Manganese Peroxidases: Molecular Diversity, Heterologous Expression, and Applications

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Abstract

Manganese peroxidases (MnPs) are a fascinating group of biocatalysts with various ecological and biotechnological implications. They are involved in the biodegradation of lignocellulose and lignin and participate in the bioconversion of other diverse recalcitrant compounds, like polycyclic aromatic hydrocarbons, chlorophenols, industrial effluents (mostly from the paper and pulp), and textile and petrochemical industries, and bioremediation of contaminated soils. This chapter presents an overview of the structural basis of the catalytic properties of MnPs and the enumeration of the molecular and protein homology characteristics of this enzyme. Multiple developments mainly pertaining to enzyme engineering for improved substrate specificity and stability of MnPs have also been highlighted. Inevitably, the progress in enzyme engineering research and the expression of MnPs have explored the vast genetic diversity of these enzymes with great interest being placed on exploiting these enzymes for a variety of industrial and scientific applications.

Keywords

Manganese peroxidases • Properties • Heterologous production • Molecular cloning • Enzyme engineering research • Crystal structure • Industrial applications

Introduction

Manganese peroxidase (MnP) [EC 1.11.1.13, Mn^{II}:hydrogen-peroxide oxidoreductase] is an extracellular heme enzyme that utilizes hydrogen peroxide (H_2O_2) as an electron-accepting cosubstrate, for catalyzing the peroxide-dependent oxidation of Mn^{II} to Mn^{III}. These enzymes have mainly been isolated from white-rot fungal species like *Phanerochaete chrysosporium, Trametes versicolor, Heterobasidion annosum, and Irpex lacteus* with the ability to degrade lignin (Eriksson et al. 1990; Cai and Tien 1993). A great majority of these enzymes contain a protoporphyrin IX (heme) prosthetic group. Lignin is a het-

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erogeneous, optically inactive polymer, consisting of phenylpropanoid subunits. These phenylpropanoid subunits cannot be cleaved by hydrolytic enzymes unlike most other natural polymers, e.g., cellulose, starch, and proteins. Interestingly, some white-rot basidiomycetes that produce peroxidases have ability to degrade lignin (Sarkanen and Ludwig 1971). Peroxidases were first discovered in *Phanerochaete chrysosporium* (Kuwahara et al. 1984; Glenn and Gold 1985; Paszcynski et al. 1985, 1986). During the last decade, work has been done on the heterologous expression of peroxidases using X-ray crystallographic studies and active-site engineering to enhance substrate specificity and the thermal stability of MnP genes (Mino et al. 1988; Benner and Gerloff 1990; Petersen et al. 1993; Li et al. 2001; Sundaramoorthy et al. 2010). Attempts have also been made to determine the regulation of MnP gene at transcriptional and translational levels (Brown et al. 1991; Gettemy et al. 1998; Johansson et al. 2002). The nonspecific and non-stereoselective nature of MnP allows it to degrade a wide range of pollutants, such as polycyclic aromatic hydrocarbons (PAHs), chlorinated phenols, polychlorinated biphenyls, dioxins, pesticides, explosives, and dyes (Levin et al. 2004).

The main objective of this chapter has been to highlight the diversity of MnPs among basidiomycetes, their heterologous production, phylogenetic analysis, structural characteristics, and molecular features. This chapter also describes attempts made to engineer this enzyme for improved substrate specificity and stability and to quantify the utility of this enzyme.

Occurrence and Phylogenetic Analysis of MnPs

Production of extracellular MnP has been mainly observed from certain basidiomycetes, and thus far, no bacterium, yeast/mold, or mycorrhiza-forming basidiomycete have been reported to produce this enzyme (Cairney and Burke 1998; Hatakka 2001). Many ecophysiological groups of basidiomycetes have been found to secrete isoforms of MnP into their microenvironments (Hatakka 1994, 2001; Heinzkill et al. 1998; Steffen et al. 2000). *C. subvermispora* have been found to produce up to 11 different isoforms of MnP (Lobos et al. 1994; Urzua et al. 1995). Various white-rot fungi, which are well characterized for their ligninolytic ability, belong to phylogenetically older families such as *Meruliaceae* (*P. radiata, P. sordida, P. chrysosporium, Merulius* sp. M15), *Coriolaceae* (*B. adusta, C. subvermispora, C. pruinosum, P. tephropora, A. biennis*), and *Polyporaceae* (*T. versicolor, T. gibbosa, T. trogii, T. hirsuta*) as well as litter decomposers of euagaric families such as *Strophariaceae* and *Tricholomataceae* have been found to have notable expression of MnP (Table 6.1). In addition, some marine-derived fungal strains and strains dwelling on decaying sea grass (Raghukumar et al. 1999), cooling-tower wood (Schmidt et al. 1997), and brown coal (Willmann and Fakoussa 1997a, b) have MnP production ability. Sequences of different MnPs were retrieved from GenBank (available at: http://www.ncbi.nlm.nih.gov). For phylogenetic analysis of all MnPs, an alignment was created with ClustalW2 multiple sequence alignment tool (available at http://www.ebi.ac.uk/Tools/msa/clustalw2/). The evolutionary history was determined using the neighbor-joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Poisson correction method (Zuckerkand and Pauling 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 53 amino acid sequences. Evolutionary analyzes were conducted in MEGA5 (Tamura et al. 2011). It can be inferred from the phylogenetic tree that MnPs are divergent among different taxonomic groups. Among the 54 amino acid sequences analyzed, 44 basidiomycetes were grouped together at the same phenetic unit, whereas apart from main basidiomycete grouping, *Coprinopsis cinerea*, *Arthromyces ramosus*, *Coprinellus disseminatus*, and *Ganoderma* sp. arose as distinct genetic groups (Fig. 6.1). The MnP variant from *Inonotus hispidus* was the single gene variation ungrouped

Table 6.1 Amino acid sequences retrieved from GenBank and used for alignment and phylogenetic analyzes

S. No.	Species	Phylum	GenBank acc. no.
1.	*Ganoderma applanatum*	Basidiomycota	BAA88392.1
2.	*Ganoderma australe*	Basidiomycota	ABB77244.1
3.	*Ganoderma formosanum*	Basidiomycota	ABB77243.1
4.	*Ganoderma lucidum*	Basidiomycota	ACA48488.1
5.	*Trametes versicolor*	Basidiomycota	CAA83148.1
6.	*Phanerochaete chrysosporium*	Basidiomycota	AAA33743.1
7.	*Pleurotus* sp. *"Florida"*	Basidiomycota	CAB51617.1
8.	*Phylloporia ribis*	Basidiomycota	ADK60897.1
9.	*Pleurotus pulmonarius*	Basidiomycota	AAY42945.1
10.	*Coprinopsis cinerea*	Basidiomycota	CAA49216.1
11.	*Lenzites gibbosa*	Basidiomycota	AEX01147.1
12.	*Bjerkandera adusta*	Basidiomycota	AAY89586.1
13.	*Ceriporiopsis rivulosa*	Basidiomycota	ABB83813.1
14.	*Trametes gibbosa*	Basidiomycota	ADW83732.1
15.	*Agrocybe praecox*	Basidiomycota	ADW41627.1
16.	*Spongipellis* sp. *FERM P-18171*	Basidiomycota	BAE79812.1
18.	*Phlebia radiata*	Basidiomycota	CAC84573.1
19.	*Hericium erinaceus*	Basidiomycota	ADK26471.3
20.	*Agaricus bisporus*	Basidiomycota	CAG27835.1
21.	*Phlebia* sp. *MG60*	Basidiomycota	BAG12560.1
22.	*Arthromyces ramosus*	Basidiomycota	BAA09861.1
23.	*Laccaria bicolor S238N-H82*	Basidiomycota	XP_001888065.1
24.	*Coprinellus disseminatus*	Basidiomycota	AAZ14938.1
25.	*Lentinula edodes*	Basidiomycota	BAG72080.1
26.	*Phlebia* sp. *b19*	Basidiomycota	ABR66918.1
27.	*Phanerochaete flavidoalba*	Basidiomycota	AAM46826.1
28.	*Phanerochaete sordida*	Basidiomycota	BAC06187.1
29.	*Dichomitus squalens*	Basidiomycota	AAF31330.1
30.	*Phellinidium ferrugineofuscum*	Basidiomycota	ACX51165.1
31.	*Lactarius rufus*	Basidiomycota	ACX51162.1
32.	*Lactarius fulvissimus*	Basidiomycota	ACX51161.1
33.	*Hypholoma fasciculare*	Basidiomycota	ACX51160.1
34.	*Hygrophorus agathosmus*	Basidiomycota	ACX51158.1
35.	*Gomphus clavatus*	Basidiomycota	ACX51157.1
36.	*Cortinarius traganus*	Basidiomycota	ACX51156.1
37.	*Cortinarius malachius*	Basidiomycota	ACX51154.1
38.	*Cortinarius infractus*	Basidiomycota	ACX51153.1
39.	*Cortinarius hinnuleus*	Basidiomycota	ACX51152.1
40.	*Cortinarius armillatus*	Basidiomycota	ACX51151.1
41.	*Inonotus hispidus*	Basidiomycota	ADK60893.1
42.	*Fomitiporia mediterranea*	Basidiomycota	ADK60890.1
43.	*Coriolopsis gallica*	Basidiomycota	AAZ16493.1
44.	*Phlebia albomellea*	Basidiomycota	ABT17238.1
45.	*Hymenochaete corrugata*	Basidiomycota	ADK60895.1
46.	*Polyporus brumalis*	Basidiomycota	AEJ38000.1
47.	*Cytidia salicina*	Basidiomycota	ABT17236.1
48.	*Phlebia chrysocreas*	Basidiomycota	ABT17228.1
49.	*Phlebiopsis gigantea*	Basidiomycota	ABT17220.1

