CHAPTER 27

HIGH REDOX POTENTIAL PEROXIDASES

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1. INTRODUCTION

High redox potential peroxidases secreted by some basidiomycete fungi are unique enzymes that play a central role in the degradation of lignin (Kirk and Cullen, 1998). Plant biomass (bryophytes and algae excluded) is mainly made up of two polysaccharides, cellulose and hemicelluloses, and the complex aromatic polymer of lignin (Fengel and Wegener, 1984). The main role of lignin in the plant cell wall is the protection of polysaccharides against the action of hydrolytic enzymes. It also contributes to stem rigidity and water transport, two important characteristics that facilitated land colonization by vascular plants. Such characteristics derive from the recalcitrance and structural complexity of lignin which includes up to three types of subunit derived from three different *p*-hydroxycinnamyl alcohols (Higuchi, 1997; Boerjan *et al*. 2003). These phenylpropanoid units are linked together by a variety of ether and C-C bonds forming a three dimensional network that confers lignin its mechanical resistance as well as an extremely high resistance to degradation.

Biodegradation of the lignin polymer has been described as "enzymatic combustion", where the aromatic units are oxidized by the hydrogen peroxide secreted by ligninolytic basidiomycetes in a reaction catalysed by high redox potential peroxidases, enzymes which are unique to this group of organisms (Kirk and Farrell, 1987). Due to the light colour of the wood decayed by ligninolytic basidiomycetes these organisms are also known as white-rot fungi (Martínez *et al*. 2005). By contrast, the so-called brown-rot basidiomycetes do not produce ligninolytic enzymes but instead transform wood into a lignin-enriched brown material. In addition to peroxidases, other fungal enzymes such as laccases and H_2O_2-producing oxidases are also involved in lignin biodegradation. Ligninolytic enzymes are of

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considerable importance for the natural degradation of lignocellulosic materials in terrestrial ecosystems, enabling the recycling of the organic carbon fixed by photosynthesis, and also for most industrial processes that utilise lignocellulosic biomass, including paper pulp manufacturing and bioethanol production both of which require the removal of lignin to exploit cellulose.

2. LIGNINOLYTIC ENZYMES

Two ligninolytic peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), were discovered in 1983–84 in the white-rot fungus *Phanerochaete chrysosporium* (order Aphyllophorales) and described as "ligninases" because of their high redox potentials which enable the oxidation of dimeric lignin model compounds (Tien and Kirk 1983; Glenn *et al*. 1983; Kuwahara *et al*. 1984). LiP is able to degrade non-phenolic lignin units (up to 90% of the polymer) or the simple compound 3,4-dimethoxybenzyl (veratryl) alcohol (Tien and Kirk, 1983), whereas MnP generates Mn^{3+} that, when chelated with oxalic or other organic acids, acts as a diffusible oxidizer of the phenolic lignin units (Glenn *et al*. 1986) or the non-phenolic units *via* lipid peroxidation reactions (Jensen *et al*. 1996). Versatile peroxidase (VP), a third type of ligninolytic peroxidase, was more recently described in *Pleurotus* and *Bjerkandera* species (from the orders Agaricales and Aphyllophorales, respectively) (Martínez *et al*. 1996; Mester and Field 1998; Ruiz-Dueñas *et al*. 1999) and combines the catalytic properties of LiP, MnP and other peroxidases (from plants and micro-organisms) that oxidise phenolic compounds (Heinfling *et al*. 1998).

Laccases are known in plants, fungi and insects, where they play a variety of roles including the synthesis of pigments, fruiting-body morphogenesis and detoxification (Thurston, 1994; Mayer and Staples, 2002) (see chapter by Alcalde in this book). Laccase production on solid media was considered to be a characteristic of white-rot fungi (Käärik, 1965), although some brown-rot fungi produce laccase in liquid cultures (Lee *et al*. 2004). However, these phenoloxidases have low redox potentials that only permit the oxidation of phenolic lignin units (often representing less than 10% of the polymer). Recently, biotechnological interest in laccases has been spurred by the discovery of their ability to oxidize high redox potential substrates in the presence of synthetic redox mediators, forming stable free radicals which act as diffusible oxidizers (Bourbonnais and Paice, 1990). The existence of natural mediators involved in lignin biodegradation has not been demonstrated despite various attempts (Li *et al*. 2001), although some lignin-derived phenols could act as laccase mediators (Camarero *et al*. 2005).

