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BRIEF COMMUNICATION

Identification of a new member of the dye-decolorizing peroxidase family from *Pleurotus ostreatus*

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Abstract Dye-decolorizing peroxidases (DyP) are atypical peroxidases showing no homology to other fungal peroxidases and lacking the typical heme binding region conserved among plant peroxidase superfamily. The gene and the corresponding cDNA encoding DyP from *Pleurotus ostreatus* have been identified on the basis of sequence homology analyses. The deduced amino acid sequence shares 43% identity with DyP from the ascomycete *Thanatephorus cucumeris* Dec 1. Analyses of the protein sequence by homology searches pointed out some properties of the DyP-type peroxidase family, which includes members from bacteria, ascomycete, and basidiomycete fungi. Some amino acids (C374, H379, and Y501 in the *P. ostreatus* DyP sequence) are proposed as candidates for the heme ligand, providing a basis for further investigations on the structure of the DyP type peroxidase family members.

Keywords Dyestuff · Fungi · Gene structure · Oxidative enzyme · Peroxidase · Proximal His

Introduction

White-rot basidiomycetes secrete two types of extracellular oxidative enzymes (peroxidases and laccases) that are involved in lignin degradation and are exploitable in the detoxification of several aromatic pollutants and synthetic toxic dyes (Pointing 2001). Fungal peroxidases include lignin peroxidase (LiP, E.C.1.11.1.14) and manganese peroxidase (MnP, E.C.1.11.1.13) that are representative members of class II peroxidases, according to the classification of plant peroxidase superfamily proposed by Welinder (1992). Catalytic mechanisms of these enzymes have been extensively studied (Kuwahara et al. 1984). LiP can catalyze the oxidation of non-phenolic compounds such as veratryl alcohol to the corresponding aryl cation radicals (Kersten et al. 1985), but substrates of other fungal and plant peroxidases are generally restricted to phenolic compounds. MnP oxidizes Mn(II) in the presence of some organic acids, and the resultant Mn(III)-organic acid complex oxidizes various phenolic compounds (Wariishi et al. 1992). Other fungal peroxidases (dye-decolorizing peroxidases, DyP) isolated from the fungi *Thanatephorus cucumeris* Dec 1 (previously named *Geotrichum candidum* Dec 1) and *Termitomyces albuminosus* have been classified as class II peroxidases, although their characteristics are different from those of any other known peroxidase (Kim and Shoda 1999; Sugano et al. 1999). DyP shows no homology to other fungal peroxidases such as LiP and MnP, and lacks the typical heme-binding region conserved among plant peroxidase superfamily (one proximal histidine, one distal histidine and one essential arginine) (Sugano et al. 1999). DyP differs from MnP because of its ability to oxidize phenolic compounds, such as 2,6-dimethoxyphenol and guaiacol, in the absence of Mn^{2+}. Moreover, DyP does not degrade non-phenolic compounds, thus differing from LiP. Another singular characteristic of DyP is its ability to degrade several synthetic dyes (Kim and Shoda 1999),

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mainly anthraquinone dyes, which are not oxidized by most of the other peroxidases. As a fact LiP, versatile peroxidase (Ruiz-Duenas et al. 2001) and manganese-independent peroxidase (Ruiz-Duenas et al. 1999) have been reported to mainly degrade azo dyes.

In this paper, we report the sequence of a gene and of the corresponding cDNA encoding a DyP from *Pleurotus ostreatus*. Homology search analyses of the deduced amino-acidic sequence allowed us to highlight properties of this peroxidase subfamily, which includes members from bacteria, ascomycete and basidiomycete fungi. Hypotheses on residues involved in heme binding have also been advanced.

Materials and methods

Organism and culture conditions

Pleurotus ostreatus (Jacq.:Fr.) Kummer (type: Florida) (ATCC no. MYA-2306) liquid cultures were carried out in 2.4% potato dextrose broth in the presence of 0.5% yeast extract, 150 μM copper sulphate and 100 μM ferric chloride. About 50 ml of a 5-day-old culture were transferred in 1 l flasks containing 450 ml broth at 28°C in the dark.