(continued)

Table 6.1 (continued)

S. No.	Species	Phylum	GenBank acc. no.
50.	*Pulcherricium caeruleum*	Basidiomycota	ABT17217.1
51.	*Cryptoporus volvatus*	Basidiomycota	ABT17214.1
52.	*Hapalopilus rutilans*	Basidiomycota	ABT17210.1
53.	*Trametes cinnabarina*	Basidiomycota	ADK60908.1
54.	*Pleurotus ostreatus*	Basidiomycota	AAA84397.1

from the main cluster as aforementioned. *Lactarius rufus* along with *Lactarius fulvissimus* formed the third additional genetic group.

The maximum parsimony (MP) method was used to analyze the evolutionary history among different basidiomycete MnPs. The bootstrap consensus tree obtained from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50 % of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein 1985). The MP tree was obtained using the close-neighbor-interchange algorithm (Nei and Kumar 2000) with search level 1 in which the initial trees were obtained with the random addition of sequences (ten replicates). Parsimony analysis revealed four distinct main evolutionary lineages among all basidiomycetes (Fig. 6.2). The MnP from *Pleurotus ostreatus* and *Pleurotus* sp. *Florida* evidenced two additional lineages as they were grouped separately from the main cluster. The MnPs from *Pleurotus pulmonarius*, *Hygrophorus agathosmus*, *Laccaria bicolor S238N-H82*, *Agrocybe praecox*, *Phellinidium ferrugineofuscum*, and *Hymenochaete corrugata* are evolutionarily closely related, and they arose as the sister group to the other remaining groups (Fig. 6.2).

Mechanism of Catalysis

Manganese peroxidase (MnP) [EC 1.11.1.13, Mn^{II}:hydrogen-peroxide oxidoreductase, MnP] is an extracellular heme enzyme that utilizes hydrogen peroxide (H_2O_2) as electron-accepting cosubstrate for catalyzing the peroxide-dependent oxidation of Mn^{II} to Mn^{III}. The Mn^{III} formed is highly active, which in turn is stabilized by fungal chelators like oxalate and malonate. These chelators act as physiological regulators to the enzyme as they enhance the enzyme activity due to their ability to facilitate the dissociation of Mn^{III} from the enzyme. The role of oxalate as an extracellular buffering agent has also been reported. It facilitates the ability of the fungus to control the pH of its environment (Timofeevski and Aust 1997; Zapanta and Tien 1997). Calcium sequestration by these chelators acts to increase the pore size of the plant cell wall and assist in the penetration of enzyme molecules. Oxidation of oxalic acid by Mn^{III} produces a formate radical (HCO_2^-) that reacts with dioxygen to form superoxide (O_2^-) and subsequently H_2O_2 (Khindaria et al. 1994; Urzua et al. 1998). Chelated Mn^{III} in turn acts as low molecular weight, diffusible redox mediator that attacks phenolic lignin structures and monomeric phenols, e.g., azo dyes, resulting in the formation of unstable free radicals that tend to disintegrate spontaneously. Characteristic features of the catalytic cycle of MnP resemble to those of other heme-containing peroxidases, such as horseradish peroxidase (HRP), lignin peroxidase, versatile peroxidase, and chloroperoxidases (Cai and Tien 1993; Magliozzo and Marcinkeviciene 1997; Longoria et al. 2008). However, MnP is unique in its ability to utilize Mn^{II} as a reducing substrate to oxidize it to Mn^{III} (Kishi et al. 1994; Sundaramoorthy et al. 1997; Youngs et al. 2000; Deguchi et al. 2002). Spectroscopic studies have revealed that the heme iron of the native enzyme is in the ferric, high-spin, pentacoordinate state and is ligated to the proximal histidine (Mino et al. 1988; Wariishi et al. 1988; Gelpke et al. 2000). The reactions

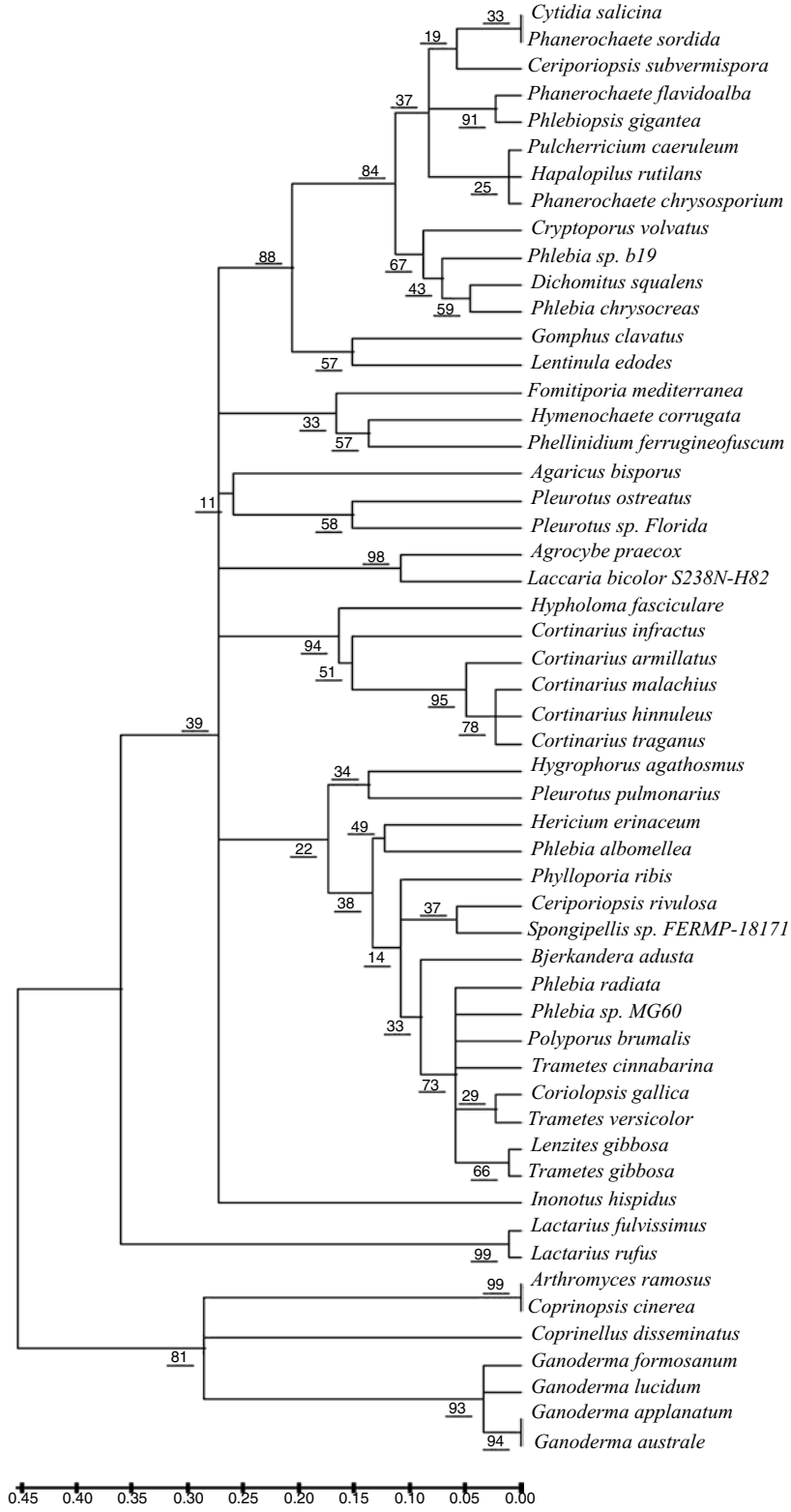

Fig. 6.1 Neighbor-joining tree of manganese peroxidases amino acid sequences. Numbers on the branches are bootstrap values (jackknife values in parentheses) obtained for 1,000 pseudoreplications

