In situ localization of manganese peroxidase production in mycelial pellets of Phanerochaete chrysosporium

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The ultrastructure of Phanerochaete chrysosporium hyphae from pellets in submerged liquid cultures was investigated in order to learn more about the interrelation between fungal architecture and manganese peroxidase (MnP) production. At day 2 of cultivation, some subapical regions of hyphae in the outer and middle zones of the pellet initiated differentiation into intercalary thick-walled chlamydospore-like cells of about 10 \( \mu \text{m} \) diameter. At the periphery of the cytoplasm of these cells, a large number of mitochondria and Golgi-like vesicles were observed. The sites of MnP production were localized at different stages of cultivation by an immunolabelling procedure. The immunomarker of MnP was mainly concentrated in the chlamydospore-like cells and principally distributed in Golgi-like vesicles located at the periphery of the cytoplasm. The apices of hyphae in the outer layer of the pellets were apparently minor sites of MnP production. Maximal MnP release into the culture supernatant coincided with apparent autolysis of the chlamydospore-like cells. Production of extracellular autolytic chitinase and protease coincided with the disappearance of these structures from the pellets. The chlamydospore-like cells observed in the mycelial pellets of P. chrysosporium could be metabolically active entities operating as an enzyme reservoir, delivering their content into the surrounding medium possibly by an enzyme-mediated autolytic process.

INTRODUCTION

Lignin peroxidase (LiP: EC 1.11.1.14), manganese peroxidase (MnP: EC 1.11.1.13) and laccase (EC 1.10.3.2) are the most important enzymes associated with the lignin degradative pathway of white rot fungi (Kirk & Farrell, 1987; Leonowicz et al., 2001). These enzymes have several potential applications in bioremediation of recalcitrant compounds including dyes, polyphenols and other xenobiotics (Lamar et al., 1992). However, commercial exploitation of these biocatalysts is often hampered because of the lack of knowledge about their production parameters in industrial conditions. Mycelial morphology in submerged cultures is usually classified into two different forms: individual filamentous mycelia (dispersed form) and spherical colonies called pellets (Prosser, 1995; Cox et al., 1998). The disadvantages of dispersed mycelial growth include increased wall growth and reduction in efficiency of mixing and oxygen supply to the cells due to increased viscosity of the medium (Schügerl et al. 1983). These problems may be solved to some extent by growth in the form of pellets, which also optimizes harvesting owing to the improved filtration characteristics of the broth (Braun & Vecht-Lifshitz, 1991).

The pellets are formed by the development of a spore inoculum into agglomerates of hyphae trapped together during germination (Gerin et al., 1993). The exact mechanism behind pellet formation is not known. The morphology of pellets may depend on many factors including the inoculum level, genetic factors, medium composition, addition of surfactants, shearing forces, etc. (Metz & Kossen, 1977).

Cultivation in the form of pellets was proposed for the industrial production of some secondary metabolites and enzymes (Braun & Vecht-Lifshitz, 1991). Because fungal morphology affects the rheological properties of the fermentation broth, control of morphology is highly desired in industrial fungal fermentation (Park et al., 2002).
Phanerochaete chrysosporium grown in agitated liquid culture typically forms pellets, and produces MnP under nitrogen limitation in the presence of Mn$^{2+}$. The size of the pellets was found to be a crucial factor for LiP and MnP production (Jaspers et al., 1994; Jiménez-Tobon et al., 1997). In order to learn more about the interrelation between fungal architecture and MnP production, we investigated the ultrastructure of a typical MnP producing culture of Phanerochaete chrysosporium mycelium. The ultrathin sections were examined with a JEM 100 C (JEOL) transmission electron microscope. The immunomarker was found only at locations where the antigen (MnP)–antibody reaction was effective. The ultrathin sections were checked that a rabbit antiserum obtained according to Jiménez-Tobon (1999). This type of structure has also been shown.

**METHODS**

**Growth conditions and enzyme assay.** Phanerochaete chrysosporium ATCC 24725 was maintained on YMPG agar (Bonnarme & Jeffries, 1990). The strain was grown at 30°C on MnP producing medium (Bonnarme & Jeffries, 1990). Conidiospore production for inoculation, MnP assay and determination of pellet size were described previously (Jiménez-Tobon et al., 1997). One unit (U) of MnP activity is defined as the amount of enzyme catalysing the oxidation of 1 µmol vanillylacetone min$^{-1}$.

