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ORIGINAL ARTICLE

Gene cloning, heterologous expression, *in vitro* reconstitution and catalytic properties of a versatile peroxidase

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Abstract

The gene of a peroxidase described as being involved in carotenoid degradation was cloned from a strain that was conserved as *Lepista irina* (CBS 458.79). Gene sequencing revealed high nucleotide and amino-acid identity with *Pleurotus eryngii* gene *vpl*, which encodes a versatile peroxidase with unique catalytic properties, and only reported in *Pleurotus* and *Bjerkandera* species. Re-identification of the supposed *L. irina* strain revealed that, in fact, it is a *P. eryngii* strain. The new *P. eryngii* peroxidase was expressed in *Escherichia coli*, and the recombinant protein folded in the presence of cofactor to obtain the active form. The purified enzyme was able to oxidize Mn^{2+}, veratryl alcohol, substituted phenols, and both low and high redox-potential dyes, demonstrating that it belongs to the versatile peroxidase family (named VPL3). These catalytic properties agreed with the presence of both Mn^{2+} and aromatic-substrate oxidation sites in its molecular structure.

Keywords: *Versatile peroxidase,* Pleurotus eryngii, *heterologous expression,* in vitro *activation, catalytic properties, lignin-degrading enzymes*

Introduction

Peroxidases catalyzing lignin oxidation by hydrogen peroxide, a process that has been described as an "enzymatic combustion" (Kirk & Farrell 1987), play a key role in carbon recycling in terrestrial ecosystems. Due to the high redox potential required for oxidative degradation of lignin, these heme proteins also have high biotechnological interest as industrial and environmental biocatalysts (Cullen & Kersten 2004).

Up to three different types of lignin-degrading peroxidases are secreted by white-rot basidiomycetes – lignin peroxidases (LiP), manganese peroxidases (MnP) and versatile peroxidases (VP) – differing in their reducing substrates. LiP oxidizes the non-phenolic aromatic units of lignin (Kirk et al. 1986) as well as recalcitrant aromatic pollutants (Hammel et al. 1986). By contrast, MnP only oxidizes Mn^{2+} that is necessary to complete the catalytic cycle (Wariishi et al. 1988). The resulting Mn^{3+} is chelated by dicarboxylic acids secreted by fungi, and acts as a diffusible oxidizer of phenolic and non-phenolic aromatic compounds, the latter in the presence of peroxidisable lipids (Wariishi et al. 1989; Bao et al. 1994). VP, the third type of lignin-degrading peroxidase more recently described in *Pleurotus* (Martínez et al. 1996) and *Bjerkandera* species (Mester & Field 1998), combines catalytic properties of LiP, MnP and other peroxidases, oxidizing substituted phenols, and, in addition, has the ability to directly oxidize high redox-potential dyes (Heinfling et al. 1998b).

The above peroxidases share the same general tertiary folding and helical topography (Gold et al. 2000; Martínez 2002), and the differences in catalytic properties are determined by certain structural details defined by a few amino acid residues. So, LiP has an exposed tryptophan responsible for non-phenolic aromatic compound oxidation (Blodig et al. 1998, 2001), whereas MnP presents a manganese binding site formed by three acidic residues near the internal propionate of heme (Kishi et al. 1996). Combination of LiP and MnP structural details in the VP structure explains some of its catalytic

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properties (Camarero et al. 1999; Ruiz-Dueñas et al. 1999b, 2007; Banci et al. 2003; Pérez-Boada et al. 2005). However, VP structural determinants responsible for oxidation of some substrates, as well as for differences in kinetic constants when compared with LiP or MnP, remain unidentified.

Structure–function studies aimed at explaining the catalytic properties of these peroxidases have been based on knowledge of their cDNA or genomic sequences, and the development of expression systems for producing variants mutated in residues potentially involved in substrate oxidation, and other studies. In the present work, we cloned the gene encoding a peroxidase described in liquid cultures of the basidiomycete *Lepista irina* (family Tricholomataceae) as involved in the biosynthesis of carotenoid-derived flavors (Zorn et al. 2003). This gene exhibits very high sequence identity with the gene *vpl* of *Pleurotus eryngii* (family Pleurotaceae) encoding a lignin-degrading VP. The final objective was to investigate if this peroxidase also exhibited VP-type catalytic properties, using heterologous expression in *Escherichia coli* and *in vitro* activation.

Materials and methods

Fungal strain, growth conditions and DNA/cDNA amplification

Strain CBS 458.79 conserved as *L. irina* was provided by the Centraalbureau voor Schimmelcultures (CBS; Utrecht, The Netherlands) in April 2004. This fungal culture was isolated by R. Kühner (20 September 1971) and conserved at the CBS from August 1979. Taxonomic re-identification by request of the authors was also performed at CBS based on ribosomal DNA sequencing.

The fungus was grown in glucose–peptone medium at 28°C and 180 rev min^{-1} for 7 days (Martínez et al. 1996). DNA was extracted (González et al. 1992) and polymerase-chain reactions (PCR) were run using *Pfu*Turbo DNA polymerase (Stratagene) and the following primers: (i) sense 5′ (5′-GGGAA TTCCATATGGCAACTTGCGCCGACGG-ACG-3′) and antisense 3′ (5′-GGAAGATCTTTACGAT CCAGGGACGGGAGG-3′) primers designed according to the cDNA sequence deposited as encoding the *L. irina* carotene-degrading peroxidase (AJ515245); and (ii) sense primer (5′-GGGAATTCCATATGTT TGCGCCATT GCTGACG-3′) designed to anneal at the promoter region of *P. eryngii vpl1* (AF007223) and the above 3′ antisense primer. Each set of primers included *Nde*I and *Bgl*II restriction sites in their 5′ ends. The PCR program included one initial cycle of denaturation (2 min at 94°C), annealing (1 min at 60°C), and extension (5 min at 68°C), followed by 25 additional cycles of denaturation (35 sec at 94°C), annealing and extension (the latter two under the initial conditions). Total RNA was isolated from CBS 458.79 cultures using Ultraspec RNA Isolation System (Biotecx). This RNA (0.5 μg) was used as template, and the above N and C terminal primers were used in a reverse transcription–polymerase chain reaction (RT-PCR) under the described conditions. The amplified DNA and cDNA were cloned into *Nde*I/*Bgl*II sites of the expression vector pFLAG1 (International Biotechnologies Inc.) and sequenced using an automated sequencer ABI377.