Other extracellular enzymes involved in lignin degradation are the H_2O_2-generating oxidases and mycelium-associated dehydrogenases. The former include aryl-alcohol oxidase (AAO), first described in *Pleurotus* and *Bjerkandera* species (Bourbonnais and Paice, 1988; Muheim *et al*. 1990; Guillén *et al*. 1992), and glyoxal oxidase found in *P. chrysosporium* and other basidiomycetes (Kersten, 1990). Fungal dehydrogenases, including aryl-alcohol dehydrogenase (AAD), are also involved in lignin degradation (Gutiérrez *et al*. 1994; Brock *et al*. 1995).

Ligninolytic peroxidases (LiP, MnP and VP) oxidize the lignin polymer in a one-electron reaction resulting in cation radical formation (phenoxy radicals from phenolic units) (Kersten *et al*. 1985). The aromatic cation radicals formed evolve through different non-enzymatic reactions resulting in breakdown of ether and C-C inter-unit linkages, demethoxylation and aromatic ring cleavage (Schoemaker, 1990; Martínez *et al*. 2005). The aromatic aldehydes released after oxidative degradation of the lignin-unit side-chains, or synthesized *de novo* by fungi (Gutiérrez *et al*. 1994), constitute the substrate for the generation of the H_2O_2 required for lignin degradation by peroxidases in *Pleurotus* species. In these and other white-rot fungi H_2O_2 is continuously formed in cyclic redox reactions involving both extracellular AAO and mycelial AAD (Guillén *et al*. 1994). Some oxidases and laccases also participate in the (direct or indirect) reduction of ferric to ferrous iron that reacts with H_2O_2 yielding the hydroxyl free radical, a very strong oxidizer involved in wood biodegradation (Evans *et al*. 1994; Guillén *et al*. 2000; Hammel *et al*. 2002). Ultimately, simple breakdown products from lignin degradation enter the fungal hyphae and are incorporated into the intracellular catabolic routes.

3. STRUCTURE AND FUNCTION OF LIGNINOLYTIC PEROXIDASES

Due to their uniqueness as industrial biocatalysts, the catalytic mechanisms of lignin-degrading peroxidases have been extensively investigated at the molecular level (Banci, 1997; Gold *et al*. 2000; Martínez, 2002). *Phanerochaete chrysosporium* LiP and MnP were the second and third peroxidases (after yeast cytochrome *c* peroxidase, CCP) whose crystal structures were solved, just ten years after their discovery (Poulos *et al*. 1993; Piontek *et al*. 1993; Sundaramoorthy *et al*. 1994); the crystal structure of the *Pleurotus eryngii* VP has been solved recently (Pérez-Boada *et al*. 2005; Piontek *et al*. 2006). As mentioned above, these peroxidases are able to catalyse the oxidation of the recalcitrant lignin units by H_2O_2. This is made possible by the formation of a high redox state oxo-ferryl intermediate after the two-electron reaction of the haem cofactor with H_2O_2. The activated enzyme oxidizes two substrate units and is reduced back to the resting state that reacts again with peroxide. This catalytic cycle, which includes the resting peroxidase and the so-called compound I (two-electron oxidized enzyme) and compound II (one-electron oxidized enzyme), is common to other peroxidases such as the well-known horseradish peroxidase (HRP) (Dunford, 1999). However, two aspects of their molecular structure provide the ligninolytic peroxidases their unique catalytic properties, namely: **i)** a haem environment conferring high redox potential to the oxo-ferryl complexes; and **ii)** specific binding sites (and mechanisms) for oxidation of their characteristic substrates including non-phenolic aromatic compounds in the case of LiP, manganous iron in the case of MnP, and both types of compounds in the case of VP. The hybrid properties of *Pleurotus eryngii* VP are illustrated in the basic catalytic cycle shown in Fig. 1A which combines the previously described cycles of LiP and MnP.

