

Enzymology and molecular genetics of the ligninolytic system of the basidiomycete *Ceriporiopsis subvermispora*

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Lignin, the most abundant renewable source of aromatic carbon on earth, consists in a highly irregular three-dimensional biopolymer of oxygenated phenylpropanoid units. In natural environments, lignin is only degraded efficiently by some fungi belonging to the group of basidiomycetes. These microorganisms secrete an array of oxidases and peroxidases for this purpose, which may be produced in various combinations. This article summarizes our studies on a particular strain called *Ceriporiopsis subvermispora*, a fungus which is highly aggressive towards lignin when growing on wood.

LIGNIN is the second most abundant deposit of organic carbon in the biosphere, being surpassed only by cellulose. It has been estimated that the amount of lignin in terrestrial plants approaches 4×10^{11} tons and that every year 3×10^{10} tons of the polymer are deposited *de novo* in plant tissues. Therefore, lignin biodegradation is a key process for the recycling of carbon in nature.

Lignin is a highly irregular biopolymer with an undefined structure. It is composed of oxygenated phenylpropanoid units which are linked among them through various types of C–C and C–O–C bonds. Due to these unusual features, the macromolecule is rather refractory to biodegradation. The term white-rot fungi has been applied to certain ligninolytic basidiomycetes with a relatively high selectivity to degrade lignin in wood. This is the only group of microorganisms with the capacity to break down lignin extensively, all the way to carbon dioxide and water. These fungi produce a set of enzymes which are directly involved in lignin decay. Among these are a phenol-oxidase termed laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP). Each species secretes a particular assortment of this enzymatic machinery to the medium in which it is growing. Thus, some strains produce LiP, MnP and lac-

case, others produce only MnP and laccase, or LiP plus laccase, etc. The components of the ligninolytic system of several strains have been isolated and characterized. In addition, some of the corresponding genes have been cloned and sequenced.

Several strains of white-rot fungi, e.g. *Phanerochaete chrysosporium*^{1,2}, *Trametes versicolor*³ and *Ceriporiopsis subvermispora*⁴, have been employed as model systems to study ligninolysis⁵. The goal of these studies has been not only to clarify the enzymatic mechanisms underlying lignin biodegradation, but also the development of some possible biotechnological applications in the pulp and paper industry. Among these, biopulping of wood chips, biobleaching of pulps and enzymatic treatment of recycling fibers appear highly promising. Moreover, the ability of white-rot fungi to mineralize xenobiotic compounds offers the perspective of their use in bioremediation processes.

Among the strains mentioned above, *C. subvermispora*⁶ (Figure 1) has been evaluated with several other fungal strains in biomechanical pulping experiments at a laboratory scale. In these assays, *C. subvermispora* consistently performed better than the other strains tested^{7–9}, which has led to the filing of patents related to the use of this fungus in biopulping. The aim of the pre-



Figure 1. *Ceriporiopsis subvermispora* growing on wood chips of radiata pine at a laboratory scale.

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sent article, which is based mainly on work conducted by our group, is to describe some general aspects of the ligninolytic system of *C. subvermispora*.

Biochemical features of the ligninolytic machinery of *C. subvermispora*

Some white-rot fungi produce their ligninolytic enzymes in response to depletion of some nutrient, i.e. during secondary metabolism. This is not the case of *C. subvermispora*, which expresses its ligninolytic machinery in a constitutive fashion¹⁰. *C. subvermispora* produces high titers of MnP and laccase, but not detectable LiP, when growing in different media⁸. MnP is a heme-containing glycoprotein that oxidizes Mn(II) to Mn(III) (ref. 11), which in turn oxidizes phenolic substrates. The wide distribution of this enzyme among white-rot fungi strongly suggests that it plays an important role in the process of lignin biodegradation¹². However, a comparison of five strains of *C. subvermispora* showed that although MnP levels in cultures were similar, significant differences were observed in their abilities to degrade lignin¹⁰.

