Isoenzymes of manganese-dependent peroxidase and laccase produced by the lignin-degrading basidiomycete *Ceriporiopsis subvermispora*

Sergio Lobos,^{1†} Juan Larraín,¹ Loreto Salas,¹ Daniel Cullen² and Rafael Vicuña¹

Author for correspondence: Rafael Vicuña. Tel: +56 2 222 1199. Fax: +56 2 222 5515.

¹ Laboratorio de Bioquímica, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago, Chile

² Institute for Microbial and Biochemical Technology, Forest Products Laboratory, Forest Service, USDA, One Gifford Pinchot Drive, Madison, Wisconsin 53705, USA

The white-rot basidiomycete *Ceriporiopsis subvermispora* produces two families of ligninolytic enzymes, namely manganese-dependent peroxidases (MnPs) and laccases, when growing in liquid cultures of defined composition. In medium containing 11 p.p.m. of Mn(II), up to seven isoenzymes of MnP and four isoenzymes of laccase were resolved by isoelectrofocusing (IEF), with pI values in the range 4·10–4·60 and 3·45–3·65, respectively. Occasionally, a fifth laccase isoform of pI 4·70 was also detected. In cultures with 25 and 40 p.p.m. of Mn(II), mainly the MnPs with higher pI values are produced. The isoenzyme pattern of MnP is not altered throughout the growth period of the fungus. MnP and laccase are also produced by *C. subvermispora* when growing on wood chips of *Pinus radiata*. Highest levels of both enzymes were obtained during the first week of incubation. A second peak of MnP activity was observed during the fourth week, whereas very low levels of laccase were extracted from the chips after the second week of growth. IEF analysis showed that the pI values of these laccases are similar to those of laccases produced in liquid cultures, being in the range 3·45–3·65. In contrast, four isoforms of MnP were resolved during the first week of incubation on wood chips, with pI values of 4·40, 4·17, 4·04 and 3·53. This profile underwent a transition during the second week of growth, at the end of which isoforms of MnP with pI values of 3·53, 3·40, 3·30 and 3·20 were resolved by IEF. Immunoblotting studies showed that the molecular mass of MnP isoenzymes from liquid cultures was about 52·5 kDa, whereas the molecular masses of MnPs extracted from wood varied from 52·5 kDa to 62·5 kDa upon ageing of the cultures. The amino terminal sequences of seven MnP isoenzymes were determined. The consensus sequences of MnPs from liquid and solid cultures were clearly distinct, although both showed homology to MnPs from related white-rot fungi.

Keywords: *Ceriporiopsis subvermispora*, manganese-dependent peroxidases, laccase, ligninolytic enzymes

INTRODUCTION

The white-rot basidiomycete *Ceriporiopsis subvermispora* is strongly ligninolytic (Otjen *et al.*, 1987; Blanchette *et al.*,

1992) and it is used extensively in pilot-scale studies of biomechanical pulping (Akhtar *et al.*, 1992, 1993). However, relative to other white-rot fungi such as *Phanerochaete chrysosporium*, *Coriolus versicolor* or *Phlebia radiata* little is known concerning the enzymology of lignin degradation by *C. subvermispora*.

Studies with liquid cultures of *C. subvermispora* have demonstrated the production of two classes of extra-cellular enzymes: manganese-dependent peroxidases

† **Permanent address:** Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile.

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); IEF, isoelectrofocusing; LiP, lignin peroxidase; MnP, manganese-dependent peroxidase; PVDF, polyvinylidene difluoride.

(MnPs) and laccases (Ruttimann *et al.*, 1992a, b). Levels of these enzymes are positively correlated with lignin mineralization in *C. subvermispora* (Ruttimann-Johnson *et al.*, 1993). Lignin peroxidase (LiP) has not been detected in cultures of this fungus grown under a wide variety of conditions (Ruttimann *et al.*, 1992a, b).

MnPs have been found in several fungal species (Orth *et al.*, 1993). In *Phanerochaete chrysosporium*, at least six isoenzymes have been identified (Leisola *et al.*, 1987). Amino-terminal sequence analyses show that they are encoded by at least four genes (Leisola *et al.*, 1987; Pease & Tien, 1992), two of which have been cloned and characterized (Pease *et al.*, 1989; Pribnow *et al.*, 1989). These isoenzymes are differentially regulated in response to nutrient limitation and culture age (Pease & Tien, 1992). MnPs have also been partially characterized from *Panus tigrinus* (Maltseva *et al.*, 1991), *Dichomitus squalens* (Péridé & Gold, 1991), *Rigidoporus lignosus* (Galliano *et al.*, 1991), *Phlebia radiata* (Karhunen *et al.*, 1990), *Lentinula edodes* (Forrester *et al.*, 1990), *Phanerochaete sordida* (Ruttimann-Johnson *et al.*, 1994) and *Coriolus versicolor* (Johansson & Nyman, 1993; Johansson *et al.*, 1993), among others. Its wide distribution among ligninolytic fungi suggests that MnP may play a key role in the process of lignin biodegradation.