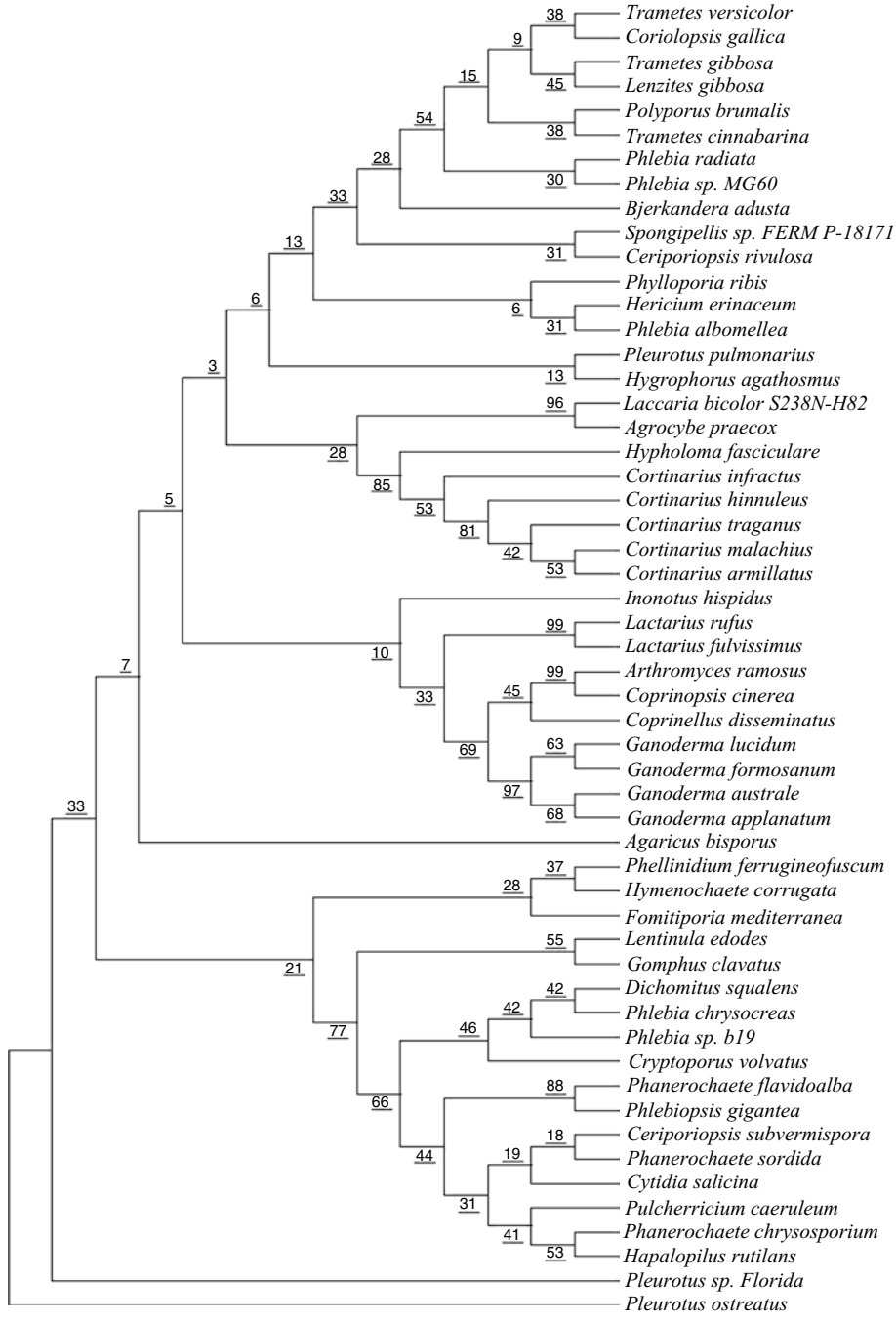

Fig. 6.2 Maximum parsimony analysis of taxa from MnP amino acid sequences. Numbers on the branches are bootstrap values (jackknife values in parentheses) obtained for 1,000 pseudoreplications

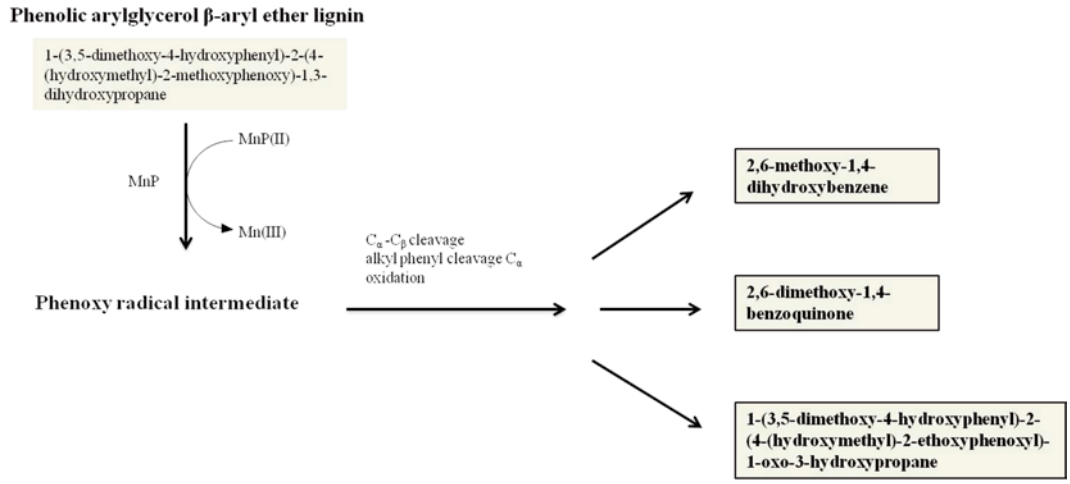

Fig. 6.3 MnP-catalyzed oxidation of phenolic arylglycerol β-aryl ether lignin model compound (Modified according to Tuor et al. 1992)

involved in the MnP catalytic cycle are (Wariishi et al. 1992; Gelpke et al. 1999)

$$MnP + H_2O_2 \rightarrow MnPI + H_2O$$

$$MnPI + Mn^{II} \rightarrow MnPII + Mn^{III}$$

$$MnPII + Mn^{II} \rightarrow MnP + Mn^{III} + H_2O$$

Catalysis of Phenolic Substrates

During the oxidation of phenolic compounds, phenoxy radical intermediates are formed which undergo rearrangements, bond cleavages, and nonenzymatic degradation to yield various breakdown products (Fig. 6.3) (Tuor et al. 1992). Mn^{III} generated by MnP is known to catalyze the oxidation of phenolic substrates, including simple phenols, amines, dyes, and also phenolic lignin substructure and dimers (Wariishi et al. 1989a; Urzua et al. 1995).