In liquid cultures of defined composition *C. subvermispora* secretes up to seven MnP isoenzymes with pI values ranging between 4.1 to 4.6. The isoelectrofocusing (IEF) pattern does not change throughout the growth period¹³. However, when the fungus is grown on wood chips, the isoenzyme pattern is dramatically affected after the second week, probably due to a shortage of nutrients. In this case, four MnP isoenzymes within a pI range of 3.2 to 3.53 are observed. Initially, four N-terminal sequences were obtained from MnP isoenzymes produced in liquid cultures and three N-terminal sequences were identified in MnPs isolated from wood chips, suggesting the expression of different genes or alleles¹³. We attempted an enzymological characterization of several of these isoenzymes, hoping to reveal differences that might be important for the process of ligninolysis. We found that the requirement for manganese by these MnPs varied according to the chemical structure of the substrate employed¹⁴. For example, in the presence of vanillylacetone, the phenolic compound routinely used in MnP assays¹⁵, the isoenzymes were activated between 12.6 to 108 fold by manganese, with a more pronounced effect of the metal observed with the isoenzymes from liquid cultures¹⁴. Also, oxidation of *p*-anisidine and *o*-dianisidine with some of the isoenzymes proceeded at significant rates in the absence of manganese. This would imply that MnPs from *C. subvermispora* behave differently from MnPs from *P. chrysosporium*, which have an absolute requirement for manganese for the completion of the catalytic cycle of the enzyme^{11,16,17}. In contrast, oxidation of guaiacol by any of the isoenzymes in the absence of manganese was negligible¹⁴.

The reactions catalysed by MnP are accelerated by chelators, which facilitate the turnover of the enzyme by removing Mn(III) from the active site and also by stabilizing Mn(III) in solution¹⁷. A typical chelator is oxalate, which is commonly secreted as a metabolite by white-rot fungi^{18–20} (Figure 2). Maximal stimulation of MnPs from *P. chrysosporium* and *C. subvermispora* takes place at concentrations of oxalate in the range of 0.5–2 mM (ref. 21), similar to its physiological concentration in liquid cultures²⁰. MnPs from *C. subvermispora* are also stimulated by oxalate in the absence of Mn(II), although at a higher concentration of this metabolite (10 mM). This effect is difficult to explain, although kinetic evidence indicates that chelators do not bind directly to the iron of the heme group, suggesting the existence of a pocket close to the heme²¹. Sundaramoorthy and coworkers have suggested that oxalate may interact with the enzyme by replacing two water molecules located near the Mn(II) binding site²².

The reason for the redundancy of MnP isoenzymes, in *C. subvermispora* as well as in several other fungi, is still uncertain. A plausible explanation could be that isoenzymes have different affinities for particular substrates. Two main kind of evidences support this hypothesis. First, at least in the case of *C. subvermispora*, each isoenzyme shows a different requirement for manganese¹⁷. On the other hand, based on the structure of the aromatic binding pocket described for the isoenzyme H4 from *P. chrysosporium*²³, we conducted an *in silico* modeling of MnP1, MnP2 and MnP3 from *C. subvermispora* (Figure 3). This exercise revealed important differences in the aminoacids located around the pocket close to the heme group. It is conceivable that these differences in primary structure could lead to particular affinities for different substrates, although there is no experimental support for this assertion. The production of recombinant isoenzymes subjected to site-directed mutagenesis could provide valuable information about this matter. In this regard, we have successfully cloned the *Cs-mnp1* gene in *Aspergillus nidulans* (see below).

As mentioned above, *C. subvermispora* also produces laccase in addition to MnP. Laccase is a copper-

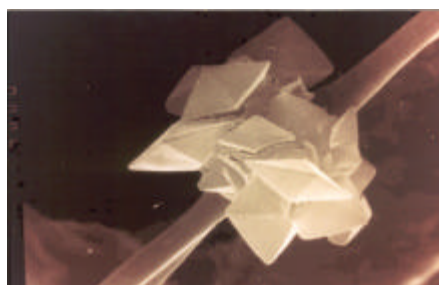


Figure 2. Electron micrograph of a hypha of *C. subvermispora* showing bright crystals of oxalate secreted by the fungus while it grows on wood.