Laccase activity has also been detected in numerous fungi, including several lignin-degraders. Among the latter, *Coriolus versicolor* (Morohoshi, 1991), *Panus tigrinus* (Maltseva *et al.*, 1991) and *R. lignosus* (Geiger *et al.*, 1986) produce multiple isoenzymes, whereas *Phlebia radiata* secretes only one species of laccase to the extracellular medium (Niku-Paavola *et al.*, 1988). Genes encoding laccase activity in several white-rot ligninolytic fungi have been cloned and characterized (Saloheimo *et al.*, 1991; Kojima *et al.*, 1990; Iimura *et al.*, 1992; Coll *et al.*, 1993).

Most studies of ligninolytic enzymes have been conducted with preparations isolated from liquid cultures of the various fungal strains utilized. Moreover, since liquid cultures are easy to manipulate, conditions have been developed in several cases for optimal enzyme production and lignin mineralization. Although this approach has been a valuable tool for understanding several aspects of the lignin biodegradation process, white-rot fungi normally exert their action while growing on solid wood. Therefore, it seems reasonable to compare the ligninolytic enzymes produced by white-rot fungi growing in liquid cultures with their counterparts produced in solid state cultures. Some knowledge has already been gained in this respect with *Phanerochaete chrysosporium*; Datta *et al.* (1991) reported that the most abundant peroxidase extracted from cultures of this organism growing on wood pulp is MnP rather than LiP.

In this work we describe multiple MnP and laccase isoenzymes from *Ceriporiopsis subvermispora* and their production in both liquid and solid state cultures.

METHODS

Organism. *C. subvermispora* strain FP-105752 was obtained from the Center for Forest Mycology Research, Forest Products

Laboratory, Madison, WI, USA. The fungus was maintained on agar slants of potato dextrose agar medium (Difco).

Growth in liquid cultures. Erlenmeyer flasks (125 ml) containing 30 ml minimal salts medium (Ruttimann *et al.*, 1992a) were inoculated with 13.5 mg of homogenized mycelium prepared as previously described (Ruttimann *et al.*, 1992a). Unless otherwise indicated, the medium contained 11 p.p.m. (195 µM) MnSO_4 and 10 mM ammonium tartrate as nitrogen source.

Fractionation of enzymes produced in liquid cultures. One hundred millilitres of 15-d cultures was centrifuged for 20 min at 10000 r.p.m. to remove the mycelium and the extracellular glucan. The supernatant was concentrated 20-fold by ultrafiltration in a 185 ml Amicon cell with a 10 kDa cut-off membrane and dialysed against 10 mM sodium acetate pH 6.0. Protein (50 mg) was loaded onto a Q-Sepharose (Pharmacia) column (0.6 cm² × 27 cm) previously equilibrated with the same solution. Enzymes were eluted with a 60 ml linear gradient of 10–600 mM sodium acetate pH 6.0 and 1 ml fractions were collected. For preparative IEF (see below), fractions that were enriched in MnP activity were pooled and concentrated by dialysis against a 40% solution of polyethylene glycol 8000 dissolved in 10 mM sodium acetate pH 6.0, to a final protein concentration of about 1 mg ml⁻¹.

Growth on wood chips. Four millilitres of a defined medium (Leatham, 1983) containing 10 mM ammonium tartrate and 2.5% (w/v) glucose, plus 6 ml water, were added to 15 g *Pinus radiata* chips (5 × 5 mm, 40% initial moisture content) in 250 ml Erlenmeyer flasks. The flasks were covered with aluminium foil and autoclaved for 45 min at 121 °C, giving a final moisture content of 60%. After cooling to room temperature, the chips were inoculated with two 1.0 cm² mycelial plugs of the fungus grown on potato dextrose agar, and vigorously mixed. Flasks were incubated at 30 °C for up to 4 weeks, being supplied with filtered and humidified air throughout this period (1.5 l h⁻¹).

Enzyme extraction from the wood chips and partial fractionation. Chips were resuspended for 1 h at 4 °C with occasional stirring in 30 ml 50 mM sodium acetate pH 4.8 (buffer A). The liquid phase was centrifuged for 1 h at 15000 r.p.m. and the supernatant was diluted twofold with water. The solution obtained was loaded onto a 2 ml Q-Sepharose column equilibrated with buffer A. Enzymes were eluted stepwise with 4 ml solutions of NaCl (0.1, 0.2, 0.3, and 0.4 M) in buffer A. Both enzymes were always recovered in the fraction containing 0.2 M NaCl. During the first week, some additional MnP activity also eluted with 0.1 M NaCl. This fractionation procedure was scaled up prior to preparative IEF.

Enzyme assays. MnP and laccase were assayed as previously described (Ruttimann *et al.*, 1992a), using vanillylacetone and ABTS (Sigma) as substrates, respectively. LiP activity was measured using two standard methods (Tien & Kirk, 1984; Archibald, 1992). Definitions of enzyme units have been reported previously (Ruttimann *et al.*, 1992a).

