reflected in the apparent non-specificity of the enzyme to its substrates and this feature lends itself to the degradation of a broad range of xenobiotics. However, commercial exploitation of the enzyme and practical applications of the fungus are limited because of the lack of knowledge about what triggers expression of the ligninolytic pathway and LiP synthesis.

In the natural environment, the fungus differentiates a thin film of mycelium to degrade lignin and metabolize cellulose. By contrast, in liquid cultures (suitable for industrial fermentation) the fungal spore inoculum develops into mycelial pellets, which are agglomerates of hyphae trapped together during germination of the spores (Gerin et al., 1993). The pellet habit represents a significant obstacle to oxygen diffusion into the hyphae (Leisola et al., 1983; Michel et al., 1992), which is essential for ligninolysis and LiP production (Kirk et al.,...
METHODS

Fungal strain and growth conditions. Phanerochaete chrysosporium, strain BKM-F-1767 (ATCC 24725), was grown in liquid culture under conditions of non-limiting nutrient nitrogen, with either glucose or cellulose (Avicel) as the carbon source at a final concentration specified in the text. The growth medium had the following composition: 20 mM NH$_4$Cl, 30 mM tartrate, 20 mM Na$_2$CO$_3$, 147 mM K$_2$PO$_4$, 2.0 mM Mg$_2$SO$_4$, 0.73 mM CaCl$_2$, 0.065 mM FeCl$_3$, 1.4 mM MgCl$_2$, 2.0 mM H$_3$PO$_4$, 64 µM nitrotriacetic acid, 1.5 mM veratryl alcohol, trace metals and an initial pH of 4.5. All chemicals were purchased from Sigma, with the exception of Avicel PH-101, which was from Fluka Chemika.

Conidiospores (2 x 10$^7$) of P. chrysosporium were used to inoculate 600 ml culture medium in 2 l Erlenmeyer flasks. ‘Semi-static’ cultures (carbon-limited) were obtained by incubating flasks in an air atmosphere at 37°C on a rotary shaker at 150 r.p.m., 2.5 cm diameter cycle, for the primary phase of growth. Culture flasks were fitted with rubber stoppers through which two glass tubes fitted with gas-sterilizing filters were inserted. When glucose was depleted, fungal pellets were concentrated threefold for the enzyme production phase by decanting off 400 ml medium as in Leisola et al. (1985). Flasks were then purged with 100 % oxygen (10 min at 1 l min$^{-1}$), sealed under a positive pressure of oxygen and incubation was continued at 60 r.p.m. to encourage the formation of mats of mycelial growth. In cellulose-maintained cultures, flasks were closed with foam stoppers, and continuously agitated at 130 r.p.m. (2.5 cm diameter cycle) (37°C) in an air atmosphere throughout growth and enzyme production phases.