Catalysis of Non-Phenolic Substrates

In contrast to LiP-catalyzed reactions, which involve electron abstraction from the aromatic ring, forming a radical cation, Mn^{III} forms reactive radicals in the presence of a second mediator during the oxidation of non-phenolic substrates (Reddy et al. 2003). The presence of thiols, such as glutathione, mediates the oxidation of substituted benzyl alcohols and diarylpropane structures to their respective aldehydes by Mn^{III} (Reddy et al. 2003). In these reactions, thiols are oxidized to thiyl radicals by Mn^{III}, which subsequently removes hydrogen from the substrate to form a benzylic radical. The latter undergoes successive nonenzymatic reactions like addition of O_2 at C1 position of benzylic radical followed by loss of $^{\bullet}OOH$, and homolytic C–O fission at C2 of benzylic radical expels a phenoxy radical that results in the formation of final products (Fig. 6.4) (Wariishi et al. 1989b, c).

Manganese peroxidase generated Mn^{III} has also been coupled with peroxidation of lipids to catalyze C_α–C_β cleavage and to β-aryl ether cleavage of non-phenolic diarylpropane and β-O-4 lignin structures, respectively (Fig. 6.4) (Bao et al. 1994; Daina et al. 2002; Reddy et al. 2003; Kapich et al. 2005). The steps involved in the mechanism are the following: firstly, hydrogen abstraction from the benzylic carbon via lipid peroxy radicals, and secondly, peroxy radicals are formed by addition of O_2 and subsequent oxidative cleavage and nonenzymatic degradation. Absence of exogenous H_2O_2 directs the enzyme to oxidize

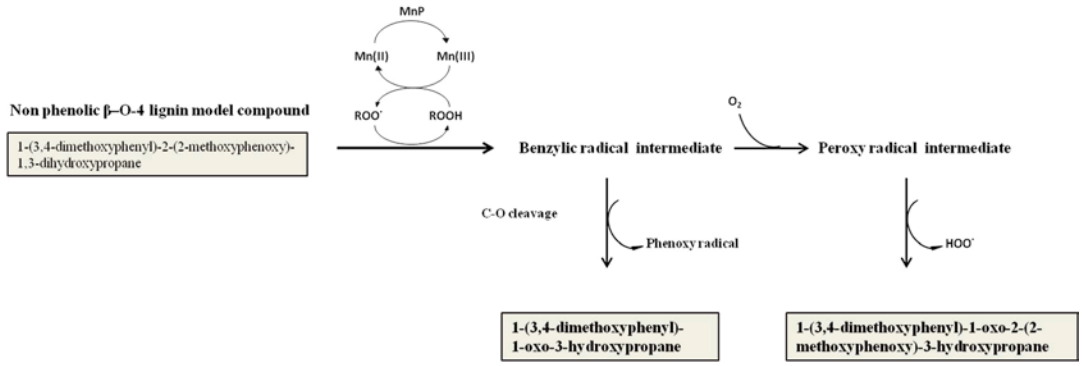

Fig. 6.4 MnP-catalyzed oxidation of non-phenolic β-O-4 lignin model compound (Modified according to Wong 2009)

nicotinamide adenine dinucleotide phosphate (NADPH) (reduced form), glutathione, dithiothreitol, and dihydroxymaleic acid to generate H_2O_2. One can infer (by observing the oxidase activity of MnP) that the H_2O_2 produced may become available for the enzyme to start the peroxidase cycle, thereby, assisting in the degradation of lignin by this fungal species (Paszczynski et al. 1986).

Molecular Cloning and Expression of MnP

In most fungi, MnP appears to be produced as a family of isoenzymes, which may be encoded by structurally related genes (Larrondo et al. 2001; Sakamoto et al. 2009). Commercial applications of MnP require significantly higher levels of extracellular enzyme production by fungal strains; however, the yield of MnP in its native hosts is too low (1.5–5 mgl^{-1}) (Stewart et al. 1996; Li et al. 2001). Therefore, improvement in the yield and reduction in the production cost are the major goals to be focused for commercial exploitation of MnP.

The first extracellular fungal MnP that was characterized and homologously expressed was obtained from *Phanerochaete chrysosporium* (Glenn and Gold 1985). It was found that high concentrations of carbon and nitrogen in the medium significantly affected the transcription of the *mnp* l gene in *P. chrysosporium* (Mayfield et al. 1994). Sakamoto et al. (2009) studied the transcriptional and translational levels of different isoforms of MnP, i.e., *lemnp*1 and *lemnp*2, in *Lentinula edodes* using sawdust medium and reported *lemnp2* as the major extracellular enzyme.

Heterologous expression of MnP from *Phanerochaete chrysosporium* has been reported in *Aspergillus niger, Pichia pastoris, P. chrysosporium adel, Aspergillus oryzae, and Aspergillus nidulans* (Pribnow et al. 1989; Mayfield et al. 1994; Stewart et al. 1996; Janse et al. 1998; Larrondo et al. 2001; Gu et al. 2003). MnPs from *Trametes versicolor*, *P. eryngii*, *P. ostreatus*, *Dichomitus squalens*, and *Ceriporiopsis subvermispora* have also been cloned and heterologously expressed in *T. versicolor* 9522-1, *Coprinus cinereus*, *P. chrysosporium*, and *Aspergillus nidulans*, respectively (Ogawa et al. 1998; Camarero et al. 2000; Li et al. 2001; Larrondo et al. 2001; Kim et al. 2005; Yeo et al. 2007). To date there have been no reports of successful expression of fungal MnPs in bacterial expression hosts.

Although the MnP production levels have often been improved significantly by expression in heterologous hosts, the reported levels are still rather low (100–400 mgl^{-1}) for use in industrial applications (Conesa et al. 2000; Punt et al. 2002; Espinosa et al. 2012). The capability of the *P. pastoris* strain to perform various posttranslational modifications, such as heme insertion, glycosylation, folding, and protein secretion, had been reported for successful

production of active MnP to a maximum yield of 120 UL^{-1} (Gu et al. 2003). As described by Yeo et al. (2007), increase in MnP activity of transformant TF6 was up to 45 % as compared to the recipient strain. A thermostable recombinant MnP from *D. squalens* has also been heterologously expressed in *P. chrysosporium* and purified. The recombinant protein appeared similar in kinetic and spectral characteristics to the wild-type MnP from *D. squalens* (Li et al. 2001), whereas the homologous expression of *P. chrysosporium* recombinant MnP resulted in 30 % of the level of MnP activity expressed under by wild-type strain (Mayfield et al. 1994). Data indicated that the addition of exogenous Mn^{II}, Cd^{II}, and Zn^{II} conferred additional thermal stability to MnP from *D. squalens and P. chrysosporium* (Li et al. 2001). *Aspergillus* species have proven to be excellent hosts for the expression of heterologous proteins such as those of *A. oryzae.* The same has been shown to be effective in the expression of *P. chrysosporium mnp1* with notable yields (Stewart et al. 1996).

Isozyme multiplicity of MnPs has been observed in various strains of *C. subvermispora* and *P. chrysosporium.* As an expression host, *Aspergillus nidulans* proved to be convenient system for MnPs. It has also been demonstrated that Mn^{II} is the key component that regulates the transcription of different recombinant MnP isoforms in carbon-limited cultures (Banci et al. 1992; Alic et al. 1997; Larrondo et al. 2001). Nutrient limitation such as Mn concentration, culture agitation, heat shock, H_2O_2 concentration, and other chemical stresses have also been reported to significantly regulate transcription of different isozymes of MnP (*mnp1, mnp2, and mnp3*) in *P. chrysosporium* (Janse et al. 1998). In contrast to manganese regulation, Tello et al. (2000) have proposed that the putative MREs (metal response elements) found in the upstream region of MnP genes in *P. chrysosporium* might have a role in the regulation of transcription of genes coding for MnP in filamentous fungi. Metallothionein genes are known to be regulated by MREs through various metals in animal cells, although these sites do not respond to manganese. Therefore, further work is required to decipher both the role of MREs in the upstream region of these genes and the mechanism of transcriptional regulation by manganese in basidiomycetes.