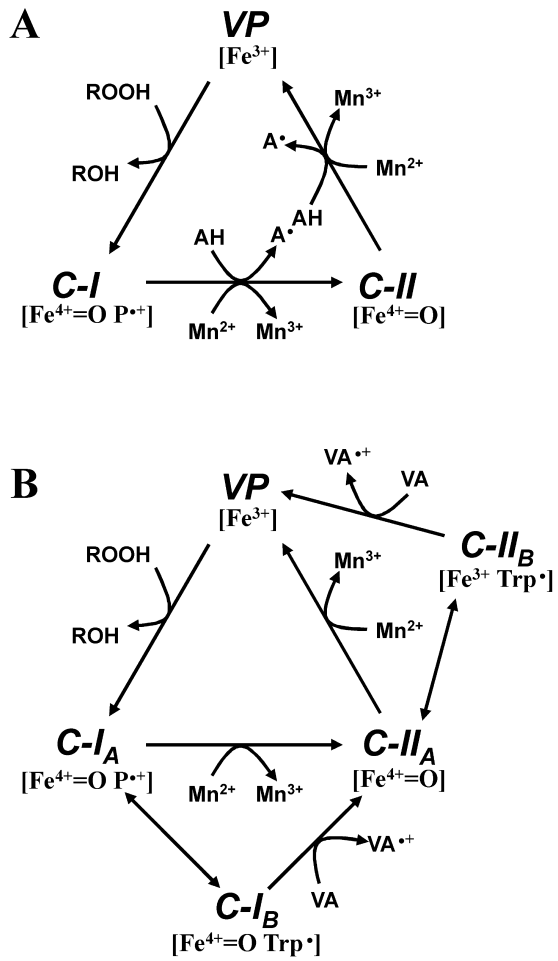

Figure 1. Catalytic cycle of the model ligninolytic peroxidase VP. **A**, Basic cycle as described by Ruiz-Dueñas *et al*. (1999), including two-electron oxidation of the resting peroxidase (VP) by hydroperoxide to yield compound-I (C-I), whose reduction in two one-electron reactions results in the intermediate compound-II (C-II) and then the resting form of the enzyme. VP can oxidize both aromatic substrates (AH) and Mn^{2+}. **B**, Extended cycle adapted from Pérez-Boada *et al*. (2005), including $C\text{-}I_B$ and $C\text{-}II_B$ involved in oxidation of veratryl alcohol (VA) and other high-redox potential aromatic substrates, where a tryptophan radical is present at position 164 corresponding to LiP W171 (low redox potential aromatic compounds are probably oxidized by both the A and B forms but for simplicity they are not included)

Similarities in the haem environment (located in the central region of the protein) between the three ligninolytic peroxidase types were evidenced by ^{1}H-NMR and confirmed by X-ray diffraction of peroxidase crystals (Banci, 1997; Banci *et al*. 2003). In NMR solution experiments it is possible to identify the signals of protons of the haem cofactor as well as those of several amino acid residues in the haem

pocket. This is possible due to the paramagnetic effects caused by the haem iron that displaces the signals of neighbouring protons outside the region where most protein protons overlap. These signals are better assigned in the spectra of peroxidase CN-adducts that yield hyperfine NMR signals. One of the main differences between the various peroxidases is the position of the histidine Nε iron ligand side chain (the so-called proximal histidine) which in the ligninolytic peroxidases is displace away from the haem (compared to HRP or CCP) contributing to the electropositivity of the oxo-ferryl complex (Poulos, 1993; Banci, 1997; Martínez, 2002). The NMR results also explain the high redox potential of MnP, despite that this peroxidase is only able to steady-state oxidize Mn^{2+}, and suggest that this is due to the absence of a specific site in MnP for the oxidation of aromatic substrates, which is present in LiP and VP. Similarly, LiP lacks the Mn-binding site described below.

4. SUBSTRATE OXIDATION SITES IN LIGNINOLYTIC PEROXIDASES

Recent studies based on peroxidase molecular structures solved by crystal X-ray diffraction are contributing to the identification of the different substrate oxidation sites in ligninolytic peroxidases. The manganese and aromatic substrate binding-sites were first identified in MnP and LiP (Doyle *et al*. 1998; Gold *et al*. 2000; Blodig *et al*. 2001), and then in VP after its crystal structure was solved. Studies on the molecular structure of VP confirmed that its novel catalytic properties are due to a hybrid molecular structure, as was suggested several years ago (Ruiz-Dueñas *et al*. 1999; Camarero *et al*. 1999).

Mn^{2+} oxidation by VP occurs at a binding site similar to that of MnP situated near one of the propionates of the haem group. The cation is bound by three acidic residues (one aspartate and two glutamates) enabling direct electron transfer to the cofactor (Fig. 2, left side). Initially it had been suggested that Mn^{2+} is oxidized by MnP at the edge of the haem in front of the classical access channel (δ-position following NMR nomenclature) (Harris *et al*. 1991). However, it has since been demonstrated that this cation uses a second access channel delimited by the three acidic residues mentioned above, situated in front of the most internal haem propionate (with respect to the main channel) (Sundaramoorthy *et al*. 1997). The functionality of this site in VP was confirmed by site-directed mutagenesis and the NMR spectra obtained during Mn^{2+} titration of the enzyme suggest that a unique Mn-binding site exists in the vicinity of the VP haem (Banci *et al*. 2003).