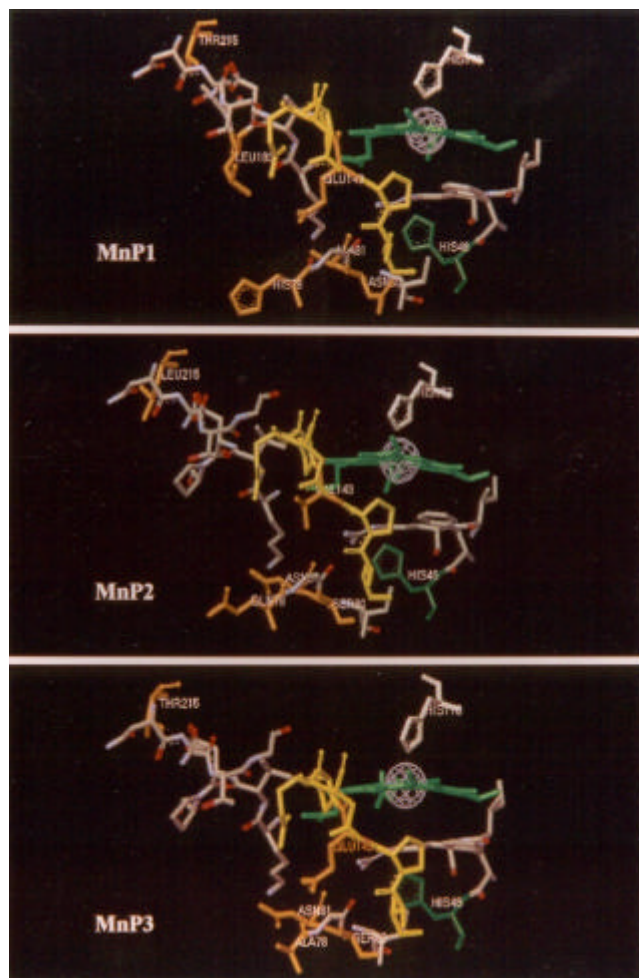


Figure 3. Molecular models of the three manganese peroxidases from *C. subvermispora* showing the corresponding active sites. In orange colour are the amino acid residues that are different in the three MnPs; the heme group is shown in green and the putative aromatic binding site is in yellow. The sphere shows the Fe atom. The modeling was carried out using the GeneQuiz Web server considering the crystallographic data from MnP H4 from *P. chrysosporium*.

containing phenoloxidase that is also able to attack indirectly non-phenolic compounds in the presence of appropriate mediators²⁵. We have shown that *C. subvermispora* secretes four isoenzymes of laccase with pIs between 3.65 and 3.45 when growing in liquid cultures of defined composition¹³. Also, a fifth isoform of pI 4.70 is occasionally detected¹³. The N-terminal sequencing of these suggested that the fungus may possess a single gene coding for this enzyme¹³. The levels of laccase increase two-fold in the presence of *p*-anisidine and are severely affected when Mn(II) or Cu(II) ions are omitted from the medium. Also, in rich medium laccase activity is ten-fold higher than in salt medium and in this case its titers are not affected by the external addition of *p*-anisidine or manganese²⁶.

With respect to LiP, we have been unable to detect it with the substrates commonly used to measure the levels of this enzyme. However, we have reported the PCR

amplification and sequencing of LiP-like genes, although we failed to detect LiP-related transcripts²⁷. The role of these LiP-like genes in *C. subvermispora* remains to be established. It is possible that these genes may be expressed under conditions that were not assayed or that their products are more susceptible to degradation enzymes such as ribonucleases and proteases.

As any other ligninolytic fungus, *C. subvermispora* requires a system to produce extracellular hydrogen peroxide to be used as an oxidant by MnPs. An enzyme called glyoxal oxidase that acts in conjunction with LiP and possesses a wide substrate specificity has been identified in *P. chrysosporium*^{28,29}. Other fungi produce glucose oxidase, pyranose oxidase, aryl alcohol oxidase or methanol oxidase for the same purpose³⁰⁻³³. We were unable to detect any of these enzymes in the extracellular fluid of cultures of *C. subvermispora*. For this reason, we considered the possibility that in this case, MnP