Heterologous expression and enzyme purification

E. coli W3110 transformed with pFLAG1 harboring the cDNA deposited as encoding the *L. irina* carotene-degrading peroxidase (AJ515245) was used for heterologous expression using Terrific Broth (Sambrook & Russell 2001). The recombinant enzyme, produced after induction of cultures with 1 mM IPTG, accumulated in inclusion bodies, and was folded *in vitro* using 0.15 M urea, 5 mM Ca^{2+}, 20 μM hemin, 0.5 mM oxidized glutathione, 0.1 mM dithiothreitol, and 0.1 mg mL^{-1} protein concentration, at pH 9.5 (Pérez-Boada et al. 2002). Active enzyme was purified by Resource-Q chromatography using a 0–0.3 M NaCl gradient (2 mL min^{-1}, 20 min) in 10 mM sodium tartrate (pH 5.5) supplemented with 1 mM $CaCl_2$.

Enzymatic activities

Oxidation of Mn^{2+} was estimated by the formation of Mn^{3+} tartrate complex (ε_{238} 6500 M^{-1} cm^{-1}) using 100 mM sodium tartrate (pH 5) and 0.1 mM $MnSO_4$. Mn-independent activity on 2 mM veratryl (3,4-dimethoxybenzyl) alcohol (veratraldehyde ε_{310} 9300 M^{-1} cm^{-1}) was estimated at pH 3.0, and on 0.1 mM Reactive Black 5 (ε_{598} 30 000 M^{-1} cm^{-1}), 0.1 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (cation radical ε_{420} 36 000 M^{-1} cm^{-1}), and 2,6-dimethoxyphenol (dimeric product ε_{469} 55 000 M^{-1} cm^{-1}) were estimated at pH 3.5 (Heinfling et al. 1998b). All activities were measured at 24°C in the presence of 0.1 mM H_2O_2, and one activity unit was defined as the amount of enzyme oxidizing 1 μmol of substrate per minute. Steady-state kinetic constants (mean and 95% confidence limits) were calculated according to oxidation of the reducing substrates, and fitting the pseudo-first order rates of product formation to the Michaelis–Menten model by double-reciprocal plots.

Protein modeling and sequence comparison

A theoretical molecular structure of the peroxidase under study was obtained by homology modeling using the atomic co-ordinates of *P. eryngii* VPL2 (PDB entry 2BOQ), *Phanerochaete chrysosporium* LiPH8 (1LLP) and LiPH2 (1QPA), and *Coprinopsis cinerea* (synonym *Coprinus cinereus*) peroxidase (CIP) (1ARP) crystal structures as templates. Comparative modeling and energy minimization were carried out with the programs ProModII and Gromos96 (Schwede et al. 2003). Sequences of basidiomycete peroxidases (a total of 48 mature proteins) were compared and phylograms were obtained by the UPGMA method from Kimura distances using the Pile-up, Distances and Tree utilities of the GCG package.

Results and discussion

Gene cloning and analysis of nucleotide and amino acid sequences

PCR amplification using template DNA from CBS 458.79, which was conserved at the CBS fungal culture collection (http://www.cbs.knaw.nl) as *L. irina*, and the sense 5′ and antisense 3′ primers designed according to the cDNA sequence deposited as encoding the carotene-degrading peroxidase of this fungus (Zorn et al. 2003), yielded a fragment of approximately 2 kb. Sequencing of this DNA product fully agreed with the sequence of the previously deposited carotene-degrading peroxidase cDNA (AJ515245) interrupted by 15 introns. Moreover, the sequence (intron–exon region) showed a very high nucleotide identity (97%) to the corresponding region of the allelic variants *vpl1* (AF007223) and *vpl2* (AF007224) of gene *vpl* encoding a VP in the lignin-degrading fungus *P. eryngii* (CBS 613.91) (Ruiz-Dueñas et al. 1999b). The new gene (called *vpl3*) and the above *vpl1* and *vpl2* nucleotide and predicted amino acid sequences were aligned for comparison (see region corresponding to *vpl3* nucleotides 1–1909 in Figure 1). The predicted sequence of the protein encoded by the new gene contains 361 amino acid residues, including a signal peptide formed by 30 amino acids that was identified after N-terminal sequencing of the mature fungal peroxidase (Zorn et al. 2003). Changes in six amino acids were observed after comparing the three sequences, although only three of them (Thr147, Lys206 and Glu309) were specific for the peroxidase encoded by the gene under study.

When PCR amplification was performed using the same DNA and a primer corresponding to a sequence situated 590 bp upstream of the ATG start codon of *P. eryngii vpl1*, together with the cDNA C-terminus primer, the amplified DNA included 549 bp of the promoter region in addition to the intron–exon sequence. A multiple alignment of the full-length sequence of the new gene and those of *P. eryngii vpl1* and *vpl2* is shown in Figure 1, which also shows the predicted amino acid sequences and some conserved elements in the promoter region. The intron and promoter sequences also showed 97% sequence identity to the corresponding regions in the two other cloned *vpl* genes, although the promoter was slightly more similar in sequence to that of *vpl2* due to the presence of a putative metal response element, which is absent from *vpl1*. No other differences were observed in the putative response elements previously reported in *vpl* (Ruiz-Dueñas et al. 1999b), which are listed in the legend of Figure 1. According to this, the regulation of expression of the new peroxidase gene should be affected by the same factors involved in transcription of *vpl* from *P. eryngii* (Ruiz-Dueñas et al. 1999a).

Due to the extremely high sequence identity at all levels (exon, intron, amino acid and promoter sequences) between the genes from *P. eryngii* and the supposed *L. irina* strain, a re-identification of the latter (CBS 458.79) based on sequence comparison of the ITS region (ITS1+5.8S+ITS2) of its nuclear ribosomal DNA (amplified and sequenced using the universal primers ITS1 and ITS4) with those of reference *L. irina* and *P. eryngii* strains (and available from databases) was performed revealing that it does not correspond to a *L. irina*, but to a *P. eryngii* strain, which maintains the same reference number in the CBS fungal culture collection. Therefore, the genomic sequence of *vpl3* was deposited in the nucleotide sequence database as a *P. eryngii* gene under accession number DQ056374. The high nucleotide identity between *vpl1*, *vpl2* and *vpl3* (Figure 1) with differences in six codons within the coding (exon) region (resulting in only three altered amino acid residues), and the highly conserved intron–exon structure suggest that *vpl3* could be a new allelic variant of gene *vpl*, although no definitive genetic evidence is available. The catalytic properties of the corresponding peroxidase, described by Zorn et al. (2003), were investigated and compared with typical *P. eryngii* VP (Heinfling et al. 1998b), after its heterologous expression as described below.

