

# Isolation and Purification of Pyranose 2-Oxidase from *Phanerochaete chrysosporium* and Characterization of Gene Structure and Regulation

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**Pyranose 2-oxidase (POX) was recovered from *Phanerochaete chrysosporium* BKM-F-1767 solid substrate culture using mild extraction conditions and was purified.  $^{13}\text{C}$ -nuclear magnetic resonance confirmed production of D-arabino-hexos-2-ulose (glucosone) from D-glucose with the oxidase. Peptide fingerprints generated by liquid chromatography-tandem mass spectrometry of tryptic digests and analysis of the corresponding cDNA revealed a structurally unusual sequence for the *P. chrysosporium* POX. Relatively high levels of *pox* transcript were detected under carbon-starved culture conditions but not under nutrient sufficiency. This regulation pattern is similar to that observed for lignin peroxidases, manganese peroxidases, and glyoxal oxidase of *P. chrysosporium*, supporting evidence that POX has a role in lignocellulose degradation.**

Pyranose oxidase (POX) (EC 1.1.3.10) is believed to have roles in ligninolysis (8, 49), production of peroxide for peroxidases, and synthesis of the antibiotic cortalcerone (3). It also is of interest for numerous biotechnological applications, analytical methods, and synthesis of novel chemicals (16). Indications are that POX is the major glucose-oxidizing enzyme in *Phanerochaete chrysosporium* ligninolytic cultures, even with the fungal strain and culture conditions for which glucose 1-oxidase (GOX) (EC 1.1.3.4) was previously reported (49). *P. chrysosporium* produces another peroxide-producing enzyme, glyoxal oxidase, which is secreted and coordinately expressed with the lignin peroxidases and manganese peroxidases (22, 24). The cloning, genomic organization, heterologous expression, site-specific mutagenesis, active-site characterization, and regulation of glyoxal oxidase have been described previously (20, 21, 23, 25, 28, 54, 55).

Several wood decay fungi in addition to *P. chrysosporium* are reported to produce POX (16), including *Oudemansiella mucida*, *Trametes versicolor* (8), *Polyporus obtusus* (36), *Phlebiopsis gigantea* (37), and *Trametes multicolor* (51). In general, POX oxidizes various aldopyranoses and disaccharides to the corresponding 2-keto sugars concomitant with the reduction of  $\text{O}_2$  to  $\text{H}_2\text{O}_2$  (16, 29). Substrates include D-glucose, D-xylose, L-sorbose, and D-glucono-1,5-lactone (2, 13). POX from *T. versicolor* oxidizes both alpha and beta anomers of glucose essentially equally well and therefore can be used in the microdetermination of glucose without the addition of mutarotase (43). POX is a FAD homotetramer with subunit molecular sizes of 68 to 76 kDa (10, 30, 48, 51), and in some instances it is glycosylated (16). The native POX purified from *P. chrysosporium* strain K3 mycelia, however, is not glycosylated, and three isoforms with pI 5.0, 5.05, and 5.15 are observed (2).

Ultrastructural and immunocytochemical studies of *P. chrysosporium* have demonstrated that the oxidase is concentrated within the periplasmic space, where it is associated with membrane-bound vesicles, other membrane structures, and extracellular slime (8, 9). Some POX activity is observed in culture fluid, but this appears to be related to cell lysis (9). POX can also be extracted from decayed birch (8, 9), and an extracellular activity is observed when the fungus is grown on the natural lignocellulosic substrate, wheat straw (52).

The purpose of the present study was to identify and characterize the glucose-oxidizing activity of *P. chrysosporium* grown on solid substrate. We used  $^{13}\text{C}$ -nuclear magnetic resonance (NMR) and  $^{13}\text{C}$ -labeled glucose to unambiguously distinguish GOX from POX. The extracellular POX was purified, the corresponding gene was identified, and a full-length cDNA was sequenced. Northern blots showed differential regulation of *pox* with transcript levels most abundant under carbon-starved ligninolytic conditions.

## MATERIALS AND METHODS

**Chemicals.** [ $^{13}\text{C}$ ]glucose, acetone- $d_6$ , and  $^{13}\text{C}$ -labeled acetone were purchased from Cambridge Isotope Laboratories, Inc. (Andover, Mass.). [ $^{13}\text{C}$ ]glucosone was synthesized from [ $^{13}\text{C}$ ]glucose (4, 35). D-glucose, D-cellobiose, D-xylose, and D-glucono-1,5-lactone were purchased from Sigma (St. Louis, Mo.).