Enzyme assays. LiP was measured in the extracellular medium with veratryl alcohol as substrate, according to Tien & Kirk (1984). One unit (U) of activity is defined as the amount of enzyme catalysing the oxidation of 1 µmol veratryl alcohol min$^{-1}$. Extracellular glucose and H$_2$O$_2$ were determined by enzyme-coupled assay based on the Trinder colorimetric method, with 4-aminophenazone as substrate, horseradish peroxidase and, for glucose determinations, glucose oxidase, which oxidized glucose with the stoichiometric production of H$_2$O$_2$. The concentration of H$_2$O$_2$ solutions in double-distilled water was determined using ε = 0.036 mM$^{-1}$ cm$^{-1}$. Residual cellulose was filtered from culture broth and from pellets extensively washed with distilled water and was estimated with K$_2$Cr$_2$O$_7$–SO$_4$– reagent according to Wood & Bhat (1988). For intracellular catalase, superoxide dismutase and protein analyses, 5–6 g mycelia was harvested, rinsed and dried with absorbent paper and mechanically ruptured in 1 ml extract buffer (50 mM potassium phosphate buffer, pH 7.5; 0.1 mM EDTA; 0.5 mM PMSF) over liquid nitrogen. Samples were clarified at 10000 g for 1 min, and assayed immediately. Catalase was measured with H$_2$O$_2$ (100 mM) as substrate in 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, by measuring the initial (100–200 s) linear rate of decrease in $\Delta$A$$_{240}$ (Chance et al., 1979). One unit of catalase was defined as that amount of enzyme able to decompose 1 µmol H$_2$O$_2$ min$^{-1}$, at 25°C, using ε = 0.036 mM$^{-1}$ cm$^{-1}$ for H$_2$O$_2$. Superoxide dismutase was assayed according to McCord & Fridovich (1969) by measuring the inhibition of reduction of nitro blue tetrazolium (NBT) by superoxide anions, generated by the xanthine/xanthine oxidase system. The assay mixture contained 0.1 mM EDTA, 0.15 mM xanthine, 0.15 mM NBT and 10 µl xanthine oxidase in 50 mM potassium phosphate buffer (pH 7.5). The rate of reduction of NBT was monitored at 560 nm. One unit of superoxide dismutase was defined as that amount of enzyme able to cause half-maximal inhibition of NBT reduction. The carbonyl content of proteins was measured according to Reznick & Packer (1993) using 2,4-dinitrophenylhydrazine. Protein-bound hydrazones were detected spectrophotometrically with a Perkin Elmer 555 spectrophotometer and the carbonyl content was calculated from the A$$_{370}$ using an absorption coefficient for 2,4-dinitro-
phenylhydrazine of 22000 M$^{-1}$ cm$^{-1}$. Protein was measured by the Bradford method (1976) with ovalbumin as standard.

Transmission electron microscopy (TEM). For TEM, samples were fixed for 4 h at room temperature in 2% (v/v) paraformaldehyde containing 2-5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 4-5). The material was washed twice with 0.1 M phosphate buffer and post-fixed in 1% (v/v) osmium tetroxide in phosphate buffer (pH 4-5) for 1 h at 4 °C, then rinsed with buffer, then distilled water, and stained in 2% (w/v) uranyl acetate in 70% (v/v) ethanol for 15 min at room temperature. Thereafter, samples were dehydrated in an ethanol series (70–100%, 10% steps for 10 min) and embedded in Agar 100 resin (Agar 100 Resin kit from Agar Scientific). The material was sectioned with a Reichert Ultracut. Hyphal sections were obtained from the outer cortex of the pellets. Initially, 2-μm-thick sections were cut and observed under a light microscope. The staining with 0-5% (w/v) methylene blue, 0-5% (w/v) azure II, 0-5% (w/v) borax revealed when sections contained fungal material, at which point thin sections for TEM studies (50–70 nm thick) were collected on nickel grids. At least 25 sections of each type of fungal material were observed under the electron microscope. Before TEM observations, grids were stained in 2% (w/v) uranyl acetate and lead citrate (Reynolds, 1963). TEM studies were carried out with a Philips 400T transmission electron microscope, using an accelerating voltage of 80 kV.

**RESULTS AND DISCUSSION**

Fungal cells were obtained from 5-d-old liquid cultures that were actively secreting LiP following their exposure to a pure oxygen atmosphere when the carbon source was depleted (Fig. 1c), and their ultrastructure was compared with cells that were not producing LiP (Fig. 2a, b). Fig. 1(a, b) typifies the appearance of cells exposed to an atmosphere of pure oxygen. Extensive degeneration of the cytoplasm is evident: the plasmalemma (p) has shrunk from the hyphal wall; half of the cytosol has disintegrated (cv); microvesicles (v), probably with autolytic functions, are recognizable on the periphery of the cell; and the cytoplasm is entirely