Isoelectrofocusing. IEF was performed over the pH range 3–6, using Servalyt Precotes 3–6 polyacrylamide gels from Serva. After applying the protein samples (0.5–1.0 µg protein per well in analytical gels, 200–400 µg in preparative gels), gels were run for 30 min at 200 V, 15 min at 400 V, 5 min at 800 V and then the voltage was raised to 1200 V until the current reached 2 mA. MnP bands were developed by soaking the gel in a solution prepared as follows: 15 mg 4-chloro-1-naphthol was dissolved in 5 ml methanol; 1 ml of this solution was mixed with 6.3 ml 20 mM Tris/HCl pH 7.5, 0.5 M NaCl. In turn, 4 ml of the

resulting solution was mixed with 25 ml 50 mM sodium tartrate pH 5.0 containing 0.2 mM MnSO_4 . Hydrogen peroxide was added to a final concentration of 50 mM. The solution for developing the bands corresponding to laccase was the same but lacked MnSO_4 and hydrogen peroxide. Occasionally, both enzymes were developed with ABTS or phenol red as substrates, which were present at final concentrations of 5 mM and 480 μM respectively. When indicated, 1 unit ml^{-1} of catalase (Sigma) was added to the developing solution of laccase activity. Standards for IEF were all from Sigma: amyloglucosidase from *Aspergillus niger* (pI 3.6), glucose oxidase from *Aspergillus niger* (pI 4.2), soybean trypsin inhibitor (pI 4.6), β -lactoglobulin A from bovine milk (pI 5.1), carbonic anhydrase II from bovine erythrocytes (pI 5.4 and 5.9) and carbonic anhydrase I from human erythrocytes (pI 6.6). Preparative IEF gels were always performed with protein which had been fractionated previously by Q-Sepharose chromatography.

Anti-MnP antibody. MnP was purified from 25-d solid state cultures. Proteins extracted from 200 g of dry wood chips were first fractionated by Q-Sepharose chromatography. Active fractions were pooled, concentrated first to 10 ml by ultra-filtration in an Amicon cell and then to 0.5 ml in a Millipore cartridge (10000 cut-off). Protein (1 mg) was applied to preparative Servalyt Precotes. The IEF gel section containing the MnP bands was excised and homogenized to a final volume of 0.6 ml with 50 mM Tris/HCl buffer pH 7.5 containing 200 mM NaCl. The suspension was extensively dialysed against PBS solution (8.1 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 , 0.8% NaCl, 0.02% KCl). A rabbit was initially injected subcutaneously with 300 μg MnP protein in complete Freund's adjuvant. Two 300 μg aliquots of MnP in incomplete Freund's adjuvant were injected 15 and 25 d after the first injection. Antibody titre was monitored by ELISA assays.

Western blots. Proteins (300 ng per track) and prestained molecular mass markers (Sigma) were first separated by SDS-PAGE and then transferred to nitrocellulose for 50 min at room temperature and 150 mA, in a BioRad chamber with a solution of 25 mM Tris/HCl pH 8.4, 192 mM glycine and 20% (v/v) methanol. The nitrocellulose was incubated with gentle agitation for 30 min at room temperature in PBS solution containing 1% (w/v) bovine serum albumin (PBS-BSA) and thereafter immersed in serum anti-MnP diluted 10-fold in PBS-BSA for an additional 30 min. The nitrocellulose was washed six times for 1 min with PBS solution containing 0.1% Tween 20 and incubated for 30 min in a 1/3500 dilution in PBS-BSA of horseradish peroxidase conjugated to antibody anti-IgG from rabbit (BioRad). The nitrocellulose was washed as before and developed by soaking it for 5–15 min in a mixture containing 10 ml 50 mM Tris/HCl buffer pH 7.4, 200 mM NaCl, 2 ml of methanol containing 6 mg 4-chloro-1-naphthol and 4 μl 30% (v/v) hydrogen peroxide.

Protein electroblotting and sequencing. After IEF, the MnP bands were excised and homogenized to a final volume of 0.2 ml with 50 mM Tris/HCl buffer pH 7.5 containing 200 mM NaCl. Suspensions were loaded onto a 8% (w/v) SDS-polyacrylamide gel and electrophoresed at 20 mA constant current. Purified isoenzymes were electroblotted to PVDF membranes (Matsudaira, 1987) and sequenced on an Applied Biosystems gas phase 475 protein sequencer at the University of Wisconsin Biotechnology Center.

Other procedures. SDS-PAGE was performed as reported by Laemmli (1970) and the protein bands were stained using a silver stain kit (Sigma). Protein concentration was measured by the method of Bradford (1976).