**Organism and culture conditions.** *P. chrysosporium* BKM-F-1767 was grown in 12-oz. wide-mouth jars (Ball Corp., Broomfield, Colo.) containing 10 g of wheat bran amended with 7 ml of 1% corn steep liquor (Sigma) and harvested after 2 weeks of growth at 30°C. *P. chrysosporium* RP78, a homokaryotic strain derived from BLM-F-1767 (40), was used for isolation of total RNA (see below).

**Enzyme purification.** Combined cultures of *P. chrysosporium* BKM-F-1767 were extracted with 5 volumes of water (based on grams of original wheat bran) at 90 rpm on ice for 30 min. The extracted proteins were separated from the wheat bran by filtration through Miracloth (Calbiochem, La Jolla, Calif.). Particulates were removed by centrifugation at  $12,000 \times g$  for 30 min. The supernatant was saturated to 85% ammonium sulfate, protein was recovered by centrifugation at  $4000 \times g$  for 10 min, and the pellet was dissolved in 10 mM phosphate buffer (pH 6.5). The sample was dialyzed against 10 mM phosphate buffer (pH 6.5). The POX was purified on an fast protein liquid chromatography unit (Pharmacia, Uppsala, Sweden) at room temperature with the sample in the appropriate buffer for each of four chromatography steps: (i) DEAE Sepharose (Sigma) eluting with 800 ml of a 0 to 0.5 M NaCl gradient in 10 mM potassium

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phosphate (pH 6.5): (ii) Hitrap affinity column with Zn (Amersham Biosciences, Piscataway, N.J.) eluting with 30 ml of a 0 to 30 mM imidazole gradient in a solution of 20 mM sodium phosphate (pH 7.2), 1 M NaCl; (iii) Sephacryl S-200-HR (Sigma) with a 20 mM sodium phosphate (pH 7.2), 1 M NaCl mobile phase; and (iv) MonoQ (Amersham Biosciences) eluting with 34 ml of a gradient from 0 to 0.5 M NaCl in 20 mM piperazine (pH 6.0). A representative purification gave yields of 100, 300, 408, 161, and 116% for the ammonium sulfate precipitation, DEAE Sepharose, Zn HiTrap, Sephacryl S-200-HR, and MonoQ purification steps, respectively. This provided approximately 1 mg of purified POX from 150 g of wheat bran culture.

**Enzyme reaction conditions.** Activity of POX was determined in 1-ml reaction mixtures containing 0.01% phenol red, 50 mM 2,2'-dimethylsuccinic acid (DMS) buffer with pH adjusted to 6.0 with NaOH, 0.01 mg of horseradish peroxidase (type 11; Sigma), and 50 mM D-glucose, D-cellobiose, D-xylose, or D-glucono-1,5-lactone. Reaction mixtures were incubated at 37°C, reactions were stopped with 50  $\mu$ l of 2 N NaOH, and absorbances at 610 nm were determined (24).

For NMR characterizations, POX reaction solutions (1.8 ml) contained 5 mM concentrations of one of [<sup>13</sup>C]glucosone through [6-<sup>13</sup>C]glucosone, 2 mM phenol red, 0.01 mg of horseradish peroxidase type II (Sigma), and 10 mM DMS buffer (pH 6). The reaction solution was flushed with O<sub>2</sub>, and O<sub>2</sub> consumption was monitored at 37°C with a Clark-type electrode. When O<sub>2</sub> was depleted the solution was flushed again. After three cycles NMR spectra were obtained.

**NMR analyses.** NMR spectra (62.9 MHz, <sup>13</sup>C) were measured at 25°C on a Bruker DPX-250 spectrometer. Acetone-d<sub>6</sub> (29.83 ppm) was used as reference signal with authentic. In enzymatic reactions, <sup>13</sup>C-acetone (30.56 ppm) in D<sub>2</sub>O (1.3-<sup>13</sup>C, 99%) was used as reference signal. Chemical shift assignments for glucosone, glucono-1,5-lactone, gluconic acid, and gluconate were determined from authentic. NMR reference data include those reported for glucose (58), aldono-1,5-lactones (53), monosaccharides (5), and glucosone (14).

**Gel electrophoresis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing were performed using the Bio-Rad Criterion system (Hercules, Calif.) according to the manufacturer's instructions. Precast 10% Tris-HCl Ready Gel and a pH 3 to 10 isoelectric focusing (IEF) Ready Gel were used for SDS-PAGE and isoelectric focusing, respectively.