Heterologous expression, purification and catalytic properties

The high sequence similarity between the peroxidase under study and those previously reported from liquid cultures of *P. eryngii* (VPL1 and VPL2) (Martínez et al. 1996; Ruiz-Dueñas et al. 1999b), as well as the results from CBS 458.79 re-identifica-

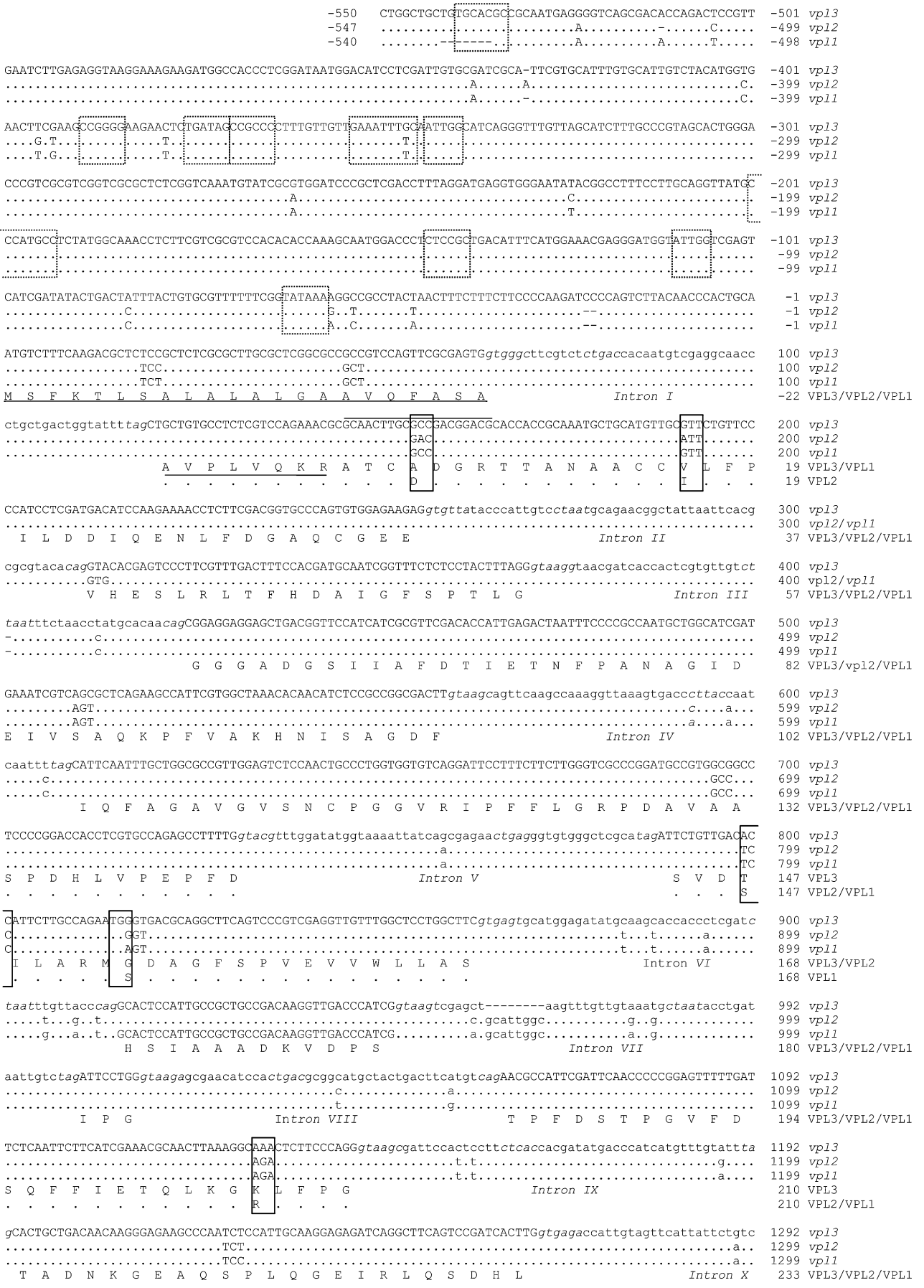

Figure 1 (Continued)