Extracellular chitinase (EC 3.2.1.14) was estimated with colloidal crab shell chitin (Xia et al., 2001). One unit (U) of chitinase activity is defined as the amount of enzyme catalysing the release of reducing sugars corresponding to 1 µmol N-acetylglucosamine min$^{-1}$.

Extracellular protease activity (EC 3.4.–.–) was measured with Azocoll as the substrate (Dosoretz et al., 1990). One enzyme unit (U) is defined as the amount of enzyme which catalyses the release of azo dye causing an $A_{520}$ change of 0.001 min$^{-1}$.

**Procedure for microscopy.** Pellet formation and growth of mycelium was monitored by phase-contrast microscopy (Docuval microscope, Carl-Zeiss).

For electron microscopy ultrathin sections of mycelium from the periphery and central parts of pellets were prepared by the procedure of Strunk (1978) modified by Kurzatkowski et al. (1991). Immunolabelling on ultrathin sections was carried out basically according to Kurzatkowski et al. (1991). For the immunodetection of MnP, a rabbit antiserum obtained according to Jiménez-Tobon (1999) was raised against a highly purified preparation of the H3 enzyme isoform (Orth et al., 1994). Preimmune serum was obtained from the same rabbit before immunization. Western blot experiments have shown that the antibodies obtained react with purified MnP, although some faint cross-reaction was observed with H8 LiP isoenzyme (Jiménez-Tobon, 1999).

Antigen–antibody complexes were visualized with goat anti-rabbit IgG–15 nm gold conjugate (BioCell). Controls were included either by omitting the antibody against MnP, or by adding pre-immune serum (dilution 1:400 in PBG; Jiménez-Tobon, 1999), followed by goat anti-rabbit IgG–15 nm gold conjugate. It was checked that the immunomarker was found only at locations where the antigen (MnP)–antibody reaction was effective. The ultrathin sections were examined with a JEM 100 C (JEOL) transmission electron microscope at 80 kV.

**RESULTS**

**MnP production pattern**

A typical curve of MnP production in the supernatant of a P. chrysosporium culture forming pellets is shown in Fig. 1. No extracellular MnP activity was observed before day 4 of cultivation, which corresponded to the end of the trophophase and entry in the idiophase (Jiménez-Tobon et al. 1997). The maximal level of MnP secreted into the extracellular medium was reached between days 7 and 8 of cultivation and thereafter decreased. Neither LiP nor laccase was produced in the cultivation procedure used here (Bonnarme & Jeffries, 1990; Jiménez-Tobon et al., 1997).

**Ultrastructure of P. chrysosporium hyphae in pellets**

With the conidial inoculation density used here the mean final diameter of the pellets was about 3.5 mm after 4 days of cultivation (Jiménez-Tobon et al., 1997). Pellets of P. chrysosporium typically have three zones: an outer layer, surrounded by its growing boundary, a middle zone with some cells undergoing a lysis process, and a central core filled with senescent cells (Jaspers et al., 1994; Jiménez-Tobon, 1999). This type of structure has also been shown.

![Fig. 1. Enzyme and biomass production in a culture of P. chrysosporium.](image-url)
for several other fungal strains forming pellets (Wittler et al., 1986; Braun & Vecht-Lifshitz, 1991).

In a transmission electron microscopy analysis (not illustrated here) we have observed that the wall of young apical cells present in the outer region of the pellet was typically composed of one thin layer with an electron-opaque cytoplasm densely packed with ribosomes. A large number of elongated mitochondria possessing numerous flat cristae were also present in the cytoplasm. Nuclei in young, growing hyphae were often elongated parallel to the long axis of the hyphae. Nuclear pores were observed in the nuclear envelope. At the nuclear envelope the extensive membrane system of an endoplasmic reticulum was present. Small vacuoles were characteristic for the young cells and the cross-walls were not closed. The cytoplasm of subapical cells was electron-opaque and contained a large number of mitochondria and nuclei. The cell wall was composed of two layers. Small vacuoles surrounded by a tonoplast could also be seen. In ageing cells present in the inner part of the pellet, the cell wall was composed of several sublayers. The cytoplasm was electron-transparent and large vacuoles surrounded by a tonoplast were noted. The developed cross-walls were accompanied by Woronin bodies.