Characteristic Features of MnP Gene

MnP genes among different basidiomycetes (starting from the ATG codon) encompass a genomic region of 1.4–1.9 kbps. Lobos et al. (1998) had shown that MnP genes contain seven short intervening sequences with sizes ranging between 52 and 60 bp. The last intron restrained by *mnp1* and *mnp2* genes of *P. chrysosporium* segregates a codon for proline to give different isozymes (Fig. 6.5) (Godfrey et al. 1990; Mayfield et al. 1994). Sequences at the intron splicing junctions adhere to the GT–AG rule. In turn, three putative internal lariat formation sites match the consensus sequence CTRAY (Padgett et al. 1989). After structural comparison of *mnp* genes from the *Cs-mnp1* gene of *C. subvermispora* and five *mnp* genes from different basidiomycetes, Lobos et al. (1994) revealed an almost perfect alignment between Cs-*mnp1* and *mnp2* of *P. chrysosporium* (Fig. 6.5), and both genes have an additional intron splitting exon 3 of *mnp1* and *mnp3* of *P. chrysosporium* at the codon for the distal histidine, H46 (Mayfield et al. 1994). The pattern of differential distribution of introns observed in the *P. chrysosporium mnp* genes might be because the *mnp2* represents the ancestral gene structure and the *mnp1*and *mnp3* genes arose with the loss of one intron each (Alic et al. 1997). In contrast, only one of the 15 introns (intron 12) in the *P. ostreatus mnp* gene aligns exactly with a *C. subvermispora mnp1* intron (intron 6), and none of the 15 introns of the *P. ostreatus mnp* gene align precisely with introns of the *P. chrysosporium mnp* genes.

The regulatory sequence of *Cs-mnp2B* contains a TATA box and an inverted CAAT (ATTG) element located 92 and 191 bp upstream of the ATG codon (Tello et al. 2000), respectively. Examination of the promoter regions of the *mnp1* and *mnp2* genes (Godfrey et al. 1990; Gold and Alic 1993; Mayfield et al. 1994) revealed the presence of putative MREs within 800 bp of the translation initiation codon. These sequences are identical

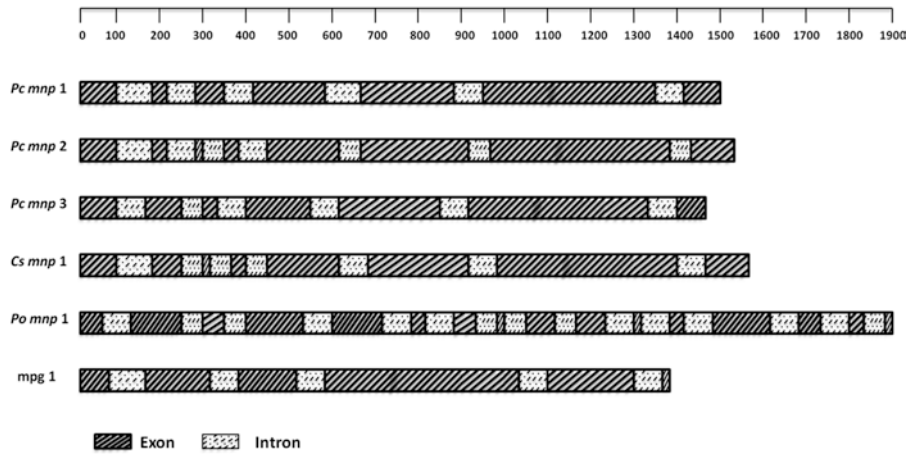

Fig. 6.5 Intron/exon structure of MnP genes *mnp1*, *mnp2*, and *mnp3* from *P. chrysosporium*, *Cs-mnp1* from *C. subvermispora*, *mnp* from *P. ostreatus*, and *mpg1* from *T. versicolor*. The exons are indicated by *open boxes*, whereas the *solid black boxes* correspond to the introns (Modified after Lobos et al. 1998)

to cis-acting MRE sequences responsible for heavy-metal induction of animal cell metallothionein genes (Gettemy et al. 1998). Interestingly, closer examination of the *mnp1* promoter region also revealed the presence of putative HSEs within 400 bp upstream of the *mnp1* translation initiation codon (Lobos et al. 1998). Mn^{2+} regulation of MnPs has been previously highlighted by different research groups (Bonnarme and Jeffries 1990). Godfrey et al. (1990) stated that 1,500 bp of sequence immediately upstream of the MnP translation start site is sufficient to regulate the *ural* reporter in a manner analogous to the regulation of the endogenous MnP genes with respect to Mn, nutrient nitrogen levels, and metabolic phase of growth.

The translocation of 48-bp fragment of promoter region of MnP isozyme 1 to a site 120 bp downstream of its original location has been shown to regulate Mn^{2+}-dependent expression of downstream genes; this suggests the possibility of the presence of at least one Mn^{2+}-responsive *cis* element in the fragment. However, deletion of a 48-bp fragment, located at 521 bp upstream of the translation start codon in the *mnp1* promoter, or replacement of this fragment with an unrelated sequence resulted in *egfp* expression under nitrogen limitation, both in the absence and presence of exogenous Mn^{2+} (Ma et al. 2004).

Orth and coworkers (1994) have analyzed the organization of the *MnP* gene family of *P. chrysosporium* BKM1767 and concluded that the λ*MP-1* and λ*MP-2* genes hybridized to 3.6 and 3.8 Mb of DNA fragments located on separate chromosomes and in contrast to five LiP genes that are localized to a dimorphic chromosome of about 3.7 and 3.5 Mb (Gaskell et al. 1991).

Crystallographic Analysis of MnP

MnP from different basidiomycetes has been crystallized and subsequently analyzed (Poulos et al. 1993; Sundaramoorthy et al. 1994a, b, 1995; Duenas et al. 1999). MnP is a glycoprotein with the molecular weight of 46 kDa and contains one heme group (Sundaramoorthy et al. 1994a, b). The structural features of manganese peroxidase (pdb 1mnp) from *P. chrysosporium* are displayed/shown in Fig. 6.6, with a resolution of 2.06 Å (retrieved from http://www.rcsb.org/pdb). The substrate-bound MnP (Mn–MnP) consists of 357 amino acids, three sugar residues, a heme prosthetic group, two structural calcium ions, substrate Mn^{II} ion, and 478 solvent molecules, including two glycerol molecules (Sundaramoorthy et al. 2010). The sequence of the heme distal helix Glu35–Ala48 is highly conserved in the key residues (Selvaggini

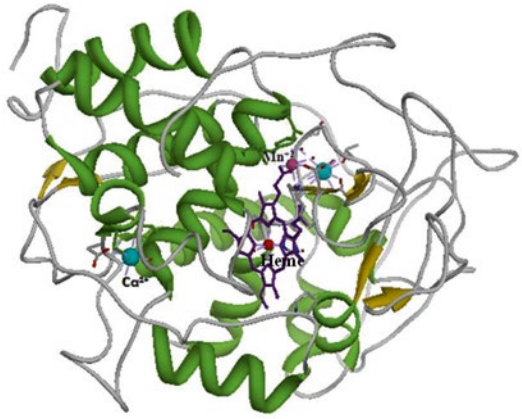

Fig. 6.6 The overall structure of *P. chrysosporium* MnP (pdb 1mnp) as analyzed by UCSF chimera 1.4.1. The cyan spheres are structural Ca^{II} ions conserved in extracellular heme peroxidases. The location of the substrate, Mn^{+2}, near the heme, is indicated. The *purple color* shows active-site structure of MnP. This architecture is highly conserved in heme peroxidase

et al. 1995). The active site consists of a proximal His ligand H-bonded to an Asp residue, and a distal side peroxide-binding pocket consisting of a catalytic His and Arg is the same among all peroxidases (Fig. 6.7). MnP differs with respect to having five rather than four disulfide bonds. The additional disulfide bond is located near the C-terminus of the polypeptide chain. The ligands constituting the Mn^{2+} binding site include Asp179, Glu35, Glu39, a heme propionate, and two water molecules. The overall structure is similar to that of two other fungal peroxidases, i.e., lignin peroxidase from *Phanerochaete chrysosporium* and *Arthromyces ramosus* peroxidase. Like the other fungal peroxidases, MnP also has two structural calcium ions and N-acetylglucosamine residues N-linked to Asn131 (Sundaramoorthy et al. 1994a, b).

Proximal pocket of MnP active site contains His173 that coordinates to heme atom and the side chain of Asp242 interacts with His173 through H bond. The Phe190 residue is positioned just below the heme ring, near the His173 residue. There is a second opening between the heme propionate residues; this region is polar and characterized by residues Arg177, Asp179, Glu35, and Glu39 (Selvaggini et al. 1995). Its ability to oxidize Mn^{2+} with high substrate affinity is related to the presence of a Mn binding site (involving Glu36, Glu40, and Asp179) which enables the oxidation of this cation by the internal heme propionate (Sundaramoorthy et al. 1997).