**Tryptic digestion of protein and LC-MS/MS analysis.** The band containing POX was cut from an SDS-PAGE gel stained with Coomassie brilliant blue R-250 (Bio-Rad). The excised gel slice was washed three times with 400  $\mu$ l of 50% acetonitrile in 25 mM NH<sub>4</sub>-bicarbonate (pH 8), dehydrated in 100% acetonitrile, and then dried in a speed-vac. The sample was submitted to the University of Wisconsin—Madison Biotechnology Center for LC-MS/MS (Agilent 1100 high-performance liquid chromatography [HPLC]/Micromass QTOF2 system; Waters, Milford, Mass.) of a tryptic digest. Data files were extracted and analyzed in Spectrum Mill MS Proteomics Workbench Rev. A.03.00.014 (Agilent Technologies, Palo Alto, Calif.).

**cDNA preparation and sequencing.** Based on LC-MS/MS peptide sequences, a putative POX gene, *pox*, was identified by tBlastn searches of the *P. chrysosporium* genome database (<http://www.jgi.doe.gov/>). Following manual corrections of *pox* gene model genewise.62.7.1, suitable primers were designed for amplification of the corresponding cDNA. Two-week-old cultures of *P. chrysosporium* BKM-F-1767 grown on wheat bran and supplemented with corn steep liquor were snap frozen with liquid nitrogen, and total RNA was extracted (21, 46). mRNA was purified by magnetic capture using Dynabeads (DynaBeads, Oslo, Norway) following the manufacturer's instructions. cDNAs were synthesized as described previously (17, 21, 46).

**Northern blots.** *P. chrysosporium* RP78, a haploid homokaryotic strain derived from BKM-F-1767 (40), was used for Northern blot hybridizations. Using QIAGEN's RNeasy Plant Mini kit (Valencia, Calif.), total RNA was isolated from mycelium harvested from (i) log phase cultures grown in B3 medium (27); (ii) ligninolytic C-limited B3 medium (39); and (iii) defined mineral salts medium (12) with 0.4% Avicel cellulose as sole carbon source (47). Twenty micrograms of RNA was size fractionated on formaldehyde gels and transferred to nylon membranes (Nytran; Schleicher & Schuell Bioscience Inc., Keene, N.H.) following Ambion's Northern Max protocol (Austin, Tex.). PCR amplicons of *pox* and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) cDNAs were partially purified (Wizard DNA Clean-up System; Promega corp., Madison, Wis.) and random primed (Stripable DNA Probe; Ambion) to specific activity of >5  $\times$  10<sup>6</sup> cpm/ $\mu$ g. PCR was as described previously (38) using primer pairs 5'-CCCCGGCCAG GTTCCCATCC-3' and 5'-ATTGTACTTACGCAGCACGAGGTT-3' for *pox* and 5'-ATGCCGGTCAAAGCAGGAATCAA-3' and 5'-CACCGCAGACAA ACATGGGGGCA-3' for *gpd*. Denatured probes were added to a final concentration of 5  $\times$  10<sup>6</sup> cpm/ml of UltraHyb solution (Ambion) and were hybridized overnight at 42°C.

**Glycosylation analyses.** The glycosylation of POX protein, separated on an SDS-PAGE gel, was determined with the Gelcode Glycosylation staining kit (Pierce Biotechnology, Rockford, Ill.) following the instructions of the manufacturer. Horseradish peroxidase and soybean trypsin inhibitor proteins were used as positive and negative controls, respectively.

**Phylogenetic analysis.** The POXs were aligned using ClustalX (45) and phylogenetic analyses of aligned amino acid sequences (TreeBase No. S1100; <http://www.treebase.org/treebase/index.html>) were performed with the Distance method as implemented in PAUP version 4.0\*10b (42) using a neighbor-joining search. Stability of clades was assessed by 1,000 bootstrap replicates.

**Nucleotide sequence accession number.** The *pox* cDNA nucleotide sequence of *P. chrysosporium* BKM-F-1767 has been deposited in the GenBank database under accession number AY522922.

## RESULTS

**Enzyme production and purification.** Our early attempts to isolate POX from *P. chrysosporium* grown on aspen sawdust were unsuccessful, although fungal growth appeared adequate (data not shown). Failure to detect activity could be due to wood extractives interfering with detection, or perhaps the enzyme was strongly associated with the natural growth substrate. We then tested wheat bran supplemented with corn steep liquor as a solid substrate, because the low extractive content would possibly minimize assay interferences and its nonwoody characteristics may allow an extracellular oxidase to be easily extracted. Accordingly, POX was recovered for purification by simple water extraction of fresh cultures of *P. chrysosporium* BKM-F-1767 (see Materials and Methods). There were dramatic increases in POX activity yields for the early chromatographic steps, suggesting that inhibitors were significant even with this isolation technique. SDS-PAGE gave a doublet band at about 70 kDa, results similar to that reported for the intracellular POX of *P. chrysosporium* K-3 (48).