RESULTS

Isoenzymes produced in liquid cultures of defined composition

Extracellular minimal medium from 15-d cultures of *C. subvermispora* was subjected to IEF as indicated in Methods. The gel was cut into four sections, which were developed under different conditions (Fig. 1). When the developing solution contained only the substrate – in this case 4-chloro-1-naphthol – several staining bands were observed between pH 3.45 and 3.65 (Fig. 1, lane 1). These bands were also observed when catalase was present in the developing solution, indicating that they correspond to isoforms of laccase. Further addition of hydrogen peroxide or Mn(II) to the developing solution did not significantly alter the band patterns (Fig. 1, lanes 2 and 3, respectively). However, when these two components were added simultaneously, several bands appeared between pH 4.1 and 4.6, strongly suggesting that the latter correspond to isoforms of MnP (Fig. 1, lane 4). Thus, IEF provides a useful tool not only to separate isoenzymes but also, in this case, to differentiate activities of MnP and laccase. The same band pattern was obtained when ABTS or phenol red were used as substrates in the developing solution.

Attempts were made to find conditions leading to preferential production of particular isoforms of each activity. For this purpose, the effects of Mn(II) and culture age were analysed. Standard cultures of *C. subvermispora* contain 11 p.p.m. of Mn(II), since at this concentration the titres of both MnP and laccase are highest (Ruttimann *et al.*, 1992a). Therefore, for comparative purposes, the fungus was grown in salt medium containing 0.3, 11, 25 and 40 p.p.m. of Mn(II) and the isoenzymes were analysed by IEF (Fig. 2). With 11 p.p.m. of Mn(II) in the medium, the expected MnP pattern was obtained (Fig. 2a, lane 2; compare with Fig. 1, lane 4). At Mn(II) concentrations of 25 and 40 p.p.m., the MnP isoforms of higher pI were preferentially produced (Fig. 2a, lanes 3 and 4). In the case of laccase, an isoform of pI 4.7 was present in the cultures

Fig. 1. IEF analysis of ligninolytic enzymes produced by *C. subvermispora* in liquid cultures of defined composition. Each gel was developed in a solution containing 4-chloro-naphthol as substrate, plus: no further addition (lane 1); hydrogen peroxide (lane 2); Mn(II) (lane 3); and hydrogen peroxide plus Mn(II) (lane 4).

Fig. 2. Isoenzyme profile of MnP (a) and laccase (b) in cultures of *C. subvermispora* in minimal salts medium containing different concentrations of Mn(II). Lanes: 1, 0.3 p.p.m.; 2, 11 p.p.m.; 3, 25 p.p.m.; 4 and 5, 40 p.p.m. Lanes 1–4 each contain 3 µg total protein. Lane 5 in each gel was loaded with the same quantity of activity units of MnP and laccase as was loaded onto the respective lane 4. The activities of MnP (units µg protein⁻¹) in the samples loaded onto lanes 1–4 were, respectively, 15.5, 24.1, 11.4 and 11.0. The activities of laccase were 3.4, 31.8, 3.4 and 2.9, respectively.

containing 0.3, 25 and 40 p.p.m. of Mn(II), but not in the culture with 11 p.p.m. of Mn(II) (Fig. 2b). In the course of these studies, this band of laccase activity was only occasionally detected in the various cultures, even in those with 11 p.p.m. of Mn(II). The conditions that favour the formation of this laccase of high pI or its relation to the low pI isoforms are not yet known. On the other hand, samples were withdrawn from standard cultures of *C. subvermispora* throughout the growth period and analysed by IEF. Both isoenzyme patterns remained unaltered until the last day tested (day 25; data not shown).

To isolate isoforms of each enzyme, extracellular fluid from liquid cultures of *C. subvermispora* was fractionated by Q-Sepharose chromatography as indicated in Methods. After applying a shallow salt gradient, broad peaks of MnP and laccase activities were obtained, with partial resolution between these two enzymes. MnP- and laccase-enriched fractions were pooled separately and each pool was subjected to IEF (Fig. 3).

Isoenzymes produced by *C. subvermispora* while growing in wood chips

When *C. subvermispora* was inoculated onto wood chips of radiata pine, fungal growth became visible on day 4. Enzymes produced by the fungus were extracted at

Fig. 3. IEF of MnP (1) and laccase (2) isoforms after fractionation by Q-Sepharose chromatography. The laccase of pI 4.7 is not observed in this sample.

Fig. 4. Activity profile of MnP (●) and laccase (○) extracted from wood chips at various times of fungal growth. Vertical bars represent a single standard error.

various times from the chips and separated by Q-Sepharose chromatography from potential inhibitory substances released from the wood (see Methods). Under the standard conditions, MnP exhibited two peaks of activity, on days 8 and 25, whereas laccase activity reached its maximum during the first week of growth (days 4–5, Fig. 4). Thereafter, the levels of laccase began to decrease, reaching very low levels beyond the second week of incubation (Fig. 4).

IEF analysis throughout the culture period showed that the pattern of MnP isoforms extracted from the chips was different from that obtained in liquid cultures. At day 4, few distinct bands were observed (Fig. 5a, lane 1). Two of

Fig. 5. IEF analysis of MnP (a) and laccase (b) extracted from wood chips at different times of fungal growth. Samples are 0.2 M sodium acetate eluates of Q-Sepharose columns loaded with extracts obtained on days 4 (lanes 1), 8 (lanes 2), 16 (lanes 3) and 25 (lanes 4).