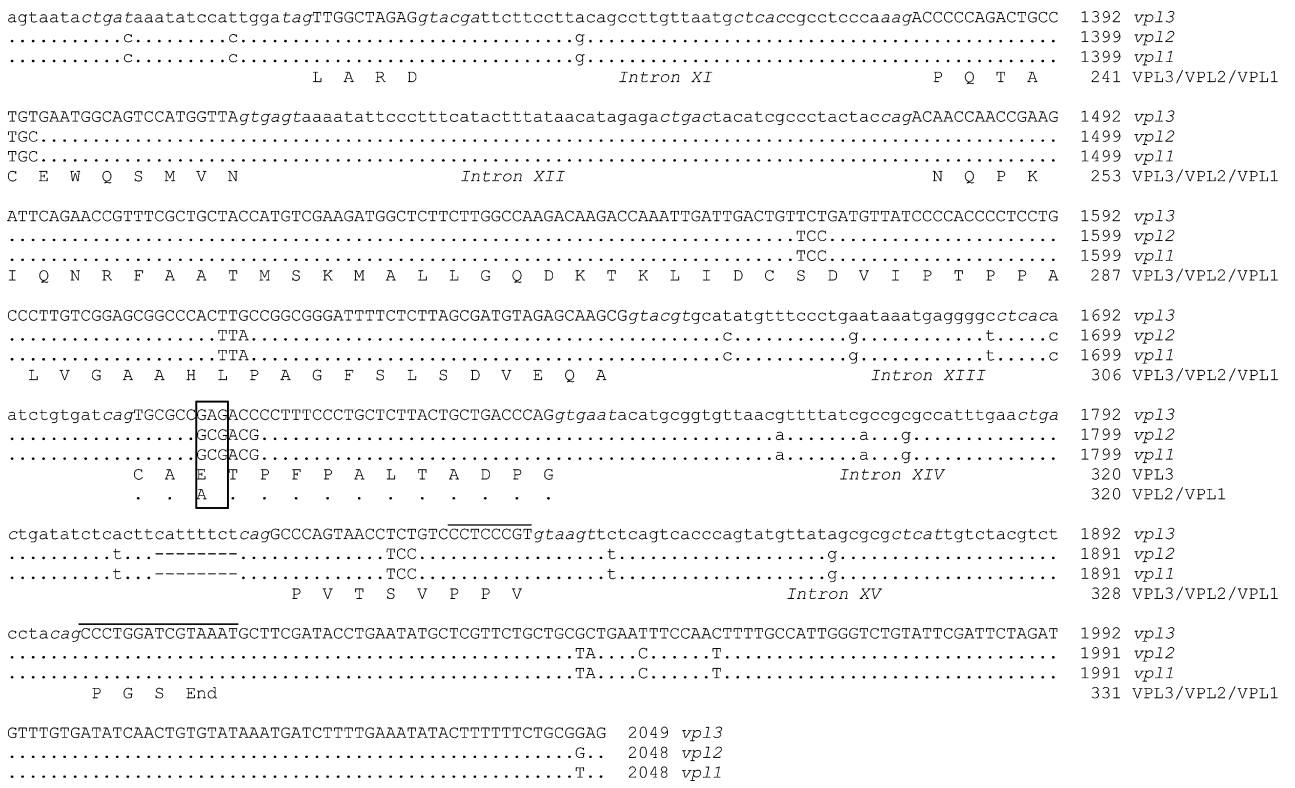

Figure 1. Comparison of the sequences of genes *vpl3* from CBS 458.79 formerly conserved as *L. irina* (DQ056374) with *vpl1* and *vpl2* from *P. eryngii* CBS 613.91 (AF007223 and AF007224, respectively), and the corresponding predicted amino acid sequences (VPL3, VPL1 and VPL2). The whole *vpl3* nucleotide sequence (introns I to XV in lowercase, with 3′ and 5′ splicing and Lariat sequences in italics) and VPL3 amino acid sequence are shown. Only those *vpl1* and *vpl2* nucleotides (or exon triplets), and predicted amino acid residues, differing from *vpl3* are indicated. Two primer annealing sites are indicated as lines above the consensus nucleotide sequence (the promoter primer site is upstream the sequence shown). Amino acid numbering starts at the first residue of the mature proteins, and the sequence of the signal peptide is underlined. Six single-nucleotide changes resulted in modification of amino acids in positions 4, 16, 147, 153, 206 and 309 (Thr147, Lys206 and Glu309 are specific for VPL3) (continuous line boxes). General transcription elements are indicated in the promoter (numbering refers to *vpl3*) including one TATA box (−63 to −58), two inverted CCAAT elements (−344 to −340 and −111 to −107) and one SPI-binding element (−370 to −365). One putative metal response element (−540 to −534), which is absent from *vpl1*, and one heat-shock element (−346 to −354) differing in one nucleotide with respect to *vpl1* and *vpl2*, together with one AP2 (−201 to −194) and two CreA (−390 to −385 and −144 to −139) binding signals are also indicated (dashed line boxes).

tion, suggested similar catalytic properties. To verify this hypothesis, the corresponding cDNA was expressed in *E. coli* W3110, and the inactive protein (present as inclusion bodies) was activated by *in vitro* folding in the presence of added hemin. The optimal urea concentration (150 mM) was much lower than reported for other fungal or plant peroxidases (Smith et al. 1990; Doyle & Smith 1996; Whitwam & Tien 1996). In contrast, the activation yield (up to 16% of protein in the folding mixture) was several-fold higher than attained with other peroxidases, and slightly higher than reported for the recombinant VPL2 (Pérez-Boada et al. 2002). The activated peroxidase was, subsequently, purified to electrophoretic homogeneity. Table I summarizes the process of activation and purification of the recombinant peroxidase from *P. eryngii* CBS 458.79 in terms of recovery of protein and peroxidase activity after the different steps.

Oxidation of five representative VP substrates (Heinfling et al. 1998b), namely Mn^{2+}, high redox potential veratryl alcohol and Reactive Black 5, and low redox potential 2,6-dimethoxyphenol and ABTS (Figure 2) by the recombinant peroxidase were investigated under steady-state conditions. As shown

Table I. Total protein and activity (estimated as H_2O_2-dependent oxidation of Mn^{2+} to Mn^{3+}) during *in vitro* activation and purification of the *E. coli*-expressed peroxidase from the fungal strain CBS 458.79, and increase of the specific activity during these processes (amounts obtained from 1 L of *E. coli* culture).

	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})
Inclusion bodies	30.7	0	0
Folding mixture	22.4	545	24
Ultrafiltration	14.3	400	28
Dialysis	2.5	376	150
Resource-Q chromatography	1.5	330	220

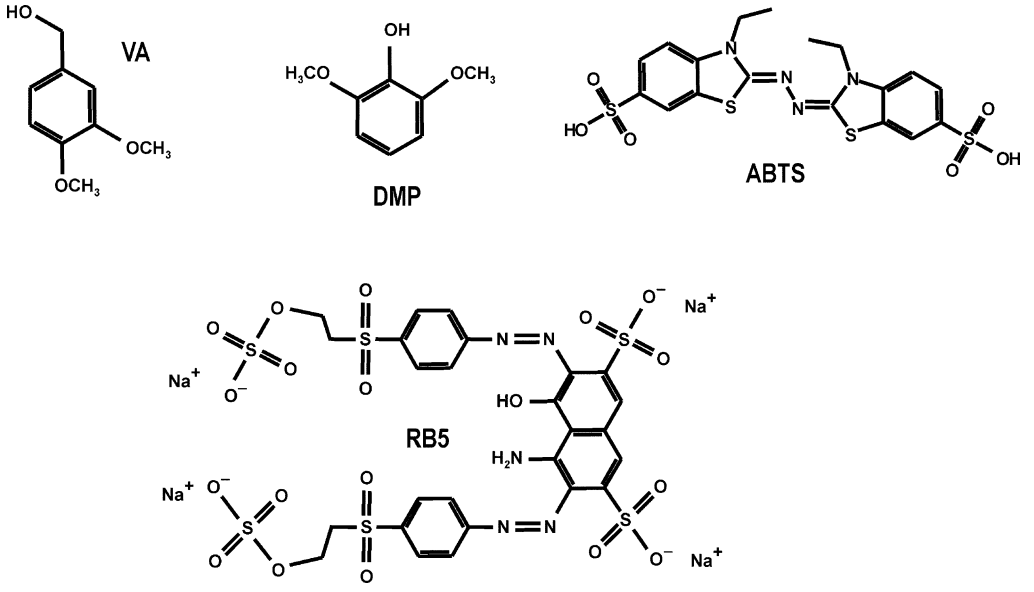

Figure 2. Chemical structures of aromatic compounds assayed (together with Mn^{2+}) as substrates of the *E. coli*-expressed and *in vitro* activated peroxidase from CBS 458.79: VA, veratryl alcohol; DMP, 2,6-dimethoxyphenol; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate; and RB5, Reactive Black 5 (see Table II for kinetic constants).