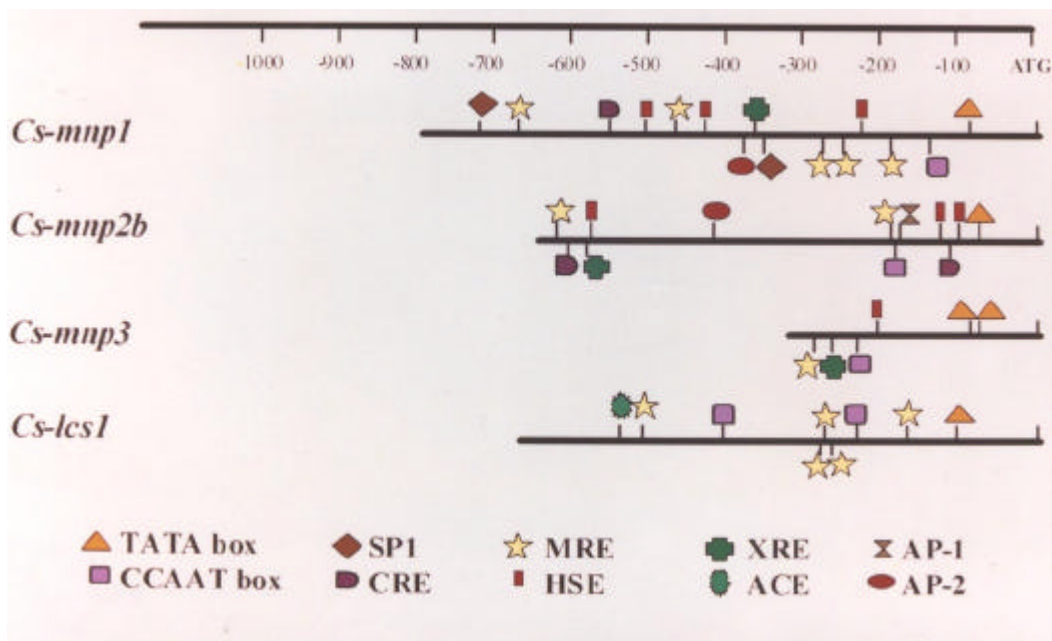


Figure 4. Putative promoter elements found in the MnP and laccase genes from *C. subvermispora*.

might act as an oxidase of certain metabolites to provide itself with hydrogen peroxide³⁴. Indeed, we detected glyoxylate and oxalate in the extracellular medium³⁴, which are known to be oxidized *in vitro* by MnP with the concomitant production of H₂O₂ and Mn(III)^{35–37}. Also, we observed a correlation between the MnP titers and mineralization of ¹⁴C-oxalate in cultures containing low concentrations of nitrogen³⁴. An additional proof in support of our hypothesis was the finding that in the presence of oxalate and glyoxylate, MnP could oxidize a lignin model substrate (phenol red) in the absence of externally added hydrogen peroxide³⁴. Due to the importance of organic chelators for the completion of the catalytic cycle of MnP, it is not surprising that compounds such as oxalate and glyoxylate are secreted by this fungus. It is likely that other as yet unidentified metabolites may also contribute to the production of hydrogen peroxide in *C. subvermispora*.

Molecular genetics of the ligninolytic system of *C. subvermispora*

We have cloned and sequenced four genes coding for MnP: *Cs-mnp1*, *Cs-mnp2A*, *Cs-mnp2B* and *Cs-mnp3* (refs 38, 39). The four genes have 5 to 7 short intervening sequences ranging in size between 53 and 67 base pairs. The splicing junctions adhere to the GT-AG rule, although some of their internal lariat formation sites do not show a perfect match with the consensus sequence CTRAY (ref. 40).

The upstream regions of the *Cs-mnp* genes contain common proximal elements, such as a TATA box, AP1 and AP2 sites, as well as putative sites for transcription regulation by metals (MRE), cAMP (CRE), xenobiotics (XRE) and heat shock (HSE) (Figure 4). To date, no functional characterization of these regulatory elements in *C. subvermispora* has been conducted. We are currently searching for nuclear factors that may bind specifically to one or more of these putative regulatory elements. Based on Northern analysis, transcription of the MnP genes *Cs-mnp2A* and *Cs-mnp2B*, but not that of *Cs-mnp3*, is activated in the presence of manganese in the culture medium. The mechanism by which manganese regulates transcription of these genes is unknown.

The corresponding mature MnP proteins contain between 364 and 366 aminoacids, preceded by leader sequences of 21–24 aminoacids. Comparative analysis of enzymes MnP2A and MnP2B suggests that genes *Cs-mnp2A* and *Cs-mnp2B* are alleles, since these proteins differ only in five residues³⁹. We have recently identified alleles of genes *Cs-mnp1* and *Cs-mnp3* as well⁴¹. In turn, the enzymes MnP1 and MnP3 differ in 25 aminoacids. All these enzymes contain the conserved proximal and distal histidines, and the distal arginine, as it is common among peroxidases since these are the residues interacting with the heme group. In turn, two glutamic acid and one aspartic acid residues that conform the manganese-binding site²² are also conserved. All four enzymes also possess ten cysteine residues at positions that are conserved in MnPs from other white-rot fungi.