Fig. 6. Immunoblot analysis of MnPs at various times of fungal growth. Lane 1, MnP from liquid cultures in salt medium; lanes 2 and 3, 0.1 and 0.2 M NaCl eluates from Q-Sepharose column obtained by fractionation of protein extracted from wood chips at day 4; lanes 4–6, MnP extracted from wood chips at days 10, 16 and 25, respectively. M, Molecular mass markers.

Fig. 7. SDS-PAGE (10% gel) of MnPs obtained on day 25, after preparative IEF. Lanes 1–4, MnPs of pI 3.20, 3.30, 3.40 and 3.53, respectively; lane 5, MnP sample prior to IEF fractionation. In this experiment, 0.5 µg protein was applied to each track. M, Molecular mass markers.

them exhibited pI values of 4.40 and 4.17, which are in the same range as MnPs from liquid cultures. However, there were two additional MnP isoforms with pI values of 4.04 and 3.53, that had not been identified previously. At day 8 and thereafter, four isoforms with pI values 3.53, 3.40, 3.30 and 3.20 were observed (Fig. 5a, lanes 2–4). These are all in the pI range of laccases isolated either from liquid cultures (Fig. 1) or from wood chip cultures (Fig. 5b, lanes 1 and 2). However, the bands observed in Fig. 5(a), lanes 2–4 can be ascribed to MnP activity, since laccases were not detected by IEF after the second week when the same samples were applied to the gel (Fig. 5b, lanes 3 and 4; see also the activity profile in Fig. 4).

Molecular mass of MnPs

Samples from a culture of *C. subvermispora* in wood chips were obtained at various times of growth and analysed by immunoblotting (Fig. 6). In this analysis, a sample from a liquid culture in salt medium withdrawn at day 11 was also included. Some of the bands developed with this technique were broad, probably due to the presence of several isoforms in the corresponding samples. However, it is clear that the molecular mass of MnPs from a liquid culture (52.5 kDa) was lower than that of MnPs from solid state culture obtained at day 25 (62.5 kDa) (Fig. 6, lanes 1 and 6). Moreover, the molecular mass of the MnPs extracted from the wood chips gradually changed during the growth period. On day 4, Q-Sepharose chroma-

(a)	A I F P A G T R V S N F V R	MnP1-C.s.
	G I f P A a T R V S N d V i S D p i p X a H	MnP2-C.s.
	G I f P A G T R V S N H V X R X F I P	MnP3-C.s.
	A I P P d G T R V S N h v d X d F I S P A q S L	MnP4-C.s.
	A I F P A G T R V S N H V . . D F I P . A	consensus 1
(b)	V T X S d G T A v P X a M X X d F I P L X X d l q s m v l q	MnP5-C.s.
	V T X S D G T A V P D A M X X D F I P L A Q X l q g x v l q	MnP6-C.s.
	V T X S D G T A V P d A M X X d F I P L X X d d	MnP7-C.s.
	V T X S D G T A V P D A M X X D F I P L X X D L Q . . V L Q	consensus 2
(c)	A I F P A G T R V S N H V . . D F I P . A	consensus 1
	V T X S D G T A V P D A M X X D F I P L X X D L Q . . V L Q	consensus 2
	A V X S Q G T R V S N A A X X A F I P L A Q D L Q - T L E M	MnP1-P.s.
	A V X P D G T X V N N E A X X A F I P L A Q D L Q E T I F M	MnP11-P.s.
	A V X P D G T A V P S T V X X D F I P L A L	MnP111-P.s.
	A T C P D G T K V N N A A C C A F I P L A Q D L Q E T I F O	MnPH3-P.c.
	A V C P D G T R V T N A A C C A F I P L A Q D L Q E T L F O	MnPH4-P.c.
	A V X H P G T X V S X A A X X X F I P L	MnPH5-P.c.
	A V C P D G T R V S H A A C C A F I P L A Q D L Q E T I F O	MnP1-P.c.
	A V X P D G T R V X N A A X X A F X P L	MnPP-P.c.
	A V G S D G T V V P D S V Q Y D F I P L A Q D L T A X L E	MnP1-L.e.

Fig. 8. Aligned N-terminal sequences of MnPs. (a, b) Isoenzymes derived from liquid and solid media, respectively. Isoenzyme pI values were: MnP1-C.s., 4.2; MnP2-C.s., 4.3; MnP3-C.s., 4.4; MnP4-C.s., 4.6; MnP5-C.s., 3.4; MnP6-C.s., 3.3 and MnP7-C.s., 3.2. Lower-case letters indicate less confident assignments and the letter X represents unknown residues. (c) The consensus sequences from *C. subvermispora* are compared to experimentally-determined sequences from: *Phanerochaete sordida* isoenzymes MnP1-P.s., MnP11-P.s., MnP111-P.s. (Ruttimann-Johnson *et al.*, 1994); *Phanerochaete chrysosporium* isoenzyme H5, MnPH5-P.c. (Pease & Tien, 1992); a *Phanerochaete chrysosporium* isoenzyme purified from aspen pulp, MnPP-P.c. (Datta *et al.*, 1991); and the *Lentinula edodes* MnP, MnP1-L.e. (Forrester *et al.*, 1990). Sequences were deduced from cDNAs for isoenzymes MnPH3-P.c. (A. Orth and others, unpublished), MnPH4-P.c. (Pease *et al.*, 1989) and MnP1-P.c. (Pribnow *et al.*, 1989) from *Phanerochaete chrysosporium*. A gap was introduced in the MnP1-P.s. sequence to optimize alignment. Residues matching the consensus are shaded.