in Table II, the K_m and k_{cat} values were, in general, in the same order of magnitude as those reported for the wild type (Martínez et al. 1996; Ruiz-Dueñas et al. 1999b) and *E. coli*-expressed VPL2 of *P. eryngii* (Pérez-Boada et al. 2002) indicating that the peroxidase under study is also a VP-type enzyme (called isoenzyme VPL3) from the point of view of its catalytic properties. Interestingly, biphasic Michaelis–Menten curves with two maximum velocity "plateaus" were observed for ABTS and 2,6-dimethoxyphenol oxidation. With these substrates, two catalytic constants in the μM and mM ranges could be calculated revealing the existence of two different binding sites, with high and low affinity, respectively. This observation is described for the first time here, but recombinant VPL2 also showed the same behavior. It may be concluded that differences in the VPL3 amino acid sequence compared with the two previously described *P. eryngii* peroxidases (VPL1 and VPL2) do not seem to affect the catalytic properties. To obtain additional information on the possible significance

Table II. Steady-state kinetic constants of the recombinant peroxidase from the fungal strain CBS 458.79 on Mn^{2+} and the aromatic substrates shown in Figure 2 (mean values and 95% confidence limits).

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Mn^{2+}	100±21	135±7	1400±200
Veratryl alcohol	3360±290	7.5±0.5	2.2±0.3
2,6-Dimethoxyphenol[a]	570±125	6.2±0.3	11±0.2
2,6-Dimethoxyphenol[b]	61200±6100	105±9	1.7±0
ABTS[a]	2.6±0.4	13.1±1	5200±500
ABTS[b]	705±53	124±10	180±10
Reactive Black 5	1.6±0.2	3.2±0.1	2040±130

[a]High affinity site; [b]low affinity site.

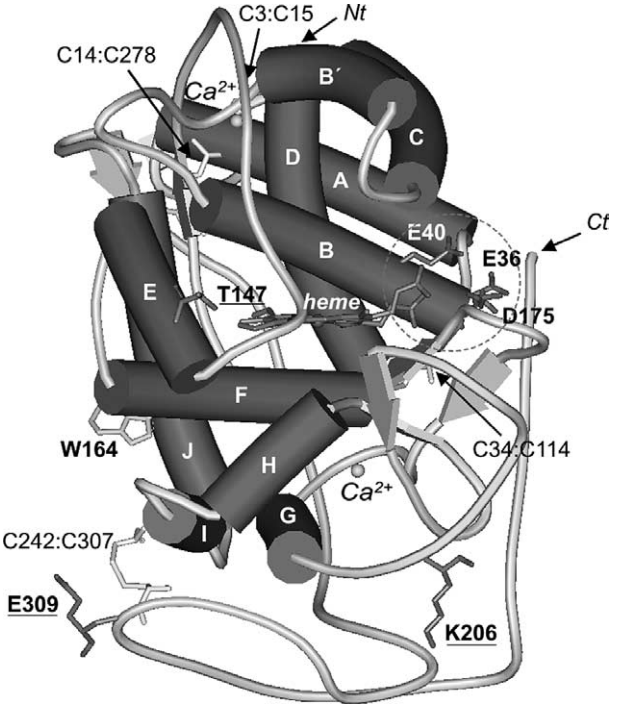

Figure 3. Schematic representation of the molecular structure of VPL3 from CBS 458.79 showing the location of those amino-acid residues (Thr147, Lys206 and Glu309, underlined) differing from *P. eryngii* VPL1 and VPL2. The N and C termini, helical structure (helices A to J), heme cofactor in the middle of two protein domains, four disulfide bonds and two structural Ca^{2+} ions are indicated. The catalytic Mn-binding site (dashed-line circle) and exposed tryptophan (Trp164) are also shown.

of these amino acid differences, a molecular model was obtained.

Molecular modeling and sequence comparison with other fungal peroxidases

A molecular model for VPL3 was built by homology modeling using crystal structures of related peroxidases as templates. As shown in Figure 3, this model showed the most characteristic structural features of VP (Martínez 2002). The putative Mn-binding site formed by three acidic residues (Glu36, Glu40, Asp175) near the internal propionate of heme (Banci et al. 2003; Ruiz-Dueñas et al. 2007), and the exposed tryptophan (Trp164) involved in high redox-potential aromatic substrate oxidation, as recently demonstrated for recombinant VPL2 (Pérez-Boada et al. 2005), are indicated. The model revealed that the three amino acid changes with respect to *P. eryngii* VPL1 and VPL2 (Thr147, Lys206, Glu309) are located at the protein surface. These differences should not affect the characteristic folding, and none of the equivalent residues in VP and other lignin-degrading peroxidases have been reported as involved in oxidation of substrates, or playing any other role (Martínez 2002). Therefore, the catalytic properties of VPL3 are in agreement with the presence of the Mn^{2+} and aromatic substrate oxidation sites mentioned above, which were not affected by the modifications observed in the amino acid sequence. The high and low affinity sites for oxidation of some substrates (2,6-dimethoxyphenol and ABTS) remain unidentified at the structural level.