Multiple sequence alignment of amino acid sequences (retrieved from www.ncbi.nlm.nih.gov/protein/) conducted on several characteristic peroxidases (*P. chrysosporium* MnP (378 amino acids) and LiP (371 amino acids), *T. versicolor* MnP (365 amino acids) and LiP (372 amino acids), *P. ostreatus* MnP (361 amino acids), *P. brasiliensis* Pb01 cytochrome c peroxidase (374 amino acids), and *P. eryngii* versatile peroxidase (370 amino acids), *Brassica rapa* TP7 (296 amino acids), and *Arthromyces ramosus* peroxidase (364 amino acids)) indicated significant conserved structural residues (Fig. 6.7). All the enzymes have the conserved proximal histidine, the distal histidine and the distal arginine. The distal arginine residue has been proposed to participate in the formation of the peroxide-binding pocket together with distal histidine, while Asn131 has been revealed to be the only carbohydrate binding site in MnP1 (Sundaramoorthy et al. 1994a, b, 2005). The iron coordination and the residues involved in the active site are the most conserved region among all the aforementioned fungal peroxidases, as well as cytochrome c peroxidase. The two glutamic acid and the aspartic acid residues present in the manganese binding site are also conserved. It could be inferred from Fig. 6.7 that heme binding sites are conserved among all the characteristic peroxidases with some variations found in the case of the turnip (*Brassica rapa*) and cytochrome c peroxidases. The distal Ca^{2+}-binding residues are conserved in all fungal peroxidases (coordination being completed by two water molecules), whereas some differences exist in the residues binding Ca^{2+} at the proximal side.

Comparative Analysis of MnP, LiP, and VP

Comparative studies were performed between *P. chrysosporium* MnP (pdb 1mnp), *T. cervina* LiP (pdb 3q3u), and *P. eryngii* VP (pdb 3fmu) on the basis of tertiary structure alignment using UCSF

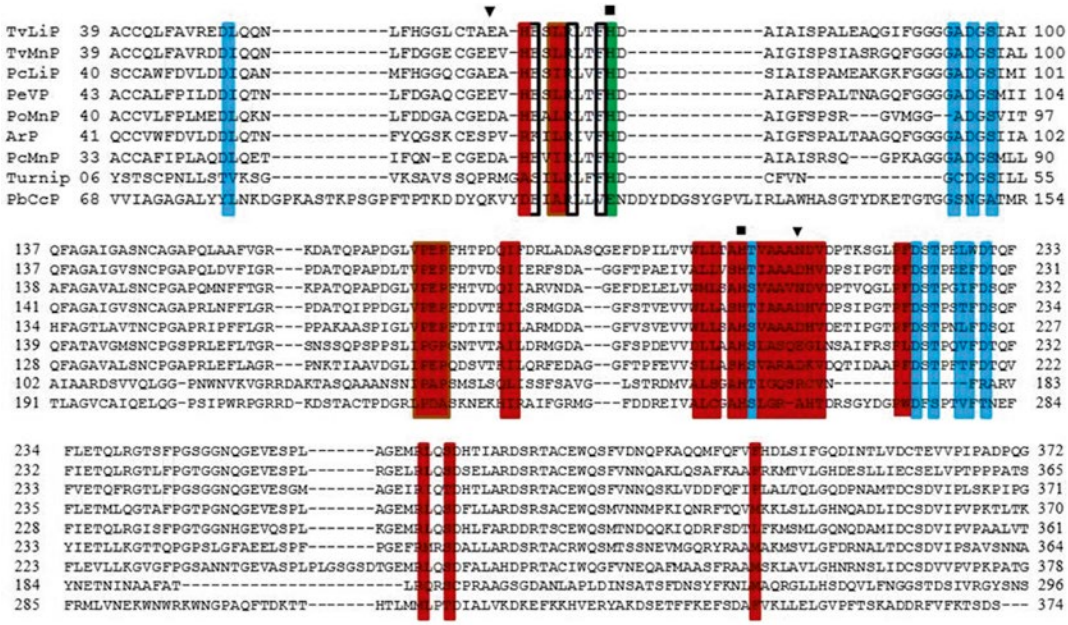

Fig. 6.7 Multiple alignments of amino acid sequences of fungal peroxidases. MnP, LiP, VP, CcP TP, and other peroxidases from *P. chrysosporium* (Pc) and *T. versicolor* (Tv), *P. ostreatus* (Po), *P. brasiliensis* Pb01 (Pb), *P. eryngii* (Pe), *Brassica rapa* turnip (turnip), and *A. ramosus* (ArP) are compared. The alignment was generated and conserved residues were identified by using Clustal X 2.0.11. The sequences highlighted in *red* denote heme binding sites; calcium binding sites are highlighted in *blue*. *Green* shows conserved residues in the substrate binding site. *Blank boxes* encompass the common residues in heme and substrate binding sites. The following marks are used in the consensus line: Mn^{2+} binding site (▼); distal and proximal histidine (■). Accession numbers of the amino acid sequences are as given below: TvLiP, AAA34049.1; TvMnP, CAA83148.1; PcLiP, AAA03748.1; PeVP, AAD54310.1; PoMnP, AAA84397.1; ArP, P28313.3; PcMnP, AAA33743.1; Turnip, P00434.3; PbCcP, EEH41729.1

chimera 1.4.1 software (Fig. 6.8). Structural alignment depicted approximately 50 % similarity of the residues among these peroxidases. Several functionally relevant structurally important features were also observed:

(a) the very close structural similarity between MnP, LiP, and VP active sites, suggesting a similar mode of hydrogen-peroxide activation. The heme prosthetic group is found embedded between the N-terminal and the C-terminal domains, along with the surrounding conserved residues H46 (MnP)/H47 (LiP)/H47(VP), H173 (MnP)/H175 (LiP)/H169(VP), and R42 (MnP)/R43 (LiP)/R43(VP);

(b) the substitution of polar residues for the hydrophobic amino acids exposed at the edge of the channel involved in substrate recognition in lignin peroxidase, suggesting that manganese peroxidase does not directly bind aromatic substrates;

(c) the location of residues potentially able to bind Mn^{2+}, spatially positioned on the side of the 3-CH_3 heme edge. The close sequence similarity of all the peroxidases strongly suggests a very similar three-dimensional fold. Lignin peroxidase was characterized by two calcium binding sites involving the side-chain residues, i.e., Asp194, Ser177, Thr196 (proximal site) and Asp65, Asp86 (distal site). The corresponding residues of aligned manganese peroxidase were Asp198, Thr199, Thr219 and Asp64, Asp85 and of versatile peroxidase were Ser195, Asp194, Lys215 and Asp65, Asp86;

(d) the high degree of conservation of the aspartic acid residue at N + 1 site position after the distal histidine suggests that the calcium distal site is involved in maintaining the integrity of