tography allowed the separation of MnPs with molecular masses of 52.5 kDa and 57.5 kDa (Fig. 6, lanes 2 and 3). Then, on day 10, the average molecular mass increased to 60.2 kDa (Fig. 6, lane 4), to reach a final value of 62.5 kDa on day 25.

After obtaining the isolated MnPs for N-terminal sequencing, their molecular masses were determined by SDS-PAGE developed by silver staining. Isoforms from liquid cultures with pI values of 4.21, 4.31, 4.43, and 4.58 had a molecular mass of 52.5 kDa (data not shown). In turn, isoenzymes from wood chips with pIs of 3.20, 3.30 and 3.40 showed a major band corresponding to a molecular mass of 62.5 kDa, whereas bands of 62.5 and 56 kDa were observed in the sample of the isoform with a pI value of 3.53 (Fig. 7).

N-terminal sequence of some MnPs

The N-terminal sequences of MnPs with pI values of 4.21, 4.31, 4.43, and 4.58 from liquid cultures, as well as of MnPs with pI values of 3.20, 3.30 and 3.40 from solid cultures were determined. The four sequences of MnPs produced in liquid medium were highly similar although distinct, most mismatches involving residues with similar

properties (Fig. 8a). In contrast, the N-terminal sequences of the MnPs extracted from the wood chips could not be confidently differentiated, although the consensus sequence was different from the N-terminal sequence of any MnP from salt medium (Fig. 8b). Pairwise comparisons with all published MnP sequences showed that MnPs from *C. subvermispora* were clearly related to those of other white-rot fungi (Fig. 8c). Database searches (SwissProt and PIR release 77) showed no significant homology to any other proteins, including the lignin peroxidases.

DISCUSSION

The basidiomycete *Ceriporiopsis subvermispora* secretes several isoenzymes of MnP and laccase when growing either in liquid cultures or in wood chips. This observation provides additional evidence that the multiplicity of ligninolytic enzymes is a common feature among white-rot fungi, although the physiological significance of this phenomenon is not yet completely understood. The MnPs isolated from solid state cultures differ from those produced in salt medium, although at this stage it would be premature to speculate which isoforms are more relevant to lignin degradation.

The isoenzyme profile remains the same during growth of *C. subvermispora* in liquid cultures, although changes in the composition of the medium influence enzyme production by this fungus. For example, variations in the concentration of Mn(II) in salt medium or growth on a solid substrate lead to different isoenzyme patterns. Moreover, the isoenzyme profile in wood chips varies upon ageing of the culture. In early cultures, while externally added nutrients are still present, MnPs produced are similar in pI and molecular mass to those from liquid cultures. Later, when the nature of the growth substrate changes as the fungus proliferates into the wood, MnPs with lower pI values and higher molecular masses are secreted.

In *Phanerochaete chrysosporium*, the most thoroughly studied ligninolytic fungus, the expression of LiP and MnP genes is finely regulated. These enzymes are produced only during secondary metabolism. In addition, different isoenzyme patterns are observed upon ageing of the cultures (Pease & Tien, 1992), changes in the composition of the medium (Leisola *et al.*, 1987; Pease & Tien, 1992; Das & Reddy, 1990), or at different concentrations of Mn(II) (Bonnarme & Jeffries, 1990; Pease & Tien, 1992). Moreover, *Phanerochaete chrysosporium* produces a specific isoform of MnP during solid substrate degradation (Datta *et al.*, 1991). It is conceivable, therefore, that at least some features of the regulatory mechanisms of the ligninolytic systems of *Phanerochaete chrysosporium* and *C. subvermispora* may be similar.

However, the ligninolytic systems of these two fungi are different: *Phanerochaete chrysosporium* produces LiP but not laccase. Systematic searches for LiP activity in defined medium (Ruttimann *et al.*, 1992a, b; Ruttimann-Johnson *et al.*, 1993) and in wood chips (this work) have failed to detect production of LiP by *C. subvermispora*. With respect to laccase, the multiple isoforms isolated from liquid cultures of *C. subvermispora* appeared similar to those extracted from the wood chips. The latter were confined to early stages of fungal growth. It is possible that this enzyme may also be produced in late cultures, but for reasons such as adherence to wood or to the mycelium, inaccessibility to the extraction buffer, proteolytic inactivation, etc., its activity is underestimated.