**Product identification.** The NMR characterizations for the enzymatic reactions with D-glucose, <sup>13</sup>C-labeled individually at each of the six positions, indicate the conversion of  $\alpha$ - and  $\beta$ -glucose to glucosone having four tautomers, designated a to d (Fig. 1). The chemical shifts we observed for glucosone are consistent with that reported by Freimund et al. (14), and our data rely on their tautomeric designations. Accordingly, the chemical shifts (in parts per million) observed at each carbon for the conversion of  $\alpha$ -glucose,  $\beta$ -glucose to a to d glucosone are the following: C-1, 92.51, 96.37 to 95.03, 95.48, 89.91, 90.53; C-2, 71.95, 74.62 to 94.01, 93.43, 97.67, 101.14; C-3, 73.25, 76.27 to 74.04, 76.85, 68.20, 76.27; C-4, 70.10, 70.06 to 69.22, 69.05, 70.34, 74.92; C-5, 71.79, 76.32 to 72.3, 76.3, 69.59, 80.87; C-6, 61.01, 61.21 to 61.37, 61.37, 63.74, 62.42, respectively. We observe relative tautomeric abundances of 46, 26, 18, and 10% for a to d, respectively, compared to values of 36, 31, 21, and 12% reported by Freimund et al. (14). A systematic offset of our data might be a result of solvent conditions and/or reference calibration. Based on these NMR results, the isolated oxidase is designated a POX.

**Enzyme characterization.** The purified oxidase showed the typical absorption maxima of flavoproteins at 275, 360, and 455 nm. Consistent with earlier reports (2, 16) of the POX from *P. chrysosporium*, the POX we isolated was not glycosylated, as indicated by the Gelcode glycosylation staining method. Three isoforms were observed at PIS of ca. 5.0, 5.05, and 5.15 by IEF electrophoresis. In contrast to a previous report that *P. chrysosporium* POX loses activity with repeated freezing and thawing

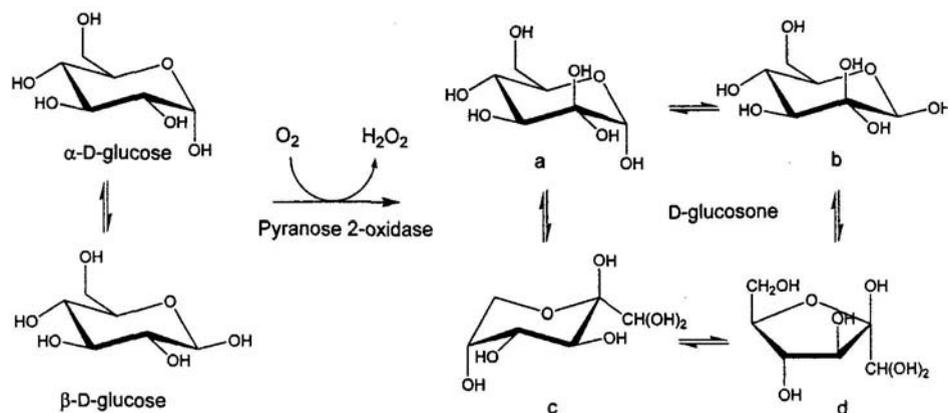


FIG. 1. Reaction catalyzed by POX. POX oxidizes both  $\alpha$ - and  $\beta$ -D-glucose at C-2 to give D-glucosone with four isomeric forms in aqueous solution. The approximate proportions of the four isomers a to d under our experimental conditions are 46, 26, 18, and 10%, respectively, based on  $^{13}C$ -NMR peak intensities.

without glycerol (2), the POX we isolated retained activity (data not shown). The relative activities of the isolated POX with 50 mM D-glucose, D-cellobiose, D-xylose, and D-glucono-1,5-lactone were 100, 17, 35, and 68%, respectively. The substrate specificity is similar to that of other pyranose oxidases, although the activity with cellobiose seems uncharacteristically high.

**Protein-gene assignment.** Sixteen distinct peptides derived from the LC-MS/MS data were correlated to one protein in the *P. chrysosporium* database, covering 42% of the predicted protein. The corresponding gene model, genewise.62.7.1, features several inaccuracies, as is often the case for automated predictions in eukaryotic genomes. Several inaccuracies occurred at the 5' and 3' termini, in part due to short exons in these regions. Nevertheless, the genewise.62.7.1 model served as a framework for designing a series of flanking primers, and a full-length *pox* cDNA was ultimately amplified.

**cDNA sequence and predicted protein.** The coding region of the *pox* cDNA is 1,866 bp long (GenBank accession no. AY522922), and alignment with the genomic sequence indicates that the gene is interrupted with 14 introns 48 to 67 bp long. Low-stringency BLAST of the *Phanerochaete* genome indicates a single POX gene in the genome.