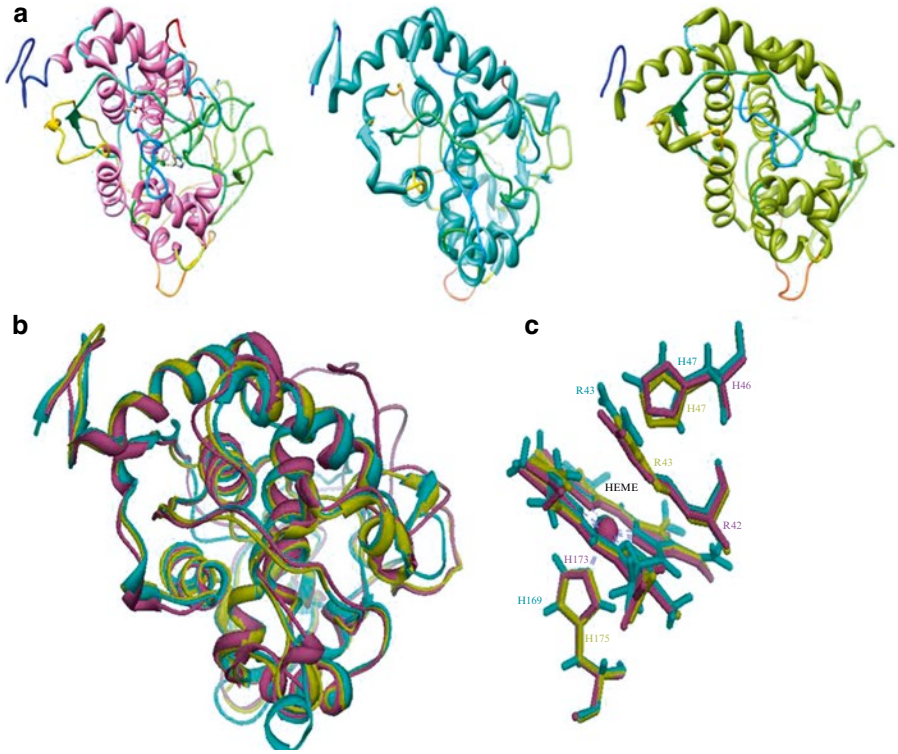

Fig. 6.8 Tertiary structural comparison of MnP, LiP, and VP using UCSF chimera 1.4.1 software. (**a**) Whole structures of *P. chrysosporium* MnP (pdb 1mnp), *T. cervina* LiP (pdb 3q3u), and *P. eryngii* VP (pdb 3fmu) are shown respectively from *left* to *right*. (**b**) Structural alignments of MnP, LiP, and VP are shown in *pink*, *cyan*, and *green*, respectively. (**c**) Structural alignment of the region surrounding heme and the MnP, LiP, and VP residue numbers are shown

the active site is in accordance with Henrisatt et al. (1990) and Welinder (1992);

(e) the sequence corresponding to the solvent-exposed a helix Ala12–Gln25, connected with other portions of the protein by disulfide bridges involving Cys3–Cysl5 and Cysl4–Cys289, is closely similar to the Ala12–Gln25 segment of LiP and VP with some closely related mutations. The coil region located at residues Ala50–Gly65 in the MnP sequence was not completely conserved in the lignin peroxidase and versatile peroxidase. The presence of glycine and proline in this region strongly suggests a coil arrangement (Benner 1989; Benner and Gerloff 1990; Branden and Tooze 1991). The region Pro163–Val175 (heme proximal helix) is highly similar to the aligned segments of lignin peroxidase and versatile peroxidase sequences except for some minor changes in this region.

Superimposition of the MnP, LiP, and VP structures clearly depicts the presence of five disulfide bonds in MnP in contrast to four disulfide bonds present in the LiP and VP. The initial four disulfide bonds in MnP, viz., Cys3–Cys15, Cys33–Cys117, Cys14–Cys289, and Cys253–Cys319, are the same as observed in Lip, VP, and *A. ramosus* peroxidase and also had perfect alignment as portrayed by superimposed structures in Fig. 6.8b. The additional disulfide bond found in MnP (Cys341–Cys348 located near the C-terminus of the polypeptide chain) aids in the formation of the Mn^{II} binding site and is responsible for pushing the C-terminus segment away from the main body of the protein (Sundaramoorthy et al. 1994a, b). LiP, VP, and MnP all have a Phe position 190, but the orientation differs. In LiP the Phe ring is nearly parallel to the proximal His imidazole ring, whereas in MnP the Phe ring is almost perpendicular to the plane of the proximal His.

Table 6.2 Helices distribution among MnP, LiP, and VP

S.No.	Helices		
	MnP	LiP	VP
1.	Ala16-Ile28	Asn11-Leu28	Asn11-Leu28
2.	Gly34-Ile49	Glu35-Val50	Gly35-Ile50
3.	Gly65-Phe70	Ser53-Ala59	Gly63-Ala79
4.	Ile83-His97	Gly69-Thr79	Ile81-His95
5.	Ser101-Ser115	Gly86-Gly102	Ser98-Asn113
6.	Ser147-Gly159	Ser104-Asn119	Ser144-Ala155
7.	Thr162-Leu170	Ser150-Asp160	Ser158-Ile171
8.	Thr199-Val205	Ser164-Ile177	Ser195-Thr201
9.	Leu239-His247	Asp184-Asp188	Gln229-Asp237
10.	Thr251-Gly257	Thr201-Leu208	Thr240-Met247
11.	Glu261-Ala277	Gln234-Asp242	Asn250-Leu267
12.	–	Thr245-Val253	Asp271-Leu275
13.	–	Asn255-Ala271	Ser279-Ile282
14.	–	Ile276-Leu280	Ser299-Val303
15.	–	Ser284-Ile287	–
16.	–	Ser306-Val310	–

Owing to the differences in orientation of the outer propionate in MnP, the distal Arg42 cannot form a hydrogen bond with the propionate, whereas in LiP, the distal arginine directly interacts with the propionate. The second (inner) propionate interacts with Mn^{2+} ion and water molecules, and the peptide NH group of a propionate together with a main chain peptide nitrogen is also found in LiP.

The residues forming the helices found to be structurally aligned in MnP, LiP, and VP models are summarized in Table 6.2.

MnP and Active-Site Engineering

Major challenges remain in understanding the role of functional domains and their structural/functional relationships. The desired features required for the commercial exploitation of these enzymes are stability, notable yields, and enhanced or superannuated activities. Site-directed mutagenesis (SDM) or active-site engineering is an invaluable tool to alter bases at precise positions in the gene. Engineered enzymes are then subjected to analysis for the above-mentioned favorable characteristics using various molecular tools. Site-directed mutagenesis is a major approach that provides opportunities to study unique structural/functional relationships in MnPs and allows the detailed characterization of the Mn^{II} binding site.

Kishi et al. (1994) developed a series of mutants (E35Q, E39Q, and E35Q-D179N) from the gene encoding manganese peroxidase isozyme 1 (*mnp*1) from *Phanerochaete chrysosporium,* using site-directed mutagenesis. The mutations demonstrated that changing any of the acidic amino acid Mn^{II} ligands, Asp179, Glu35, or Glu39, significantly affects the oxidation of Mn^{II}, most probably by decreasing the affinity of the enzyme for Mn^{II}. Asp179, Glu35, and Glu39 residues at the catalytic site are essentially required for Mn^{II} oxidation, since the double mutation, i.e., E35Q–D179N, had almost completely resulted into the loss of Mn^{II} oxidation (Sundaramoorthy et al. 1994a, b). The coordination of Mn^{II} at this site is octahedral, which is typical of Mn^{II} coordination complexes (Demmer et al. 1980). It has been postulated that Glu39 is a Mn ligand and its precise geometry within the Mn binding site of MnP is essential for the efficient binding, oxidation, and release of Mn by this enzyme and that mutation of this ligand decreases both the Mn binding and the rate of Mn oxidation (Martınez 2002; Li et al. 2001).

Miyazaki and Takahashi (2001) found that the site-directed mutagenesis of oxidizable Met273

located near the H_2O_2-binding pocket to a non-oxidizable Leu had resulted into improved stability of IMnP, as it retained more than 60 % of its initial activity in presence of 1 mM H_2O_2 and more than 30 % at a concentration of 3 mM H_2O_2 as compared to wild type that was completely inactivated by 1 mM H_2O_2. Stability in the presence of hydrogen peroxide may be attributed to the above-mentioned mutation that makes it resistant to oxidation by the conformational stabilization around the H_2O_2-binding pocket. Manganese peroxidase (MnP) is susceptible to thermal inactivation due to the loss of calcium. Engineering of a disulfide bond near the distal calcium binding site of MnP by double mutation A48C and A63C showed the improvement in thermal stability as well as pH stability in comparison to native MnP. The disulfide bond adjacent to the distal calcium ligands Asp47 and Asp64 stabilizes the recombinantly expressed MnP against the loss of calcium (Reading and Aust 2000).