In general, multiple isoforms may result from the expression of different genes, the expression of alleles of the same gene or post-translational processing. An example of the first possibility is provided by the LiPs from *Phanerochaete chrysosporium* (Cullen & Kersten, 1992). In contrast, the presence of allelic variants encoding isoenzymes with different pI values has been shown for glyoxal oxidase from *Phanerochaete chrysosporium* (P. Kersten & D. Cullen, unpublished). Finally, differential glycosylation of the same gene product may give rise to enzymes with different pI values. The N-terminal sequences of four isoenzymes of MnP produced by *C. subvermispora* in liquid cultures were highly similar, but distinct. Thus, these isoenzymes may represent the products of separate genes, although allelic variation cannot be formally excluded in a basidiomycete such as *C.*

subvermispora. In contrast, since the three MnP isoenzymes isolated from wood chips appear to exhibit the same N-terminal sequence, the difference in their pI values may reflect variations in their carbohydrate moieties. On the other hand, their higher molecular mass compared to the MnPs from liquid cultures also points to the presence of more than one gene. To date, a multiplicity of MnP genes has been demonstrated only in *Phanerochaete chrysosporium* (Pease & Tien, 1992; Pease *et al.*, 1989; Pribnow *et al.*, 1989). The role and interaction of these genes in lignin degradation remains to be established.

ACKNOWLEDGEMENTS

This work was financed by grants from National Science Foundation (NSF-INT 9121955), FONDECYT (0758/91) and the US Department of Energy (DE-FG02-87ER13712).

REFERENCES

- Akhtar, M., Attridge, M. C., Myers, G. C., Kirk, T. K. & Blanchette, R. A. (1992). Biomechanical pulping of loblolly pine with different strains of the white-rot fungus *Ceriporiopsis subvermispora*. *Tappi J* **75**, 105–109.
- Akhtar, M., Attridge, M. C., Myers, G. C. & Blanchette, R. A. (1993). Biomechanical pulping of loblolly pine chips with selected white-rot fungi. *Holzforschung* **47**, 36–40.
- Archibald, F. S. (1992). A new assay for lignin-type peroxidases employing the dye Azure B. *Appl Environ Microbiol* **58**, 3110–3116.
- Blanchette, R. A., Burnes, T. A., Eerdmans, M. M. & Akhtar, M. (1992). Evaluating isolates of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora* for use in biological pulping processes. *Holzforschung* **46**, 109–115.
- Bonnarme, P. & Jeffries, T. W. (1990). Mn(II) regulation of lignin peroxidases and manganese-dependent peroxidases from lignin-degrading white rot fungi. *Appl Environ Microbiol* **56**, 210–217.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Coll, P. M., Taberner, C., Santa-María, R. & Pérez, P. (1993). Characterization and structural analysis of the laccase I gene from the newly isolated ligninolytic basidiomycete PM1 (CECT 2971). *Appl Environ Microbiol* **59**, 4129–4135.
- Cullen, D. & Kersten, P. (1992). Fungal enzymes for lignocellulose degradation. In *Applied Molecular Genetics of Filamentous Fungi*, pp. 100–131. Edited by J. R. Kinghorn & G. Turner. London: Chapman and Hall.
- Das, S. B. & Reddy, C. A. (1990). Characterization of extracellular peroxidases produced by acetate-buffered cultures of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *FEMS Microbiol Lett* **69**, 221–224.
- Datta, A., Bettermann, A. & Kirk, T. K. (1991). Identification of a specific manganese peroxidase among ligninolytic enzymes secreted by *Phanerochaete chrysosporium* during wood decay. *Appl Environ Microbiol* **57**, 1453–1460.
- Forrester, I. T., Grabski, A. C., Mishra, C., Kelley, B. L., Strickland, W. N., Leatham, G. F. & Burgess, R. R. (1990). Characteristics and N-terminal amino acid sequence of a manganese peroxidase purified from *Lentinula edodes* cultures grown on commercial wood substrate. *Appl Microbiol Biotechnol* **33**, 359–365.
- Galliano, H., Gas, G., Seris, J. L. & Boudet, A. M. (1991). Lignin degradation by *Rigidoporus lignosus* involves synergistic action of

two oxidizing enzymes: Mn peroxidase and laccase. *Enz Microb Technol* **13**, 478–482.

Geiger, J. P., Rio, B., Nandris, D. & Nicole, M. (1986). Laccases of *Rigidoporus lignosus* and *Pbellinus noxius*. *Appl Biochem Biotechnol* **12**, 121–133.

Imura, Y., Takenouchi, K., Nakamura, M., Kawai, S., Katayama, Y. & Morohoshi, M. (1992). Cloning and sequence analysis of laccase genes and its use for a expression vector in *Coriolus versicolor*. In *Biotechnology in the Pulp and Paper Industry*, pp. 427–431. Edited by M. Kuwahara & M. Shimada. Tokyo: UNI Publishers.

Johansson, T. & Nyman, P. O. (1993). Isozymes of the lignin peroxidase and manganese (II) peroxidase from the white-rot basidiomycete *Trametes versicolor*. I. Isolation of enzyme forms and characterization of physical and catalytic properties. *Arch Biochem Biophys* **300**, 49–56.