The translate tool from ExPasy (<http://www.expasy.org/>) predicts a protein of 621 amino acids, molecular size of 69 kDa, and pI 6.03 from POX cDNA sequence. Reanalysis of the LC-MS/MS data against the predicted protein indicates 2 additional matching peptides, giving a total of 18 covering 40% of the protein. Notably, one of the peptides was 56 amino acids upstream of the N terminus reported by Artolozaga et al. (2) (Fig. 2). This is consistent with the POX that we isolated being slightly larger (70 kDa by SDS-PAGE) than the 65-kDa result reported by Artolozaga et al. (2).

**Northern analysis.** Figure 3 shows that the relative transcript level of *pox* was highest in carbon-starved media, with very low transcript levels in log phase cultures in B3 media or media with Avicel cellulose as sole carbon source. These results suggest a basal level of expression of POX and activation due to carbon starvation in *P. chrysosporium*. This is consistent with POX production during idiophasic growth of *P. chrysosporium* (13).

## DISCUSSION

The glucose oxidase described here is classified as a POX based on the NMR analyses of the enzymatic product from glucose and on substrate specificity. Although GOX can be distinguished from POX by  $^{13}C$ -NMR using  $[1-^{13}C]$ glucose as substrate (11), here we show that the products exactly match the four glucosone tautomers at all six carbons. Label at C-1 or C-2 alone is highly diagnostic, and the method has distinct advantages because no derivatization or product isolation is required. Relatively low concentration of glucose substrate is sufficient because of the sensitivity afforded by the enriched  $^{13}C$  label, and therefore oxygen limitation is not problematic for exhaustive consumption of the carbon substrate. This method not only allows the characterization of glucosone as the final product with purified oxidase but also as an intermediate in reactions with crude enzyme preparations (11).

The isolation technique (simple  $H_2O$  extraction) that we used for POX suggests an extracellular location for the oxidase when grown on wheat bran. However, analysis of the cDNA sequence by SignalP V1.1 (31) (<http://www.cbs.dtu.dk/services/Signalp>) does not indicate a typical secretion signal. Consequently, although secretion of proteins without a secretion signal is known (for example, see reference 38), autolysis may best account for the easy extraction of the enzyme. The stability to freeze-thaw cycles of the POX described here, in contrast to that of the enzyme isolated from mycelia (2), prompted us to test for glycosylation as a possible maturation step, but no glycosylation was detected. However, the POX reported here has significant N-terminal sequence (see below) that apparently is lacking from freeze-thaw-sensitive POX. Whether this N-terminal peptide contributes to the stability of the enzyme is not known.

Recently, pyranose oxidase was identified as a member of the glucose-methanol-choline oxidoreductase (GMC-OXRED) family of proteins based on *Coriolus versicolor* POX (CvPOX) and *Trametes hirsutus* POX (ThPOX) sequence analyses (1). The POXs show only very low, nonsignificant identity to individual GMC-OXRED members, but structure-based multiple sequence alignment shows distant homology. Fifteen strictly conserved residues and three major conserved regions (dis-