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**Fig. 1.** (a, b) TEMs of 5-d-old hyphae of *P. chrysosporium* maintained on 0-2% (w/v) glucose (initial concentration) under semi-static culture conditions and exposed to a pure O$_2$ atmosphere to trigger LiP synthesis (see Fig. 1c). m, Mitochondrion; cv, cytoplasmic voids; p, plasmalemma; v, microvesicles. Bar, 0.5 μm. (c) Production of LiP by semi-static liquid cultures of *P. chrysosporium* grown on 0-2% (w/v) glucose (initial concentration) in an air atmosphere and exposed to a pure O$_2$ atmosphere on day 3 (arrow). Values are means of three replicates; error bars indicate standard deviations.
coagulated. Only mitochondria (m), the sites for oxygen reduction to water, were consistently found to have retained their internal organization. These features were observed in at least 25 different sections, suggesting that they were not restricted to the oldest parts of the mycelium, but were widespread throughout the cortex of mycelial pellets. By contrast, when samples were taken from non-ligninolytic cell populations that had not been exposed to pure oxygen, over the time frame of 3–5 d growth in either 0–2% (w/v) or 0–4% (w/v) glucose, two distinct fungal growth conditions were revealed. The section shown in Fig. 2(a) is typical of hyphae of the first morphological type. The cytoplasm is homogeneously electron-dense, there are few vacuoles and vesicles, and the plasmalemma is seen to adhere to the cell wall. Numerous mitochondria (m) confined at the periphery of the cytoplasm can be identified by the presence of well-defined cristae which are arranged parallel to the long axis of the organelle. Fig. 2(b) typifies sections from the second morphological type representing cells undergoing autolysis. Fungal cells within a single mycelium are known to autolyse to provide nutrients to ensure growth or maintenance in the absence of external nutrients (Trinci & Righelato, 1970; Trinci & Thurston, 1976). Fig. 2(b) shows the presence of numerous vacuoles (V) and vesicles (v), and, in the cytoplasm, autolytic regions (cv) and protein aggregates. An elongated mitochondrion (m) is visible along the cell wall, with an identifiable internal orthodox organization. Multi-membrane complexes (mc) are also visible, possibly representing a reservoir of material for membrane-delimited cytoplasmic structures (Trinci & Righelato, 1970; Markham, 1995). Despite a search to show the contrary, cells from non-ligninolytic cultures never reached the extreme level of appearance that was observed for cells exposed to a pure oxygen atmosphere, typified by Fig. 1(a, b).

These results confirmed the toxic effects of exposing cultures to pure oxygen. Exposure to pure oxygen, apart from triggering LiP synthesis, caused an alteration of cellular architecture. This is consistent with reports of a flux of nitrogen into the extracellular medium by cultures exposed to pure oxygen to induce ligninolysis (Jeffries et al., 1981), the development of conidiospores in the mycelium (Bonnarme et al., 1993), and extracellular presence of H₂O₂ (Tonon & Odier, 1988; Kern, 1989).

In light of these results, it was of interest to investigate the intracellular architecture of cultures that synthesized LiP without exposing them to a pure oxygen atmosphere. For this purpose, we selected liquid cultures agitated in air with cellulose as the carbon source. Sections were taken from the outer region of pellets

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**Fig. 2.** (a, b) TEMs of non-ligninolytic hyphae of *P. chrysosporium*. Two morphological types are depicted: cells with continuous cytoplasm and no signs of degeneration (a) and cells undergoing autolysis (b). These figures were prepared from cultures after 4 d growth in an air atmosphere with 0.4% (w/v) glucose (initial concentration). cw, Cell wall; V, vacuole; v, vesicles; m, mitochondrion; cv, cytoplasmic void; mc, multi-membrane complex. Bar, 0.5 μm.
Ultrastructure and lignin peroxidase production

Fig. 3. (a, b) TEMs of a 4-d-old hypha of *P. chrysosporium* agitated in liquid culture in an atmosphere of air with cellulose (0.4%, w/v, initial concentration) as the carbon source (see Fig. 3c). Note the cytoplasmic voids (cv) and the coagulated proteins in the cytosol. The presence of a nucleus (n) suggests that the cell is representative of the younger part of the mycelium. m, Mitochondrion. Bar, 0.5 µm. (c) Kinetics of LiP appearance in agitated liquid cultures of *P. chrysosporium* grown with cellulose (0.4%, w/v, initial concentration) under an atmosphere of air. Cultures were not exposed to an atmosphere of pure O₂. Values are means of six replicates; error bars indicate standard deviations.