Johansson, T., Welinder, K. G. & Nyman, P. O. (1993). Isozymes of the lignin peroxidase and manganese (II) peroxidase from the white-rot basidiomycete *Trametes versicolor*. II. Partial sequences, peptide maps and aminoacid and carbohydrate compositions. *Arch Biochem Biophys* **300**, 57–62.

Karhunen, E., Kantelinen, A. & Niku-Paavola, M.-L. (1990). Mn-dependent peroxidase from the lignin-degrading white-rot fungus *Phlebia radiata*. *Arch Biochem Biophys* **279**, 25–31.

Kojima, Y., Tsukuda, Y., Kawai, Y., Tsukamoto, A., Sugiura, J., Sakaino, M. & Kita, Y. (1990). Cloning, sequence analysis and expression of ligninolytic phenol oxidase genes of the white-rot basidiomycete *Coriolus hirsutus*. *J Biol Chem* **265**, 15224–15230.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Leatham, G. F. (1983). A chemically defined medium for the fruiting of *Lentinus edodes*. *Mycologia* **75**(5), 905–908.

Leisola, M. S. A., Kozulic, B., Meussdoerffer, F. & Fiechter, A. (1987). Homology among multiple extracellular peroxidases from *Phanerochaete chrysosporium*. *J Biol Chem* **262**, 419–424.

Maltseva, O. V., Niku-Paavola, M.-L., Leontievsky, A. A., Myasodova, N. M. & Golovleva, L. A. (1991). Ligninolytic enzymes of the white-rot fungus *Panus tigrinus*. *Biotechnol Appl Biochem* **13**, 291–302.

Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* **262**, 10035–10038.

Morohoshi, N. (1991). Laccases from the ligninolytic fungus *Coriolus versicolor*. In *Enzymes in Biomass Conversion*, ACS Symposium Series 460, pp. 207–204. Edited by G. Leatham & M. E. Himmel. Washington, DC: American Chemical Society.

Niku-Paavola, M.-L., Karhunen, E., Salola, P. & Raunio, V. (1988).

Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. *Biochem J* **254**, 877–884.

Orth, A., Royse, D. J. & Tien, M. (1993). Ubiquity of lignin-degrading peroxidases among various wood-degrading fungi. *Appl Environ Microbiol* **59**, 4017–4023.

Otjen, L., Blanchette, R., Effland, M. & Leatham, G. (1987). Assessment of 30 white-rot basidiomycetes for selective lignin degradation. *Holzforschung* **41**, 343–349.

Pease, E. A. & Tien, M. (1992). Heterogeneity and regulation of manganese peroxidases from *Phanerochaete chrysosporium*. *J Bacteriol* **174**, 3532–3540.

Pease, E. A., Andrawis, A. & Tien, M. (1989). Manganese-dependent peroxidases from *Phanerochaete chrysosporium*: primary structure deduced from cDNA sequence. *J Biol Chem* **264**, 13531–13535.

Péridé, F. H. & Gold, M. H. (1991). Manganese regulation of manganese peroxidase expression and lignin degradation by the white-rot fungus *Dichomitus squalens*. *Appl Environ Microbiol* **57**, 2240–2245.

Pribnow, D., Mayfield, M. B., Nipper, V. J., Brown, J. A. & Gold, M. H. (1989). Characterization of cDNA encoding a manganese peroxidase from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *J Biol Chem* **264**, 5036–5040.

Ruttimann, C., Schwember, E., Salas, L., Cullen, D. & Vicuña, R. (1992a). Ligninolytic enzymes of the white-rot basidiomycetes *Phlebia brevispora* and *Ceriporiopsis subvermispora*. *Biotechnol Appl Biochem* **16**, 64–76.

Ruttimann, C., Salas, L. & Vicuña, R. (1992b). Studies on the ligninolytic system of the white-rot fungus *Ceriporiopsis subvermispora*. In *Biotechnology in the Pulp and Paper Industry*, pp. 243–248. Edited by M. Kuwahara & M. Shimada. Tokyo: UNI Publishers.

Ruttimann-Johnson, C., Salas, L., Vicuña, R. & Kirk, T. K. (1993). Extracellular enzyme production and synthetic lignin mineralization by *Ceriporiopsis subvermispora*. *Appl Environ Microbiol* **59**, 1792–1797.

Ruttimann-Johnson, C., Cullen, D. & Lamar, R. (1994). Manganese peroxidases of the white-rot fungus *Phanerochaete sordida*. *Appl Environ Microbiol* **60**, 599–605.

Saloheimo, M., Niku-Paavola, M.-L. & Knowles, J. K. C. (1991). Isolation and structural analysis of the laccase gene from the lignin-degrading fungus *Phlebia radiata*. *J Gen Microbiol* **137**, 1537–1544.

Tien, M. & Kirk, T. K. (1984). Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization and catalytic properties of a unique H₂O₂-requiring oxygenase. *Proc Natl Acad Sci USA* **81**, 2280–2284.

Received 16 March 1994; revised 5 May 1994; accepted 12 May 1994.