At day 2 of cultivation, some subapical regions of hyphae apparently started a process of differentiation into spherical cells of about 10 μm diameter morphologically similar to chlamydospores (Fig. 2a, b). These intercalary chlamydospore-like cells grew in number to culminate between days 3 and 4 of cultivation. Disappearance of these structures apparently started between days 4 and 5 and was nearly complete after 7 days of cultivation. Chlamydospore-like cells have also been observed in cultures not producing MnP but apparently never engaged in an autolytic process without the addition of extra Mn²⁺, which is necessary for enzyme induction (not shown).

The diameter of the chlamydospore-like spherical cells observed in the outer and middle zones of the pellet varied from 8 to 13 μm. Fig. 2(c) shows a typical cell observed after 4 days cultivation which was surrounded by a thick wall with a cytoplasm densely packed with mitochondria and nuclei. Golgi-like vesicles 50–200 nm in diameter, produced from the endoplasmic reticulum, were visible and apparently had a tendency to be distributed to the periphery of the cell.

**Immunolocalization of the sites of MnP production**

The sites of MnP production were localized at different stages of cultivation. MnP-reacting antibodies were found concentrated mainly in the chlamydospore-like cells and secondarily at the apical ends (Fig. 3a, b). In chlamydospore-like cells from 3 and 4 day cultures, the immunomarker was found to be predominantly concentrated in the Golgi-derived vesicles located at the peripheral part of the cytoplasm (Figs 3b and 4a). In chlamydospore-like cells

![Figure 2](http://mic.sgmjournals.org) Chlamydospore-like cells produced by *P. chrysosporium*. (a, b) Cells observed by phase-contrast microscopy in subapical regions of hyphae obtained from the outer layer of pellets after 4 days cultivation. Bars, 30 μm (a) and 10 μm (b). (c) Transmission electron micrograph of an ultrathin section of a typical chlamydospore-like cell present in the outer layer of 4 day pellets of *P. chrysosporium* (cw, cell wall; M, mitochondrion; N, nucleus; v, vesicles; V, vacuoles). Bar, 1 μm.
from 6 day cultures the immunomarker density was lower and distributed in approximately equal proportions between the cell wall and the Golgi-derived vesicles (Figs 3b and 4b). A much lower density of the immunomarker was always detected in the other cell organelles and in the cytoplasm. Cells in the senescent central core of the pellet exhibited only a very faint immunoreaction (Fig. 3c).

**Extracellular autolytic enzymes produced by *P. chrysosporium***

From the above-described results it appears that maximal MnP secretion coincided with apparent autolysis of the chlamydospore-like cells. These could thus act as an enzyme reservoir, delivering their content into the culture supernatant by a process of autolysis. In order to support this assumption we examined the extracellular enzyme profile of autolytic hydrolases involved in disruption of organelle and cell wall structure (White *et al.*, 2002; Pocsi *et al.*, 2003) (Fig. 1). Extracellular chitinase activity started to increase at day 3, which corresponded to the deceleration phase preceding the end of the trophophase. The enzyme reached a plateau at day 5 of cultivation. Proteolytic activity started to increase during the trophophase at day 2, reached a maximum on day 4 and decreased to a lower level on day 7. A second smaller peak of proteolytic activity was attained between days 9 and 10.

**DISCUSSION**

The relationships between culture morphology and LiP and MnP production by *P. chrysosporium* have not as yet been well documented. Using a sandwiched mode of solid culture, and an immunodetection procedure, Moukha *et al.* (1993) suggested that peroxidases (LiP and MnP) were initially secreted only at the apex of secondary growing hyphae and later slowly released into the surrounding medium. No other sites of enzyme production were detected in their study. An apical mode of enzyme production was also shown here in *P. chrysosporium* pellets, but was apparently minor compared to production by the chlamydospore-like cells. The mycelial morphology in a submerged culture is nevertheless different from that of a plate culture. Particular situations of nutrient and oxygen concentrations in a pellet might create a physiological context encouraging a structural differentiation of the idiophasic hypha of the fungus. Fragmentation of idiophasic fungal hyphae is under metabolic and environmental control (Gow, 1994) and may result in differentiated cell