Lignin-degrading peroxidases are produced only by white-rot basidiomycetes. Figure 4 shows a comparison of the deduced amino acid sequences of 48

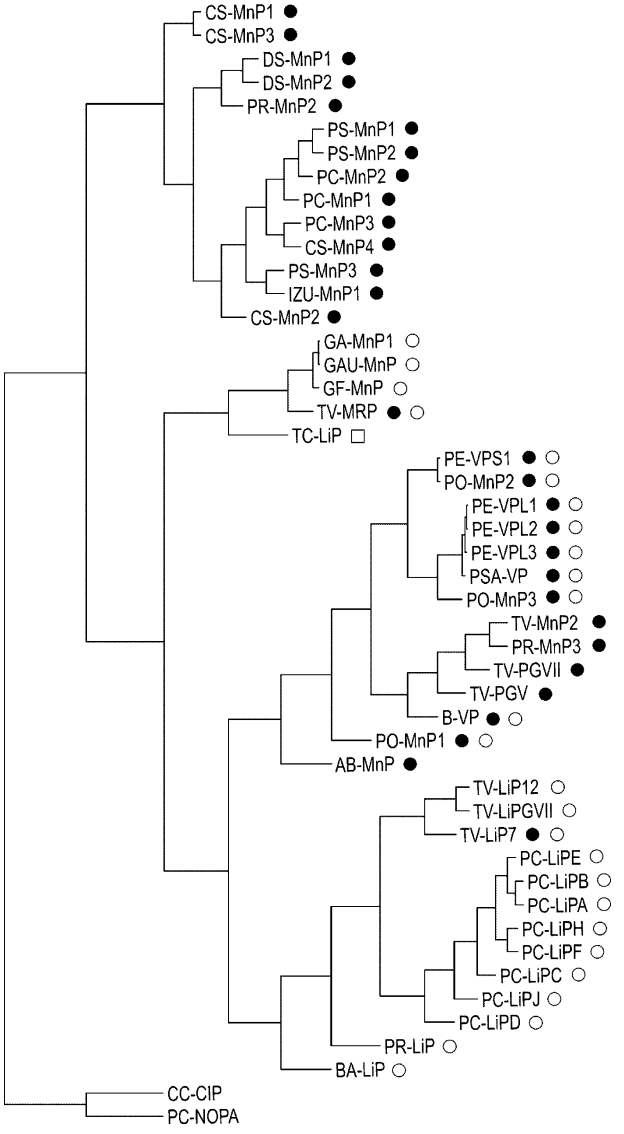

Figure 4. *(Continued)*

Figure 4. Dendrogram from amino acid sequence identity between 48 peroxidases (mature proteins) from 18 basidiomycetes. The comparison shows LiP, MnP and VP-type isoenzymes (including three forms of *P. eryngii* VPL, and four forms of *P. chrysosporium* LiPH8), together with *C. cinerea* peroxidase (CIP), the so-called manganese-repressed peroxidase (MRP) of *T. versicolor*, the *nopa*-encoded "hybrid peroxidase" of *P. chrysosporium* (NOPA), three putative MnP from *Ganoderma* species, and several hypothetical proteins such as PGV and PGVII of *T. versicolor*. Those sequences including a Mn-binding site (●) or an exposed tryptophan involved in aromatic substrate oxidation (○) are indicated (as well as a *T. versicolor* LiP including an exposed tyrosine, □). Abbreviations for fungal species: AB, *A. bisporus*; B, *Bjerkandera* sp; BA, *B. adusta*; CC, *C. cinerea*; CS, *C. subvermispora*; DS, *Dichomitus squalens*; GA, *Ganoderma applanatum*; GAU, *Ganoderma australe*; GF, *Ganoderma formosanum*; IZU, unidentified basidiomycete IZU-154; PC, *P. chrysosporium*; PE, *P. eryngii*; PO, *P. ostreatus*; PR, *P. radiata*; PS, *Phanerochaete sordida*; PSA, *P. sapidus*; TC, *Trametes cervina*; and TV, *T. versicolor*. Protein sequence entries: AB-MnP, CAG27835; B-VP, AAO47909; BA-LiP, 1906181A; CC-CIP, CAA50060; CS-MnP1, AAB03480; CS-MnP2, AAD43581; CS-MnP3, AAD45725; CS-MnP4, AAO61784; DS-MnP1, AAF31329; DS-MnP2, AAF31330; GA-MnP1, BAA88392; GAU-MnP, ABB77244; GF-MnP, ABB77243; IZU-MnP1, no entry available but sequence taken from the literature (Matsubara et al. 1996); PC-LiPA (isoenzyme H8), AAA53109; PC-LiPB (isoenzyme H8), AAA33741; PC-LiPC (isoenzyme H10), AAA33739; PC-LiPD (isoenzyme H2), CAA33621; PC-LiPE (isoenzyme H8), AAA33738; PC-LiPF, AAA33736; PC-LiPH (isoenzyme H8), AAA 56852; PC-LiPJ, AAD46494; PC-MnP1, AAA33744; PC-MnP2, AAA33745; PC-MnP3, AAB39652; PC-NOPA, AAU82081; PE-VPL1, AAD01401; PE-VPL2, AAD01404; PE-VPL3, CAD56164 (and DQ056374); PE-VPS1, AAD 54310; PO-MnP1, AAA84396; PO-MnP2, CAB51617; PO-MnP3, BAA33449; PR-LiP, P20010; PR-MnP2, CAC 85963; PR-MnP3, CAC84573; PS-MnP1, BAC06185; PS-MnP2, BAC06186; PS-MnP3, BAC06187; PSA-VP, CAJ 01576; TC-LiP, AB191466; TV-LiP12, AAA34049; TV-LiP7, CAA83147; TV-LiPGII, CAA53333; TV-MnP2, CAA 83148; TV-MRP, AAB63460; TV-PGV, CAA54398; and TV-PGVII, CAA91043.

peroxidases (mature proteins) from 17 white-rot (lignin-degrading) basidiomycetes, and one soil basidiomycete (*C. cinerea*). The groups obtained were similar to those reported by Hildén et al. (2005), although some differences were observed in the groups from the latter authors due to the clustering method used (neighbor-joining vs. UPGMA), inclusion of signal peptide sequences in the comparison, and different number of sequences analyzed. VPL3 grouped together with other VP found in *P. eryngii* liquid cultures, a putative VP from *Pleurotus sapidus* (deposited by one of the authors) and two *Pleurotus ostreatus* peroxidases. Although the latter were described as MnP (Giardina et al. 2000), they show VP-type structural characteristics, namely the above-mentioned manganese-binding site and exposed tryptophan (indicated on the dendrogram by black and white circles, respectively). This group clustered first with a different VP expressed in lignocellulose cultures of *P. eryngii* (VPS1) (Camarero et al. 1999, 2000), and the similar peroxidase from *P. ostreatus*, and then with several peroxidases (from *Trametes versicolor, Phlebia radiata,* and *Agaricus bisporus*) as well as with VP from *Bjerkandera* sp (Moreira et al. 2005). The two other main clusters correspond, respectively, to MnP sequences (top) all including a Mn-binding site (black circle), and LiP sequences (bottom) all including an exposed tryptophan (white circle). In a small cluster, five peroxidases form an apparently heterogeneous group. Three correspond to *Ganoderma* (Synonym: *Elfvingia*) peroxidases described as MnP (Maeda et al. 2001), although they do not present one of the residues of the manganese-binding site but an exposed tryptophan, and the two others are unusual *Trametes* peroxidases. In addition to those mentioned above, three putative VP (TV-MRP, PO-MnP1, TV-LiP7) were identified by the presence of both the Mn-binding site and exposed tryptophan. Finally, the putative "hybrid peroxidase" encoded by gene *nop* (Larrondo et al. 2005), recently identified in the complete genome of *P. chrysosporium* (Martínez et al. 2004), seemed unrelated to lignin-degrading peroxidases, and clustered together with a peroxidase from the soil fungus *C. cinerea* (Baunsgaard et al. 1993).