Timofeevski et al. (1999) have described that a single mutation (S168W) in rMnP added veratryl alcohol oxidase activity to the enzyme without significantly affecting Mn^{2+} oxidase activity. This surface tryptophan residue, present in various LiP isoenzymes but absent in MnP, may be the site of VA binding and oxidation by LiP. Other research conducted observed how the hydrophobicity of the heme pocket could affect the reactivity of compound I (formed during MnP catalysis). Leu169 and Ser172 were mutated and converted Phe and Ala, respectively. Steady-state kinetics characterization indicated that the Leu169Phe mutation had little effect on activity, whereas the Ser172Ala mutation decreased *k*cat to **45 s^{-1}** as compared to wild type (449 s^{-1}) and also the specificity constant (*k*cat/*K*m) of Ser172Ala mutant decreased from $1.1 \times 10^7\ M^{-1}s^{-1}$ to $5.3 \times 10^5\ M^{-1}s^{-1}$ for Mn^{2+}, but not H_2O_2. It has been shown/demonstrated that compound II is the most sensitive to changes in the heme environment when compared to compound I (Balay et al. 2000).

The role of the axial ligand hydrogen-bonding network on heme reactivity was analyzed by Whitwam et al. (1999); D242 is hydrogen bonded to the proximal His of MnP, in other peroxidases, and this conserved Asp, in turn, is hydrogen bonded to a Trp. In MnP and other fungal peroxidases, the Trp is replaced by a Phe (F190). Both residues are thought to have a direct influence on the catalytic center of the enzyme. Mutagenesis of D242 and F190 has shown that these residues affect the reactivity of the heme active site. The changes in the axial ligand H-bonding network largely influence the reactivity of compound II (Fe^{4+}) and have little influence on the reactivity of compound I (porphyrin cation radical).

Zhang et al. (2009) described the role of Arg42 and Asn131 in the oxidation of 2,6-DMP after performing site-directed mutagenesis with in vitro synthesis. As previously described, R177A and R177K mutants of *P. chrysosporium* had specifically altered binding of Mn, whereas the rate of electron transfer from Mn^{2+} to the oxidized heme was apparently not affected (Gelpke et al. 1999). Whitwam et al. (1997) had suggested that Arg177 may anchor to the carboxylate of Glu35 in the Mn^{2+}-occupied closed configuration of the protein. Shortening the side chain of this residue by one methylene in the E35D mutant probably does not affect the salt bridge to Arg177, but it may restrain the carboxylate of this ligand from making a strong bond with the Mn^{II} atom. This results in a disruption of the ligation for Mn^{II} and hence in the electron-transfer rate as observed by Gelpke et al. (1999).

MnPs and Industrial/Commercial Applications

Industrial applications for manganese peroxidases that have been proposed include bleaching of unbleached kraft pulp in pulp and paper industries, treatment of textile industry effluents, in generating natural aromatic flavors in foods, degradation of environmental pollutants like polyaromatic hydrocarbons (PAHs), azo dyes, TNT, and DTT. Manganese peroxidase is versatile and energy saving, and its ability to bioremediate displays the capacity of this enzyme to be a significant tool for a number of eco-friendly commercial applications.

Ecotoxic organic chemicals generated from textile, pulp, and paper industry effluents are major contributors for the environmental pollution and are the major health hazards for a number of vertebrates and to human population. Detoxification of these compounds poses an immense technical challenge (Evans et al. 2004; Brar et al. 2006). Conventional methods for treatment of various industrial effluents include physical (adsorption, membrane filtration, ion exchange, irradiation, etc.) and chemical (oxidation, coagulation, electrochemical, etc.) processes; these methods have earlier been reviewed extensively (Hao et al. 2000; Forgacs et al. 2004; Joshi et al. 2004; Kuhad et al. 2004). The major drawbacks of physicochemical approaches are that these are prohibitively expensive, less efficient, not versatile, and have interference by other wastewater constituents. Biological methods consisting of biosorption, biodegradation, and enzymatic processes are eco-friendly, simpler, and cost-effective and are receiving greater attention for treatment of industrial effluents (Kuhad et al. 2004; Kaushik and Malik 2009).

Manganese peroxidases are the part of the extracellular oxidative system which evolved in white-rot fungi for lignin degradation (Kirk and Cullen 1991; Hatakka 1994; Vares and Hatakka 1996). Lignin is a heterogeneous, optically inactive polymer consisting of phenylpropanoid interunits, which are linked by several covalent bonds (e.g., aryl-ether, aryl–aryl, carbon–carbon bonds) (Hofrichter 2002). The structure of the lignin polymer implies that lignolytic enzymes possess the ability to oxidize substrates of high redox potential in a nonspecific manner. Paper and pulp industries employ a combination of chlorine-based chemicals and alkaline extraction multistage procedures for bleaching of the kraft pulp. However, chemical bleaching procedures end up with chlorinated organic substances as by-products which contain toxic, mutagenic, and carcinogenic polychlorinated dioxins, dibenzofurans, and phenols. Discharge of these organic compounds into the effluent generates serious environmental concern. Partially purified manganese peroxidase in the presence of oxalate preparations is known to be effective in decolorizing kraft effluents and oxidizing a broad range of xenobiotic compounds (Harazono et al. 1996; Sasaki et al. 2001). In vitro depolymerization studies using LiP and MnP showed that the enzymes were able to degrade to a variety of aromatic substrates (Conesa et al. 2002).

MnP also demonstrated the ability to decolorize a range of azo and anthraquinone dyes, as well as textile industry effluents in aqueous cultures and in packed bed bioreactors (Robinson et al. 2001; Mielgo et al. 2001; Kasinath et al. 2003; Shin 2004; Yang et al. 2004; Snajdar and Baldrian 2007; Asgher et al. 2008; Sedighi et al. 2009). Susla et al. (2008) have evaluated the contribution of MnP from *D. squalens* and laccase in degradation of azo, anthraquinone, phthalocyanine, and oligocyclic aromatic dyes. MnP has been observed to be capable of decolorizing the mixture of azo dyes at a concentration range of 10–200 mgl^{-1} each (Singh and Pakshirajan 2010), and also MnP from *P. chrysosporium* sp. HSD has been reported to rapidly decolorize a higher concentration (up to 600 mgl^{-1}) of azo dyes (Hailei et al. 2009).

MnP has been shown to have the mineralization ability for many environmental contaminants. Besides having ability to degrade azo, heterocyclic, reactive, and polymeric dyes (Champagne and Ramsay 2005), it can degrade 1.1.1-trichloro-2.2-bis-(4-chlorophenyl) ethane (DDT), 2.4.6-trinitrotoluene (TNT), and polycyclic aromatic hydrocarbons (PAHs) too (Maciel et al. 2010).

Manganese peroxidase (MnP) from two metabolically distinct fungi *Phanerochaete chrysosporium* BKM-F-1767 (ATCC 24725) and *Bjerkandera* sp. BOS55 (ATCC90940) has the ability to degrade (98 %) anthracene to generate anthraquinone in organic solvent mixtures after 6 h of operation under optimal conditions (Eibes et al. 2005). Utilization of MnP (from the basidiomycete *Bjerkandera adusta)* for acrylamide polymerization has also been reported (Iwahara et al. 2000). MnPs from *Phanerochaete chrysosporium* have also been employed in styrene degradation, an important industrial polymer used as a raw material for wrapping and transporting goods. Its disposal poses serious environmental concerns (Soto et al. 1991; Lee et al. 2006).

MnP a redox enzyme has the potential of directly transferring the electrons to the electrodes.

This enables the use of this enzyme for various applications in the development of biosensors, designing effective biofuel cells, and for selective bioorganic synthesis (Maciel et al. 2010).

Future Perspectives

This review highlights the various developments related to the molecular features, cloning, heterologous production, crystal structure refinements of MnPs, and its possible industrial and biotechnological applications. MnPs are promising enzymes and an eco-friendly alternative to the conventional physicochemical processes as presently employed for various such as the pulp and paper, textile, pharmaceutical, and for food industries. However, a major challenge in the commercialization of the MnPs is due to its lower thermal stability. Despite recent progress, our understanding of the process is still limited due to its substrate complexity and because of multiplicity of the peroxidases. Manganese peroxidases currently generated are so far not promising enough for their commercial scale exploitation. Although efforts have been made for improving the thermal stability of MnPs, further efforts are required for the development of designer enzymes with desired levels of thermal stability for its industrial applications. Therefore, the tailor-made enzymes can be designed using a combination of molecular approaches like site-directed mutagenesis, saturation mutagenesis, and directed evolution enabling the industrial exploitation of the unique catalytic abilities of these biocatalysts. The various developments in enzyme engineering research open a wide spectrum of possible applications in the near future.

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