By contrast, it has been shown that veratryl alcohol and other lignin model substrates are oxidized by the ligninolytic peroxidases at the surface of the protein by a long-range electron transfer mechanism that initiates at an exposed tryptophan residue (Fig. 2, right side). The rationale for this mechanism is that most LiP and VP aromatic substrates, including the lignin polymer, cannot penetrate inside the protein to transfer electrons directly to the cofactor in the haem pocket. Thus, they are oxidized at the enzyme surface and the electrons travel to the haem by a

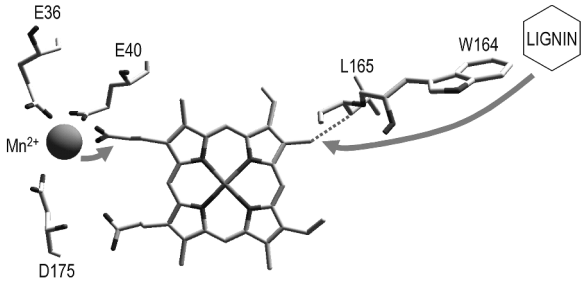

Figure 2. Detail of substrate oxidation sites in the model ligninolytic peroxidase VP including: **i)** Mn^{2+} oxidation site (left) where this cation is bound by the side-chains of E36, E40 and D175 and the internal propionate of haem that directly receives one electron from the substrate; and **ii)** high redox potential aromatic substrate (such as lignin and veratryl alcohol) oxidation site (right) where one electron travels *via* a long-range electron transfer pathway formed by an exposed tryptophan residue (W164, in the form of a tryptophanyl radical in the H_2O_2-activated VP) and a leucine residue connected to the haem (axial view from the proximal side of the haem group)

protein pathway. The tryptophan residue where the protein radical is expected to be produced was identified in LiP to be W171, which is involved in the oxidation of veratryl alcohol (Doyle *et al*. 1998) and a tetrameric lignin model compound (Mester *et al*. 2001). The formation of a protein radical was suggested by indirect evidence (Blodig *et al*. 1999). The homologous residue in VP (W164) has been identified, and two other putative long electron transfer pathways proposed for LiP have been discounted by site-directed mutagenesis studies. Simultaneously, the existence of a tryptophanyl protein radical was directly detected for the first time in a ligninolytic peroxidase using low-temperature EPR of H_2O_2-activated VP (Pérez-Boada *et al*. 2005; Pogni *et al*. 2005). Moreover, using a combination of multi-frequency EPR and DFT (density-functional theory) calculations it has been possible to identify the VP protein radical as a neutral tryptophanyl radical and confirm that it corresponds to W164 as suggested by site-directed mutagenesis (Pogni *et al*. 2006). On the other hand, it has been found that W171 in wild LiP (isoenzymes H2 and H8), as well as in *Escherichia coli*-expressed LiP treated with several H_2O_2 equivalents, is C_β-hydroxylated (Blodig *et al*. 1998; Choinowski *et al*. 1999, 2001). This finding was related to the formation of the above-mentioned protein radical, however, no trace of W164 hydroxylation was found in the atomic resolution structures of recombinant VP treated with H_2O_2 or in wild VP (Pérez-Boada *et al*. 2005). This result demonstrates that hydroxylation is not an immediate consequence of the formation of the tryptophan radical in ligninolytic peroxidases (the evolution of this reactive radical in the absence of reducing substrate probably depends on its protein environment that is different in VP and LiP).

Finally, several pieces of evidence suggest that similarly to HRP (Henriksen *et al*. 1999) and the low redox potential fungal peroxidase from litter-decomposing *Coprinus* species (Tsukamoto *et al*. 1999), some low redox potential substrates –

including substituted phenols and dyes like 2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) – can be oxidized at the main haem access channel of VP and in some cases of LiP (Doyle *et al*. 1998).