Conclusions

We clarified the unexpected description of a peroxidase that showed very high sequence identity with two *P. eryngii* VP, although it was produced by a fungal culture conserved at the CBS culture collection as an unrelated basidiomycete (*L. irina*). Cloning and sequencing of the corresponding gene showed that the high identity also included the promoter and introns. Due to this observation, which could be explained only by a hypothetical horizontal gene transfer, the identity of the supposed *L. irina* was investigated, and ribosomal DNA sequencing revealed that it was a *P. eryngii* instead of *L. irina*.

When the catalytic properties of the protein product of the new gene were investigated on selected substrates, no significant differences from typical VP from *P. eryngii* were observed. This was in agreement with the structural information provided by the molecular model obtained, which showed the presence of putative substrate oxidation sites, and location of those residues differing from related VP. The present characterization, from molecular to kinetic and structural levels, indicated that this peroxidase should be considered as a new peroxidase isoenzyme in the VP family.

VP are enzymes of biotechnological interest because of their different activities, and the ability to directly oxidize some recalcitrant compounds, such as polycyclic aromatic hydrocarbons, high redox-potential dyes, and pesticides (Heinfling et al. 1998a; Wang et al. 2003; Dávila-Vázquez et al. 2005). It is important to mention that other lignin-degrading peroxidases can only oxidize some of these compounds in the presence of mediators or do not oxidize them at all. Therefore, the description, cloning and characterization of new VP forms is important for identifying new peroxidases with industrial and environmental potential.

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References

Banci L, Camarero S, Martínez AT, Martínez MJ, Pérez-Boada M, Pierattelli R, Ruiz-Dueñas FJ. 2003. NMR study of Mn(II) binding by the new versatile peroxidase from the white-rot fungus *Pleurotus eryngii*. J Biol Inorg Chem 8:751–760.

Bao WL, Fukushima Y, Jensen KA, Moen MA, Hammel KE. 1994. Oxidative degradation of non-phenolic lignin during lipid peroxidation by fungal manganese peroxidase. FEBS Lett 354:297–300.

Baunsgaard L, Vind J, Dalboge H. 1993. The sequence of *Coprinus cinereus* peroxidase gene *cip1*. In: Welinder KG,

Rasmussen SK, Penel C, Greppin H, editors. Plant peroxidases: Biochemistry and physiology. Geneva: University of Geneva. pp. 239–242.

Blodig W, Doyle WA, Smith AT, Winterhalter K, Choinowski TH, Piontek K. 1998. Autocatalytic formation of hydroxy group at Cβ of Trp171 in lignin peroxidase. Biochemistry 37:8832–8838.

Blodig W, Smith AT, Doyle WA, Piontek K. 2001. Crystal structures of pristine and oxidatively processed lignin peroxidase expressed in *Escherichia coli* and of the W171F variant that eliminates the redox active tryptophan 171. Implications for the reaction mechanism. J Mol Biol 305:851–861.

Camarero S, Sarkar S, Ruiz-Dueñas FJ, Martínez MJ, Martínez AT. 1999. Description of a versatile peroxidase involved in natural degradation of lignin that has both Mn-peroxidase and lignin-peroxidase substrate binding sites. J Biol Chem 274:10324–10330.

Camarero S, Ruiz-Dueñas FJ, Sarkar S, Martínez MJ, Martínez AT. 2000. The cloning of a new peroxidase found in lignocellulose cultures of *Pleurotus eryngii* and sequence comparison with other fungal peroxidases. FEMS Microbiol Lett 191:37–43.

Cullen D, Kersten PJ. 2004. Enzymology and molecular biology of lignin degradation. In: Brambl R, Marzulf GA, editors. The Mycota III: Biochemistry and Molecular Biology. Berlin: Springer-Verlag. pp. 249–273.

Dávila-Vázquez G, Tinoco R, Pickard MA, Vázquez-Duhalt R. 2005. Transformation of halogenated pesticides by versatile peroxidase from *Bjerkandera adusta*. Enzyme Microb Technol 36:223–231.

Doyle WA, Smith AT. 1996. Expression of lignin peroxidase H8 in *Escherichia coli*: Folding and activation of the recombinant enzyme with Ca^{2+} and haem. Biochem J 315:15–19.

Giardina P, Palmieri G, Fontanella B, Rivieccio V, Sannia G. 2000. Manganese peroxidase isoenzymes produced by *Pleurotus ostreatus* grown on wood sawdust. Arch Biochem Biophys 376:171–179.

Gold MH, Youngs HL, Gelpke MD. 2000. Manganese peroxidase. Met Ions Biol Syst 37:559–586.

González R, Ramón D, Pérez González JA. 1992. Cloning, sequencing analysis and yeast expression of the *egl1* gene from *Trichoderma longibrachiatum*. Appl Microbiol Biotechnol 38:370–378.

Hammel KE, Kalyanaraman B, Kirk TK. 1986. Oxidation of polycyclic aromatic hydrocarbons and dibenzo[p]-dioxins by *Phanerochaete chrysosporium* ligninase. J Biol Chem 261:16948–16952.

Heinfling A, Martínez MJ, Martínez AT, Bergbauer M, Szewzyk U. 1998a. Transformation of industrial dyes by manganese peroxidase from *Bjerkandera adusta* and *Pleurotus eryngii* in a manganese-independent reaction. Appl Environ Microbiol 64:2788–2793.