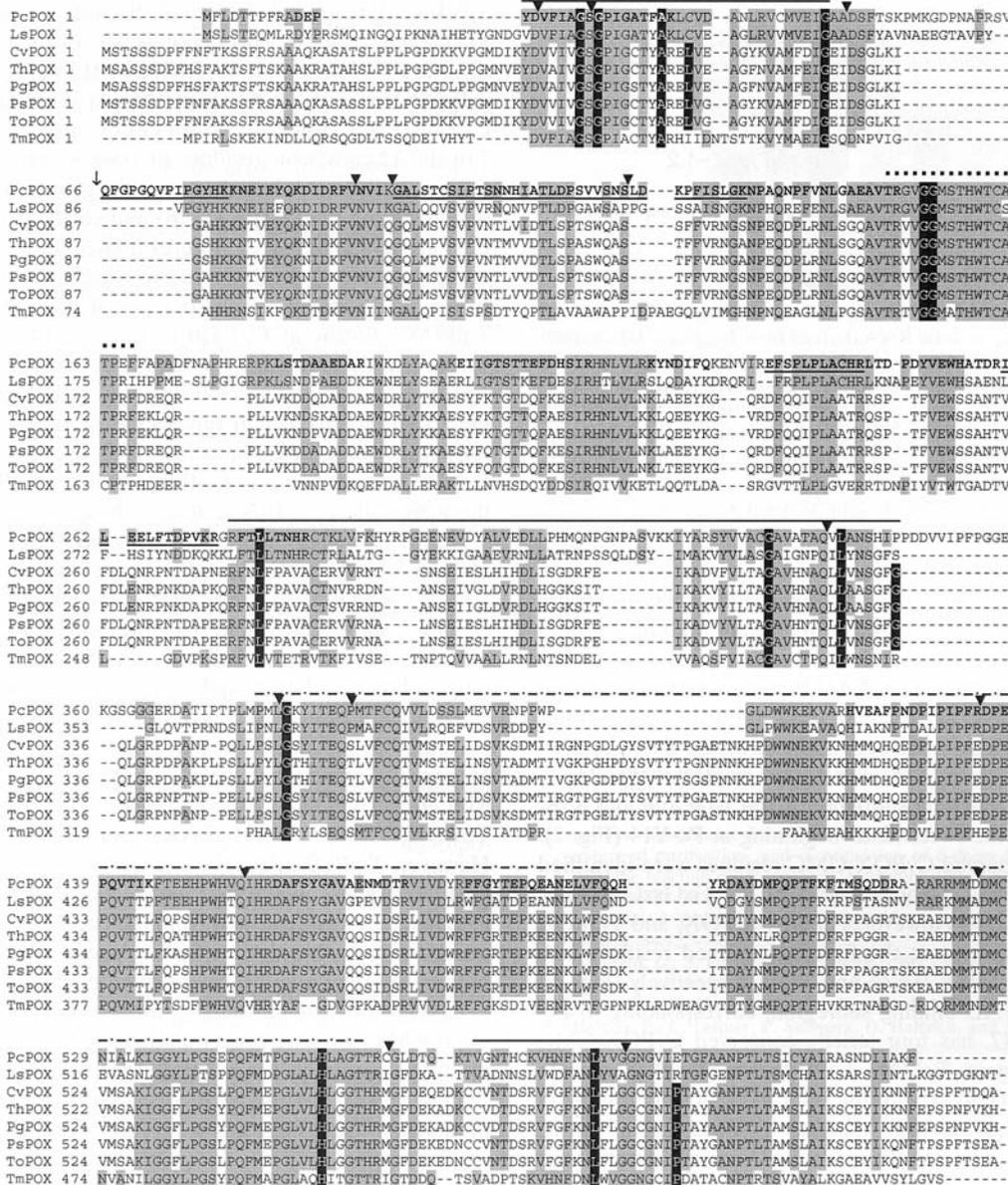


FIG. 2. Alignment of *P. chrysosporium* BKM-F-1767 POX (PcPOX accession no. AY522922) translated from cDNA sequence, with the POXs from *Lyophyllum shimeji* (LsPOX; accession no. gi |44886072|), *Corioliolus versicolor* (CvPOX accession no. gi |25091018| [33]), *T. hirsuta* (ThPOX; accession no. gi |25091016| [7]), *Peniophora gigantea* (PgPOX; accession no. gi |34452037|), *Peniophora* sp. (PsPOX; accession no. gi |27436422|), *Trametes ochracea* (ToPOX, accession no. gi |31044224|) and *Tricholoma matsutake* (TmPOX; accession no. gi |25553433| [44]). Grey shaded amino acids indicate homology with PcPOX; black shaded amino acids with white typeface indicate CvPOX-GMC consensus sequences (see the text). Solid lines above sequences indicate FAD-binding domains; dotted lines above sequences indicate flavin attachment loops; dashed lines above sequences indicate substrate binding domains. Boldface indicates peptides identified by LC-MS/MS; underlined and boldface type distinguishes separate peptides within a continuous sequence. ▽, intron positions; ↓, the start of the N terminus reported by Artolozaga et al. (2) for the POX of *P. chrysosporium* K3.

discussed below) were identified. *P. chrysosporium* POX (PcPOX) shows only moderate identity with CvPOX (41%) and ThPOX (41%) according to National Center for Biotechnology Information tBlastn (<http://www.ncbi.nlm.nih.gov/BLAST/>), and only 12 of the 15 strict consensus GMC-OXRED residues (1) are conserved (Fig. 2). Therefore, for the purposes of discussion, the strictly conserved residues identified by Albrecht and Langauer (1) are referenced here only in the restrictive context in which they were defined and termed CvPOX-GMC residues.

Other POXs (Fig. 2) also show only moderate identity with PcPOX, e.g., *Lyophyllum shimeji* POX (LsPOX; 53%), *Peniophora gigantea* POX (PgPOX; 41%), *Peniophora* sp. POX (PsPOX; 41%), *Trametes ochracea* POX (ToPOX 41%), and *Tricholoma matsutake* POX (TmPOX, 36%). As a subgroup, *C. versicolor*, *T. hirsuta*, *P. gigantea*, *Peniophora* sp., and *T. ochracea* POXs show high identity with each other (82 to 99%) and also conform to strict CvPOX-GMC consensus.