The distinctive feature of all these MnPs from *C. subvermispora* is the insertion of four aminoacids following the glycine 226 residue³⁹.

In order to obtain the product of a single gene free of contaminating activities, as well as to be able to conduct site-directed mutagenesis, we used an heterologous expression system with *Aspergillus nidulans* as a host. This microorganism has been previously described to produce recombinant peroxidases that are secreted to the extracellular medium⁴². We transformed *A. nidulans* with the cDNA of *Cs-mnp1* under the control of the α -amylase promoter from *A. oryzae* and the sequence for transcription termination from the glucoamylase gene, both from *Aspergillus awamori*⁴³. Analysis of transformants by Southern hybridization were consistent with tandem duplications at multiple loci. A peak of activity of recombinant MnP1 was observed at 48 h after inoculation and the yield of enzyme varied between 3 and 6 mg/l. The highest titers were obtained with 5% maltose as carbon source and in the presence of 0.5 mg/ml of hemin added at the time of inoculation. Surprisingly, IEF of rMnP1 showed several distinct bands. These were not due to proteolytic cleavage, since the protein migrated as a single band in polyacrylamide gels run under denaturing conditions. This clearly indicates that expression of a single gene may give rise to isoenzyme multiplicity, as measured by IEF. The nature of the posttranslational modifications responsible for this pattern is presently unknown. It is likely that it consists of differential phosphorylation, since sugar moieties do not influence the isoelectric point of proteins in a significant fashion.

On the other hand, a gene coding for laccase in *C. subvermispora* (*Cs-lcs1*) has also been cloned and sequenced. Southern blot analysis showed that *Cs-lcs1* is the only gene coding for laccase in this fungus⁴⁴. *Cs-lcs1* contains an open reading frame of 2,215 base pairs, encoding a mature protein of 499 aminoacids with a signal peptide of 21 residues. The gene possesses 11 introns with splicing junctions and internal lariat forming sites adhering to the GT-AG and CTRAY rules, respectively. Comparison of the intron/exon structure of several laccase genes from basidiomycetes indicates a high degree of conservation, being *Cs-lcs1* most similar to the laccase genes *C.h.-po* from *Coriolus hirsutus*⁴⁵, *PM1 lac1* from basidiomycete PM1 (ref. 46) and *I-62 lcc1* from basidiomycete CECT 20197 (ref. 47). The upstream region of *Cs-lcs1* contains a TATA box, two CAAT sites, five putative MREs and an ACE1 element. The latter has been identified in *Saccharomyces cerevisiae* as a recognition element for a transcription factor responding to copper and silver, but not to zinc. In agreement with this evidence, transcription of *Cs-lcs1* is activated by copper and silver, as shown by Northern blot and reverse transcription followed by DNA amplification analysis⁴⁴.

Future work

Several aspects, most of them related with regulation of the ligninolytic system of *C. subvermispora*, will be the focus of our attention in the near future. Our first priority is to solve the mechanism by which manganese regulates the expression of MnP. The same applies to the regulation of laccase expression by copper. In the first case, manganese might act at the level of transcription, although it could also exert some influence on the stability of the mRNAs transcribed from the *Cs-mnp* genes. The latter assumption is based on a similar role described for iron and magnesium in other biological systems⁴⁸⁻⁵⁰. In contrast, if manganese is affecting the level of transcription of *Cs-mnp* genes, we would have to look for the elements located in the upstream region of these genes that are involved in this effect. At the same time, we will have to isolate and characterize the transcription factors acting at these sites. To date, transcription factors acting in response to manganese have not been described. On the other hand, the presence of a putative ACE box in the promoter region of *Cs-lcs1* strongly suggests that copper is acting at the level of transcription. For this reason, we are now concentrating our efforts on the identification of protein(s) binding specifically to this ACE site by means of electromobility shift assays. Preliminary experiments conducted by incubating crude extracts with a DNA probe containing the ACE sequence are already showing promising results.

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