Heinfling A, Ruiz-Dueñas FJ, Martínez MJ, Bergbauer M, Szewzyk U, Martínez AT. 1998b. A study on reducing substrates of manganese-oxidizing peroxidases from *Pleurotus eryngii* and *Bjerkandera adusta*. FEBS Lett 428:141–146.

Hildén K, Martínez AT, Hatakka A, Lundell T. 2005. The two manganese peroxidases Pr-MnP2 and Pr-MnP3 of *Phlebia radiata*, a lignin-degrading basidiomycete, are phylogenetically and structurally divergent. Fungal Genet Biol 42:403–419.

Kirk TK, Farrell RL. 1987. Enzymatic 'combustion': The microbial degradation of lignin. Annu Rev Microbiol 41:465–505.

Kirk TK, Tien M, Kersten PJ, Mozuch MD, Kalyanaraman B. 1986. Ligninase of *Phanerochaete chrysosporium*. Mechanism of its degradation of the non-phenolic arylglycerol β-aryl ether substructure of lignin. Biochem J 236:279–287.

Kishi K, Kusters-van Someren M, Mayfield MB, Sun J, Loehr TM, Gold MH. 1996. Characterization of manganese(II) binding site mutants of manganese peroxidase. Biochemistry 35:8986–8994.

Larrondo L, Gonzalez A, Perez AT, Cullen D, Vicuna R. 2005. The *nop* gene from *Phanerochaete chrysosporium* encodes a peroxidase with novel structural features. Biophys Chem 116:167–173.

Maeda Y, Kajiwara S, Ohtaguchi K. 2001. Manganese peroxidase gene of the perennial mushroom *Elfvingia applanata*: Cloning and evaluation of its relationship with lignin degradation. Biotechnol Lett 23:103–109.

Martínez AT. 2002. Molecular biology and structure–function of lignin-degrading heme peroxidases. Enzyme Microb Technol 30:425–444.

Martínez MJ, Ruiz-Dueñas FJ, Guillén F, Martínez AT. 1996. Purification and catalytic properties of two manganese-peroxidase isoenzymes from *Pleurotus eryngii*. Eur J Biochem 237:424–432.

Martínez D, Larrondo LF, Putnam N, Gelpke MD, Huang K, Chapman J, Helfenbein KG, Ramaiya P, Detter JC, Larimer F, Coutinho PM, Henrissat B, Berka R, Cullen D, Rokhsar D. 2004. Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nat Biotechnol 22:695–700.

Matsubara M, Suzuki J, Deguchi T, Miura M, Kitaoka Y. 1996. Characterization of manganese peroxidases from the hyperligninolytic fungus IZU-154. Appl Environ Microbiol 62:4066–4072.

Mester T, Field JA. 1998. Characterization of a novel manganese peroxidase–lignin peroxidase hybrid isozyme produced by *Bjerkandera species* strain BOS55 in the absence of manganese. J Biol Chem 273:15412–15417.

Moreira PR, Duez C, Dehareng D, Antunes A, Almeida-Vara E, Frère JM, Malcata FX, Duarte JC. 2005. Molecular characterisation of a versatile peroxidase from a *Bjerkandera* strain. J Biotechnol 118:339–352.

Pérez-Boada M, Doyle WA, Ruiz-Dueñas FJ, Martínez MJ, Martínez AT, Smith AT. 2002. Expression of *Pleurotus eryngii* versatile peroxidase in *Escherichia coli* and optimisation of *in vitro* folding. Enzyme Microb Technol 30:518–524.

Pérez-Boada M, Ruiz-Dueñas FJ, Pogni R, Basosi R, Choinowski T, Martínez MJ, Piontek K, Martínez AT. 2005. Versatile peroxidase oxidation of high redox potential aromatic compounds: Site-directed mutagenesis, spectroscopic and crystallographic investigations of three long-range electron transfer pathways. J Mol Biol 345:385–402.

Ruiz-Dueñas FJ, Guillén F, Camarero S, Pérez-Boada M, Martínez MJ, Martínez AT. 1999a. Regulation of peroxidase transcript levels in liquid cultures of the ligninolytic fungus *Pleurotus eryngii*. Appl Environ Microbiol 65:4458–4463.

Ruiz-Dueñas FJ, Martínez MJ, Martínez AT. 1999b. Molecular characterization of a novel peroxidase isolated from the ligninolytic fungus *Pleurotus eryngii*. Mol Microbiol 31:223–236.

Ruiz-Dueñas FJ, Morales M, Pérez-Boada M, Choinowski T, Martínez MJ, Piontek K, Martínez AT. 2007. Manganese oxidation site in *Pleurotus eryngii* versatile peroxidase: A site-directed mutagenesis, kinetic and crystallographic study. Biochemistry 46:66–77.

Sambrook J, Russell DW. 2001. Molecular cloning. Cold Spring Harbor, NY: CSHL Press.

Schwede T, Kopp J, Guex N, Peitsch MC. 2003. Swiss-Model: An automated protein homology-modeling server. Nucleic Acids Res 31:3381–3385.

Smith AT, Santama N, Dacey S, Edwards M, Bray RC, Thorneley RNF, Burke JF. 1990. Expression of a synthetic gene for

horseradish peroxidase C in *Escherichia coli* and folding and activation of the recombinant enzyme with Ca^{2+} and heme. J Biol Chem 265:13335–13343.

Wang YX, Vázquez-Duhalt R, Pickard MA. 2003. Manganese–lignin peroxidase hybrid from *Bjerkandera adusta* oxidizes polycyclic aromatic hydrocarbons more actively in the absence of manganese. Can J Microbiol 49:675–682.

Wariishi H, Akileswaran L, Gold MH. 1988. Manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*: spectral characterization of the oxidized states and the catalytic cycle. Biochemistry 27:5365–5370.

Wariishi H, Valli K, Renganathan V, Gold MH. 1989. Thiol-mediated oxidation of nonphenolic lignin model compounds by manganese peroxidase of *Phanerochaete chrysosporium*. J Biol Chem 264:14185–14191.

Whitwam R, Tien M. 1996. Heterologous expression and reconstitution of fungal Mn peroxidase. Arch Biochem Biophys 333:439–446.

Zorn H, Langhoff S, Scheibner M, Nimtz M, Berger RG. 2003. A peroxidase from *Lepista irina* cleaves β,β-carotene to flavor compounds. Biol Chem 384:1049–1056.