In light of the above information the basic cycle of VP (Fig. 1A), as a model ligninolytic peroxidase, can be now completed in the part corresponding to the oxidation of veratryl alcohol and other high redox potential substrates (Fig. 1B). This modification, which also agrees with the mechanisms proposed in LiP, implies the existence of two additional peroxidase forms, compounds I_B and II_B, where one oxidation equivalent would be in the form of a tryptophan radical. These two forms would be in equilibrium with the classical intermediate compounds (now I_A and II_A), where the oxidation equivalent is in the form of a porphyrin cation radical and a Fe^{4+}-oxo, respectively. The percentage of forms A and B in the two equilibrium reactions could be variable, and their involvement in VP catalysis will depend on the substrate. Thus the "classical" compounds I_A and II_A will be predominant during oxidation of Mn^{2+}, whereas a certain percentage of the protein radical forms will be present during oxidation of veratryl alcohol. EPR results have shown that VP compound I_B (including W164 radical) is present in the compound I equilibrium (representing around 25% under the experimental conditions used) (Pérez-Boada *et al*. 2005; Pogni *et al*. 2006). The existence of compound II_B, which in CCP represents 10% of total compound II at pH 5 (Ho *et al*. 1983), is still to be directly demonstrated in VP, though its presence is required for catalysis as explained above.

5. INDUSTRIAL BIOCATALYSTS

The use of enzymes as industrial biocatalysts to remove lignin-derived compounds responsible for the dark colour of paper pulps was introduced several years ago to substitute chlorine-containing bleaching reagents that cause negative environmental impact (Viikari *et al*. 1994; Bajpai 2004). The first enzymes used in the bleach plant were hemicellulose-degrading xylanases (see Chapter 5 of section A) since the positive effects of xylanases in pulp bleaching were first shown (Kantelinen *et al*. 1990) at a time when barely any information was available on ligninolytic enzymes. However, xylanases have limited efficiency in bleaching as they only contribute to remove natural xylan-lignin complexes and lignin fragments that co-precipitate with xylan after cooking. Thus, xylanases are mainly used in so-called elementary chlorine free (ECF) bleaching in combination with chlorine dioxide. In order to develop new enzyme-based totally chlorine free (TCF) sequences the use of lignin-degrading enzymes is required.

In addition to xylanases, ligninolytic enzymes have been being studied in recent years for pulp bleaching application and include the use of both peroxidases and laccases (Paice *et al*. 1995; Bajpai 2004; Sigoillot *et al*. 2005). Laccases have been known for decades and are already commercially produced in high yields using heterologous expression systems. By contrast, ligninolytic peroxidases are not currently commercially available despite considerable efforts devoted to improve

their expression in fungal hosts (Conesa *et al*. 2000, 2002; Gu *et al*. 2003; Lú-Chau *et al*. 2004). As already mentioned, laccases have a low redox potential and can degrade lignin only in the presence of synthetic redox mediators, the so-called laccase-mediator system (see Chapter by Alcalde in this volume). However, it is important to note that it is more difficult to reduce the costs associated with obtaining the chemically synthesised mediators than those of the enzymes obtained by genetic engineering. This together with the potential risks of mediator release to the environment, are currently the main obstacles to the use of laccase-mediator systems at pulp mill scale.

By contrast, ligninolytic peroxidases do not require mediators to degrade high redox-potential compounds which represents a significant advantage compared to laccases. Indeed, they should be the enzymes of choice for removing lignin or transforming high-redox potential aromatic compounds in different industrial applications. Hence the rapid acquisition of knowledge on the structure and function of these enzymes over the last few years. The information currently available has been used to modulate their catalytic (substrate specificity) and operational properties (including enzyme stability) using site-directed mutagenesis as illustrated in various studies (Yeung *et al*. 1997; Wilcox *et al*. 1998; Timofeevski *et al*. 1999; Reading and Aust 2000; Celik *et al*. 2001; Mester and Tien 2001; Feng *et al*. 2003). In those cases where the structural basis of the property to be improved is unknown, or too difficult to be predicted, directed evolution is the approach of choice (Cherry *et al*. 1999; MiyazakiImamura *et al*. 2003). Moreover, gene fusion permits the introduction of cellulose binding domains to improve peroxidase efficiency (Levy *et al*. 2003) or the generation of functional multienzymatic systems. It is to be expected that genetic engineering techniques will also contribute to overcoming the main drawback for the industrial utilization of ligninolytic peroxidases, i.e. their low yields in heterologous expression. In addition to increasing the expression levels of the target genes (e.g. by changing promoters or signal peptides, gene co-expression or fusion, etc.) an alternative approach that is currently being explored is to engineer an already commercial (i.e. high-yield expressed) peroxidase by introducing the ability to oxidize high-redox potential compounds by protein engineering. Using a combination of site-directed mutagenesis, directed evolution and other techniques such as chemical stabilization, tailor-made biocatalysts will be available in the future enabling industrial exploitation of the unique characteristics of the high redox peroxidases produced by lignin-degrading fungi.

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