Isolation and sequencing of DyP cDNA

Total RNA was extracted from lyophilized mycelia, harvested from 7-day cultures, as described by Lucas et al. (1977). Reverse transcription reaction was performed using Super Script II Rnase H$^-$ Reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and the oligonucleotide dT-NotI as primer. PCR experiments were performed using the oligonucleotides GGHCCNGAYGAYCCNGC and GGRTGNGTRTARTCGATNCC (Y = T/C, R = G/A; H = T/C/A) as primers, at 65°C as annealing temperature. The amplified 800 bp cDNA fragment was cloned in the pGEM-T plasmid (Promega, Madison, WI, USA) and sequenced.

To complete the 3′ and the 5′ terminal cDNA region, amplification experiments were performed using as primers dT-NotI—CTCGGCTCCGGGTTCC and ATGCGCTGGTGGACTACCTG—CGGGCAGCTCTGCGGGG oligonucleotide couples, designed on the basis of the determined gene sequence.

Isolation and sequencing of DyP gene

Amplification experiments of the gene fragment were performed at 60°C annealing temperature using specific oligonucleotides designed on the basis of the cDNA sequence: CCCGGCGATGCGGCTGG and CCCTGGTCAGCGCGGGG. The amplified gene fragment, labelled by the random priming method, was used as probe to screen a *P. ostreatus* genomic library (Giardina et al. 1995). Two positive clones were selected and their sequences, overlapping for 100 bp, encompassed the complete coding sequence, as well as 400 bp upstream and 1,800 bp downstream.

Sequencing by the dideoxy chain-termination method was performed by PRIMM Sequencing Service (Milan, Italy) using universal and specific oligonucleotide primers.

Nucleotide sequence accession number

The sequence of DyP gene reported in this paper has been entered in the EMBL Data Library with accession number AM283034.

Results

RT-PCR experiments performed on *P. ostreatus* mRNA allowed us to amplify a cDNA fragment whose encoded amino acid sequence is homologous to that of DyP from *T. cucumeris* Dec 1 (Kim and Shoda 1999). The corresponding gene fragment was amplified and used as probe to screen a *P. ostreatus* genomic library (Giardina et al. 1995) and the whole gene was isolated and sequenced. Three putative start codons were identified in the 5′ gene region. cDNA amplification experiments, performed using primers corresponding to these positions, allowed the identification of the actual start codon. Comparison of the cDNA and gene sequences showed that the coding sequence is interrupted by nine introns. TATA (position 280 of AM283034 deposited sequence) and CAAT boxes (position 64) were identified in the 5′ gene-flanking region, extending 400 bp upstream of the ATG. Stretches that closely match consensus sequences of regulatory elements, such as stress responsive element (STRE, positions 367 and 413) and catabolic responsive element (Cre-A, position 141) were also recognized in that region (Soden and Dobson 2003).

The isolated cDNA encodes a putative protein, named *Po*DyP, of 516 amino acid residues with a M_r of 57,256 Da. Homology search analyses showed that *Po*DyP is a member of the so-called DyP-type peroxidase family (InterPro at EMBL-EBI web site), that comprises 174 proteins either from prokaryotes and eukaryotes. Among the 13 eukaryotic members, nine

are proteins from fungi. In Fig. 1, the alignment of *Po*DyP with some of these sequences, sharing an identity in the range 33–44%, is shown. Intron positions of the proteins, whose gene sequences are available, are also highlighted. The best-characterized member of the DyP family is that from *T. cucumeris* Dec 1 (Kim and Shoda 1999), that shares 43% identity with *Po*DyP. Analysis of the DyP-type peroxidase family sequences pointed out some conservative residues as putative candidates for the proximal ligands (indicated by arrows in Fig. 1).