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**Fig. 3.** Quantitative distribution of the MnP immunomarker in organelles of *P. chrysosporium* cells at different times of cultivation. The mean values reported represent the number of gold-conjugated particles per μm² of hyphal ultrathin sections from the periphery and central parts of pellets. Five pellet sections were examined. (a) Apical extensions; (b) chlamydospore-like cells; (c) central cores of pellets.
structures having particular metabolic properties: for example yeast-like cells synthesizing the antibiotic cephalosporin in *Acremonium chrysogenum* (Bartoshevich et al., 1990; Sandor et al., 1998). In the case of *P. chrysosporium* it has been reported that a hyperoxidant state of growth, caused by limiting respirable carbon, induces a disorganization in the intracellular architecture of LiP-secreting hyphae in mycelial pellets, which in turn may promote enzyme production (Zacchi et al., 2000). Production of metabolically active chlamydospore-like cells by *P. chrysosporium* might therefore result from a particular physiological context in the MnP-producing pellet medium. However, the exact nature of these chlamydospore-like cells remains undefined at present. Chlamydospore-like cells and 'pseudo-chlamydospores', distinct from 'true' chlamydospores, have been reported occasionally in fungi, for example in *Fusarium* (Leslie, 2000) and *Tremella* sp. (Chee-Jen & Oberwinkler, 2000). Further studies are required to elucidate their structure, such as transmission electron microscopy to determine the thickness of their outer cell walls and whether they contain reserves of glucans and lipids. *P. chrysosporium* and its anamorph *Sporotrichum pulverentulum* are known to produce several differentiated structures, including typical chlamydospores (Larone, 1995; St-Germain & Summerbell, 1996; Wu, 1998). *P. chrysosporium* 'true' chlamydospores typically have diameters of up to 60 μm and contain abundant reserves of glucans and lipids (St-Germain & Summerbell, 1996; de Hoog et al., 2000). This was apparently not the case for the chlamydospore-like cells, which have a mean diameter of about 10 μm and several characteristics of metabolically active structures.

These cells might be related to yeast-like forms and intermediate structures between hyphae and chlamydospores in other fungi (Bermejo et al., 1981; Dominguez et al., 1978). Moreover in certain fungal species, chlamydospores were shown to be fully functional cells, morphologically and physiologically active and independent from mycelium (Vidotto et al., 1996).

MnP production in *P. chrysosporium* chlamydospore-like cells was found mainly associated with Golgi-derived vesicles produced from the endoplasmic reticulum and located in the peripheral part of the cytoplasm. Mitochondria and nuclei were characteristically co-located with the vesicles, which suggests a cooperative metabolic activity. LiP and MnP were also found mainly associated with Golgi-derived vesicles in apical cells of secondary growing hyphae (Bonnarme et al., 1994).

Release of MnP in the culture supernatant coincided with the phase of disappearance of the chlamydospore-like cells from the mycelial pellets of *P. chrysosporium*, and with maximal activity of extracellular chitinase and protease. Chitinases and proteases in fungi were identified as autolytic enzymes involved in a variety of functions (Gooday, 1997a; Rao et al., 1998), including disruption of organelle and cell wall structure (Gooday, 1997b; White et al., 2002; Pocsi et al., 2003). Production of proteases is a common feature among fungi including Basidiomycetes (Rao et al., 1998). Chitinases have apparently been reported only occasionally in Basidiomycetes (Tracey, 1955; Ohtakara, 1988) but, as far we are aware, not previously in *P. chrysosporium*. The presence of protease and chitinase

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**Fig. 4.** Immunoelectron microscopy of distribution of the MnP immunomarker in chlamydospore-like cells. (a) Distribution pattern of the gold-conjugated particles in a chlamydospore-like cell from the periphery of a 4-day-old pellet. At the cell wall (cw) a vesicle (v) is packed with immunomarker of MnP. Surrounding this vesicle, smaller vesicles are seen showing enzyme immunomarkers in the interior. Bar, 0.5 μm. (b) Distribution pattern for a cell from a 6-day-old pellet. Bar, 0.1 μm.
activities in the culture supernatant of *P. chrysosporium* might reflect their participation in hyphal autolysis steps, possibly including the disappearance of chlamydospore-like cells. For example, age-related extracellular chitinases have been shown to play a crucial role in both autolysis and fragmentation of *Penicillium chrysogenum* (Sami et al., 2001). In this context, the first peak of extracellular protease produced by *P. chrysosporium* could be involved in combination with chitinases (and possibly other enzymes) in the lysis of the chlamydospore-like cells, whereas the protease produced during late idiophase would play a role in the decline of MnP, as was suggested for LiP (Dosoretz, 1990; Dass, 1995). The exact role of these lytic enzymes has to be demonstrated in future in vivo enzyme inhibition experiments using for example allosaminid (Sami et al. 2001). From all the various data collected in this study, we conclude the chlamydospore-like structures of *P. chrysosporium* apparently show the characteristics of metabolically active entities acting as an enzyme reservoir and delivering their content into the surrounding medium by an autolytic process.

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