sampled on day 4 when LiP was detected in the extracellular medium (see Fig. 3c). The representative micrographs in Fig. 3(a, b) illustrate that these hyphae showed a remarkable similarity to those exposed to pure oxygen. Irregularly shaped electron-translucent areas or voids (cv) in the cytoplasm that are not surrounded by a membrane are evident, as well as electron-dense particles, possibly cytoplasmic protein aggregates. Cyto- logically ‘normal’ hyphae as depicted in Fig. 2(a, b) were never observed. Mitochondria (m) maintained an orthodox organization, as before, with the cristae visible as lamellae parallel to the long axis of the organelle, in a configuration typical of filamentous fungi (Markham, 1995). In this micrograph, the presence of a nucleus (n) indicates that the cell is part of the younger mycelium, and not of an ageing hyphal network.

The disrupted intracellular architecture in the agitated cultures maintained on cellulose suggested that a hyperoxidant state of growth, caused by limiting respirable carbon, might have arisen in these cultures (Hansberg & Aguirre, 1990). Properties diagnostic of a hyperoxidant condition were therefore investigated both in the cultures maintained on cellulose and, for comparison, in cultures maintained under the same conditions but with glucose as the carbon source, which had shown a more organized ultrastructure (see Fig. 2). Fig. 4 shows that levels of intracellular catalase were up to 20-fold higher by the fourth day of growth [8.8 ± 4.3 U (µg protein)⁻¹] in the cellulose-maintained cultures compared to glucose [0.413 ± 0.3 U (µg protein)⁻¹], reaching 10.4 U (µg protein)⁻¹ by the fifth day of growth. In glucose-grown cultures, catalase levels increased as extracellular glucose levels declined, a similar trend to observations reported for catalase in *Escherichia coli* (Hassan & Fridovich, 1978), but never reached the levels recorded in cellulose-maintained hyphae. Marked differences were also found in levels of extracellular H₂O₂ between
glucose- and cellulose-maintained cultures (see Fig. 5). On the third day of growth the level of \( \text{H}_2\text{O}_2 \) in cellulose-maintained cultures \((0.17 \pm 0.051 \text{ mM} ; n = 3 ; \pm s_d) \) was more than double that found in glucose-sufficient cultures \((0.07 \pm 0.008 \text{ mM} ; n = 3 ; \pm s_d) \), but declined sharply with the appearance of LiP to \(0.03 \pm 0.007 \text{ mM} (n = 3 ; \pm s_d) \). This is in line with the catalytic activity of LiP in reducing \( \text{H}_2\text{O}_2 \) to water. In both cultures, the level of superoxide dismutase was similar, increasing only slightly from 100 \(\pm 10 \text{ U} \) (mg protein)\(^{-1}\) on the third day of growth to approximately 160 U (mg protein)\(^{-1}\) as the carbon source from each culture was depleted. These relatively constant levels infer that levels of this enzyme were not limiting, in line with its importance in protection against the potentially damaging effects of superoxide radicals (McCord & Fridovich, 1969). When the carbonyl content of intracellular proteins was examined, no difference could be detected between non-stressed and stressed cells up to the fifth day of growth \([3–5 \text{ nmol} (\text{mg protein})^{-1}\] for days 3–5]. However, on the sixth day of growth the level recorded for the cellulose-maintained cultures doubled to 10 \(\pm 1.3 \text{ nmol} (\text{mg protein})^{-1}\) compared to the control. The increase detected in carbonyl content is much later than the appearance of LiP (day 4), inferring irreversible breakdown of the cell at this time.

Both these results and the observations of cellular architecture suggest that cultures agitated with cellulose as the carbon source developed a hyperoxidant state. The most likely explanation is that the rate of supply of carbon from cellulose hydrolysis was insufficient to maintain the intracellular NADH pool at a concentration required for oxygen homeostasis during air agitation. Significantly, these conditions also led to the appearance, in the extracellular medium, of LiP. LiP is also produced in cultures exposed to a pure oxygen atmosphere, which was demonstrated in this paper to inflict massive cellular damage. These data together suggest that LiP may be an enzyme that is induced under conditions of oxidant stress to reduce the levels of extracellular \( \text{H}_2\text{O}_2 \) as well as, in \textit{vivo}, to degrade lignin to access further sources of carbon.

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REFERENCES


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