Discussion

The ability of *P. ostreatus* to decolorize dyes, such as Remazol Brilliant Blue R (RBBR), has been demonstrated by several research groups. The involvement of a H_2O_2-dependent oxidative activity in the RBBR decolorization was demonstrated during solid state fermentation, by Vyas and Molitoris (1995). On the other hand, RBBR decolorization experiments by *P. ostreatus* liquid cultures indicated laccases as the main agents of the dye transformation process, even if the production of a dye peroxidase activity was detected in these conditions (Palmieri et al. 2005). Production and purification of a *P. ostreatus* RBBR-decolorizing peroxidase has been described by Shin et al (1997). This enzyme had a molecular mass of about 73 kDa, a large glycosylation moiety (38% by weight) and a blocked N terminus. Comparison between this enzyme and the protein encoded by the gene we isolated and sequenced seems to indicate an identity between them. If the protein undergoes a proteolytic processing after the signal peptide removal, in a way similar to the *T. cucumeris* Dec 1 (Sugano et al. 2000) or *T. albuminosus* homologous enzymes (Johjima et al. 2003), N65 could represent the new N terminal aminoacid, which could then cyclize to form a pyroglutamic acid residue. This could justify the

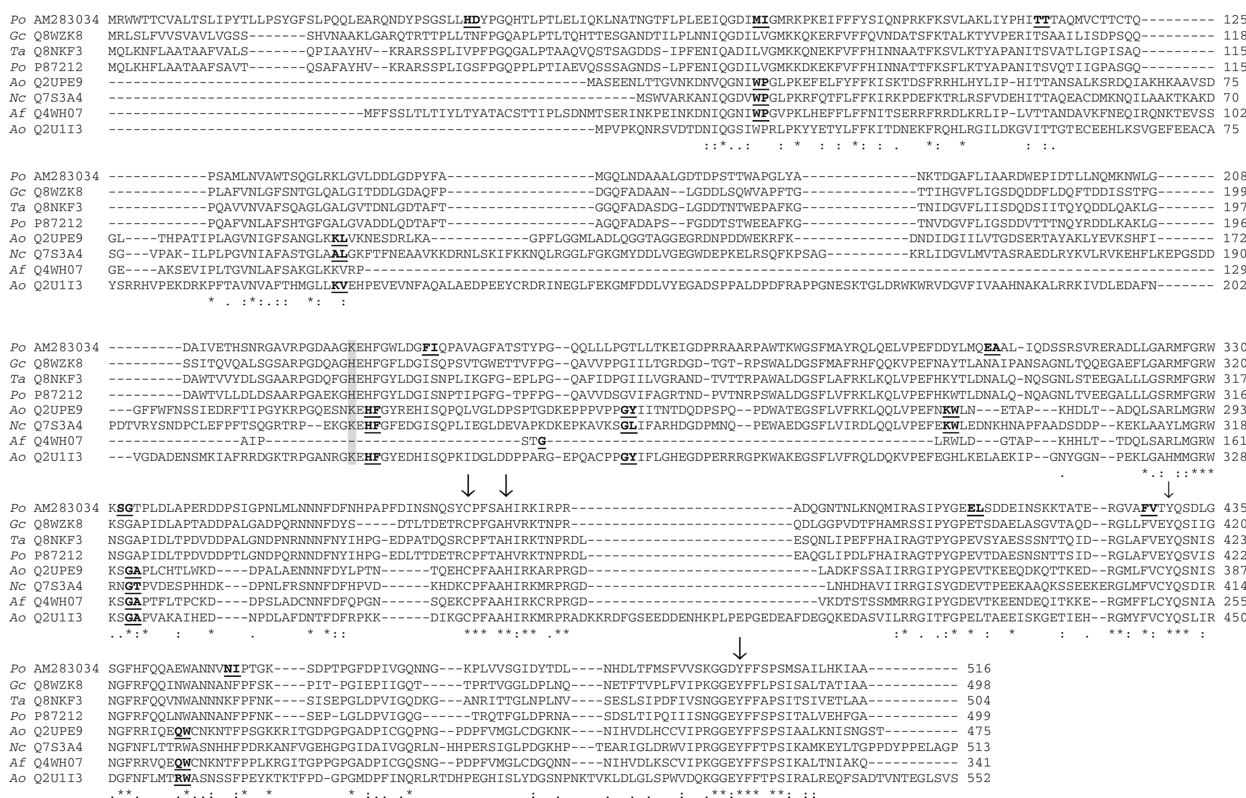

Fig. 1 Multiple alignment of selected fungal peroxidases. *Po* AM283034: Dye-decolorizing peroxidase from *Pleurotus ostreatus*; *Gc* Q8WZK8: DyP from *Thanatephorus cucumeris* Dec 1; *Ta* Q8NKF3: Peroxidase TAP from *Termitomyces albuminosus*; *Po* P87212: Peroxidase from *Polyporaceae sp*; *Ao* Q2UPE9: Predicted iron-dependent peroxidase from *Aspergillus oryzae*; *Nc* Q7S3A4: Hypothetical protein from *Neurospora crassa*; *Af* Q4WH07: Peroxidase, putative from *Aspergillus fumigatus*; *Ao* Q2U1I3: Predicted iron-dependent peroxidase from *Aspergillus oryzae*. *Asterisks* indicate conserved residues. Intron flanking positions are highlighted in *bold and underlined.* Conservative candidate residues for the proximal ligands in the fungal peroxidase sequences, are indicated by *arrows*, and those conserved in the majority of the other sequences of the whole DyP family are indicated by *bold arrows*. The position corresponding to H 164 of *T. cucumeris* Dec 1 is highlighted in *grey*

blocked N-terminus of the *P. ostreatus* enzyme found by Shin et al. (1997). The calculated M_r of the putative mature protein (49,982 Da) is consistent with that of the RBBR-decolorizing peroxidase, taking into account its glycosylation moiety.

The gene from *P. ostreatus* contains a higher number of introns than the homologous genes from other fungi, as already verified for other *P. ostreatus* genes, such as those coding for laccases (Giardina et al. 1995; Palmieri et al. 2003) and MnPs (Giardina et al. 2000). Comparison of the DyP gene structures shows one intron position fully conserved, while several other positions are conserved among at least three genes. This result further supports the idea that these genes are members of the same family, and suggests that they could have been originated from a common ancestor.