The three conserved regions identified for CvPOX (1) were

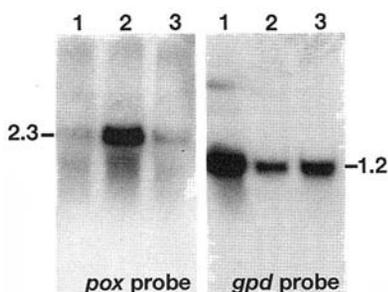


FIG. 3. Northern blots showing differential regulation of POX. Lanes contain 20  $\mu$ g of total RNA derived from log phase B3 medium (lanes 1), carbon starved B3 medium (lanes 2), and defined salts medium with Avicel cellulose as sole carbon source (lanes 3). Following washes, the blot probed with the *gpd* housekeeping probe was exposed to XAR film (Kodak) for 5 h without an amplifying screen. The *pox*-probed blot was exposed overnight with an amplifying screen. Autoradiograms were scanned, and the digitized images were labeled in Photoshop Illustrator (Adobe, San Jose, Calif.).

correlated with glucose 1-oxidase of *Penicillium amagasakiense* (PaGOX) because of obvious functional relatedness and because crystal X-ray diffraction data are available (57). The conserved regions are (i) the FAD-binding site with four subregions, (ii) the flavin attachment loop, and (iii) the substrate-binding region. Here, we deduce these structure-based regions from alignments of CvPOX with the other POXs (Fig. 2).

The first FAD-binding subregion is the ADP-binding  $\beta\alpha\beta$ -fold motif near the N terminus beginning at Pc-Y14 (Fig. 2). Five strictly conserved CvPOX-GMC residues were identified for this subregion (1) and are part of the GxGxxGxxxAxxL  $\beta\alpha\beta$ -fold motif (56). However, PcPOX, LsPOX, and TmPOX break from consensus at the fourth conserved residue, with Cv-L replaced by Pc-C, Ls-C, and Tm-I, respectively (Fig. 2).

The second FAD-binding subregion, corresponding to Pc-R275 to Pc-P347, has four strictly conserved CvPOX-GMC residues (1). Again, PcPOX, LsPOX, and TmPOX deviate from consensus (Fig. 2), with the last CvPOX-GMC residue G replaced with unconservative residues Pc-P, Ls-S, and Tm-R, respectively (Fig. 2). The G at Pc-G333 in this subregion is conserved by all POXs and CvPOX-GMC proteins, and this corresponding glycine in PaGOX is between the diphosphate and ribose of FAD (1, 26). This G is also the beginning of the GMC-OXRED signature 2 (Prosite PS00624) (6). PcPOX and CvPOX each mismatch 3 of the 10 consensus residues for a total of 5 mismatches, indicating that this signature, [GS]-[PSTA]-x(2)-[ST]-[PS]-x-[LIVM](2)-x(2)-S-G-[LIVM]-G, is not very diagnostic in POXs.

The third FAD-binding subregion, from Pc-V567 to Pc-E589, has two strictly conserved CvPOX-GMC residues (1). However, the strict CvPOX-GMC consensus at Cv-P is broken by Pc-E and Ls-R, again not conservative substitutions. The fourth region has no strictly conserved CvPOX-GMC residues and forms the C-terminal helix. The POX sequences show strong homology at the beginning of this short subregion.

The flavin attachment loop, located from Pc-T149 to Pc-F166 (Fig. 2), is highly conserved among the POXs, and they all conform to the strict CvPOX-GMC consensus for this region (i.e., at PcG153-G154). The FAD of *T. multicolor* (synonymous with *T. ochracea*) was shown to be of the  $8\alpha$ -(N<sup>3</sup>-histi-

dyl)-FAD linkage type attached through the histidine of the STHW domain (18). This region is part of the GMC-OXRED signature 1 given by [GA]-[RKNC]-x-[LIV]-G(2)-[GST](2)-x-[LIVM]-N-x(3)-[FYWA]-x(2)-[PAG]-x(5)-[DNESH] (Prosite PS00623) (6). However, both PcPOX and CvPOX deviate at 7 of the 12 consensus residues of GMC-OXRED signature 1.