It has been reported that DyP from *T. cucumeris* Dec 1, as all the members of the DyP family, lacks the typical heme-binding region conserved among the other members of plant peroxidase superfamily (Sugano et al. 1999). The conserved residues, considered to be essential for peroxidase activity, are one His residue (proximal histidine) which acts as the axial ligand for the heme, a second His (distal histidine) and an Arg (essential arginine) that are considered to be involved in charge stabilization during the reaction between heme and H_2O_2 (Conesa et al. 2002). DyP-type peroxidases do not have similar heme-binding regions. Sugano et al. (2004) suggested that H164 of DyP from *T. cucumeris* Dec 1 could play the role of the proximal histidine, on the basis of preliminary X-ray analysis of the native enzyme (Sato et al. 2004) and characterization of the H164A mutant. As a fact, the specific activity and Reinheitzahl (RZ) value (A_{407}/A_{280}) of the H164A mutant were negligible with respect to those of the wild type. As shown in Fig. 1, H164 is replaced by lysine in *Po*DyP, in the proteins from *Aspergillus oryzae* (Q2UPE9, Q2U1I3), from *Neurospora crassa* (Q7S3A4), and in several other members of the DyP family (data not shown). According to these observations, two hypotheses are conceivable. Results of Sugano et al. (2004) could not indicate a direct involvement of H164 in heme coordination, but point out a severe destabilization of the protein structure decreasing its heme-binding affinity, due to H164 substitution. If this is the case, another amino acid residue should be involved in heme coordination in DyP-type peroxidases. The second hypothesis could be that enzymes from different sources are not identical with respect to heme iron ligands, thus identifying at least two sub-classes of dye peroxidases with different heme-coordinating amino acids. Johjima et al. (2003) proposed, as candidates for the proximal ligands, ten residues of His, Tyr and Cys conserved among DyPs from *T. albuminosus*, *T. cucumeris* Dec 1 and *Polyporaceae* sp. Among these, H166 of DyP from *T. cucumeris* Dec 1, although conserved in the majority of the sequences of the family, was demonstrated to be not essential for peroxidase activity by Sugano et al. (2004). Among the other candidate residues, four are conserved in the fungal peroxidase sequences and three of them in the majority of the sequences of the whole DyP family (indicated by arrows in Fig. 1). On the basis of these observations, new site-directed mutagenesis experiments can be planned to verify the role of these candidate residues for heme-binding ligands in the DyP subfamily, through structural and functional characterizations of the mutants. Analyses of these proteins along with those of different members of the DyP family can throw light on these atypical peroxidases.

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