The contiguous substrate-binding region is the least similar region among the GMCs (1, 26) and corresponds here to Pc-P377 to Pc-T557 (Fig. 2). Interestingly, there is a high degree of identity among the POXs in this region, but it is interrupted with a 21- to 26-amino-acid deletion in PcPOX, LsPOX, and TmPOX, starting at Pc-P410 in the case of PcPOX. Strictly conserved CvPOX-GMC residues are observed at Pc-G381 and Pc-H554. The conserved histidine is thought to be part of the active site involved in general base catalysis-accepting protons from the glucose substrate (26). POX alignments with PaGOX indicate only one other possible residue common in the active site (1). This residue corresponds to Pc-Y85 (Cv-Y97) and is in a region of high POX homology (Fig. 2). As with CvPOX and ThPOX, no other active-site residues are suggested for PcPOX as revealed by alignments with PaGOX (data not shown). Reliable structure-function analyses will require structure determinations of the POXs, for which there is only preliminary data available in the case of *T. multicolor* (19).

The distinctions based on GMC structure-function of the various POXs, as discussed above, are reflected in the corresponding phylogenetic tree (Fig. 4). As expected, the POXs with all 15 CvPOX-GMC consensus residues (CvPOX, PsPOX, ToPOX, ThPOX, and PgPOX) group as a separate clade. However, TmPOX, PcPOX, and LsPOX break from CvPOX-GMC consensus in the first and second FAD-binding subregions and are distinguished from the consensus group. PcPOX and LsPOX further deviate from consensus in the third FAD-binding subregion and form a separate clade (Fig. 4).

Besides the distinguishing features of PcPOX as it relates to other GMCs, there are several other aspects worth highlighting in comparison with the other POX sequences. For example, PcPOX has a 22-amino-acid insert at position 54 and a 14-amino-acid insert at position 345 which are not observed with

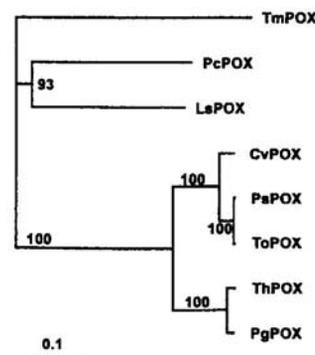


FIG. 4. Phylogenetic analysis of POX. Distance analysis and tree construction of amino acid sequence data were done by the neighbor-joining method in PAUP version 4.0\*10b. Numbers indicate the percent support for nodes after 1,000 replications of bootstrap analysis. The bar represents a 10% estimated sequence difference. Refer to the legend to Fig. 2 for abbreviations and gi numbers.

the other POX sequences. The N terminus reported by Artolozaga et al. (2) for native POX from *P. chrysosporium* K3 (QFGPGQVPIPGYSKNNEIEYQKDIDRFVNV) is within the 22-amino-acid insert and is encoded by the fourth and fifth exons starting at position 66 (Fig. 2). A 2-amino-acid discrepancy is observed (out of 31 amino acids) that may be due to fungal strain differences. The Spectrum Mill analysis of our LC-MS/MS data identified a peptide beginning at position 10, indicating that the protein we isolated has significant N-terminal sequence not observed by Artolozaga et al. (2). This would explain why Leitner et al. (29) observed no apparent homology of the N terminus of *P. chrysosporium* with those of *T. multicolor* and *T. versicolor*; i.e., the corresponding sequence was absent. *P. chrysosporium* POX has the smallest N-terminal sequence preceding the FAD-binding region 1. Specifically, there are only 13 amino acids with PcPOX, in contrast to 46 with CvPOX. The CvPOX is presumably synthesized as a preproenzyme and is localized in the periplasmic space after processing of 38 amino acids (33). The maturation mechanism for these different POXs is clearly different considering the lack of homology in this region.

Northern blots show that pox of *P. chrysosporium* is strongly regulated with relatively high transcript levels under C limitation (Fig. 3). This is consistent with POX activity profiles observed for *P. chrysosporium* in response to carbon limitation (13). In contrast, constitutive activity is observed in *T. versicolor* (32) and *O. mucida* (34) liquid cultures. This suggests different and/or multiple roles for POX in these various fungi. Importantly, the regulation pattern observed here with *P. chrysosporium* pox is similar to that observed for lignin peroxidases, manganese peroxidases, and glyoxal oxidase (41), supporting evidence that POX has a role in lignocellulose degradation.

One role for POX may be peroxide production for the extracellular peroxidases. However, extracellular glyoxal oxidase, for which a secretion signal has been identified (23), would seem to have a more direct role in extracellular metabolism. An alternate or additional role for POX could be related to further metabolism of the glucosone. *P. chrysosporium* has a pyranosone dehydratase (PYD) for subsequent conversion of glucosone to the antibiotic cortalcerone (15, 50). No PYD have been reported for the other fungi, for which there is POX cDNA sequence (Fig. 2). Many other wood decay fungi possessing POX apparently lack a PYD (3). This observation raises questions regarding the metabolic fate of glucosone during wood decay.

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