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A Novel White Laccase from *Pleurotus ostreatus**

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Two laccase isoenzymes (POXA1 and POXA2) produced by *Pleurotus ostreatus* were purified and fully characterized. POXA1 and POXA2 are monomeric glycoproteins with 3 and 9% carbohydrate content, molecular masses of about 61 and 67 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis, of about 54 and 59 kDa by gel filtration in native conditions, and of 61 kDa by matrix-assisted laser desorption ionization mass spectrometry (only for POXA1) and pI values of 6.7 and 4.0, respectively. The N terminus and three tryptic peptides of POXA1 have been sequenced, revealing clear homology with laccases from other microorganisms, whereas POXA2 showed a blocked N terminus. The stability of POXA2 as a function of temperature was particularly low, whereas POXA1 showed remarkable high stability with respect to both pH and temperature.

Both enzymes oxidize syringaldazine and ABTS (2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) together with a variety of different substituted phenols and aromatic amines with the concomitant reduction of oxygen, but POXA1 is unable to oxidize guaiacol. Both enzymes were strongly inhibited by sodium azide and thioglycolic acid but not by EDTA.

UV/visible absorption spectra, atomic adsorption, and polarographic data indicated the presence of 4 copper atoms/mol of POXA2 but only one copper, two zinc, and one iron atoms were found/mol of POXA1.

The neutral pI and the anomalous metal content of POXA1 laccase render this enzyme unique in its structural characteristics. The lack of typical absorbance at 600 nm allows its classification as a "white" laccase.

White rot Basidiomycetes are microorganisms able to efficiently degrade lignin. However the different degradation degree of lignin with respect to other wood components depends very much on the environmental conditions and the fungal species involved.

It is now clear that there is not a unique mechanism to achieve the process of lignin degradation and that the enzymatic machinery of the various microorganisms are different (1). *Pleurotus ostreatus* belongs to a subclass of lignin-degrading microorganisms that produce laccases, manganese peroxidases, and veratryl alcohol oxidases but no lignin peroxidases.

The oxidative enzymes (laccase, manganese peroxidase) do catalyze the formation of radical intermediates from high molecular weight lignins, but the intermediates produced can recondense, shifting back the reaction course (2). It has been shown that prevention of the repolymerization is achieved by the reduction of the formed radicals; this reducing process is carried out by some flavin adenine dinucleotide-dependent oxidases such as veratryl alcohol oxidase (3). Laccases and manganese peroxidases are able to oxidize only phenolic residues of lignin, whereas lignin peroxidases have been shown to be effective in the oxidation of the nonphenolic residues of the polymer (4). However, in the presence of mediators, the substrate range of laccases can be extended (5).

Laccases have been isolated from various fungi (6). They belong to the class of the blue oxidases containing 4 copper atoms/molecule distributed in three different copper binding sites (7, 8). The type 1 (or blue copper) site is responsible for the intense blue color of the enzymes, presumably due to a ligand-to-metal charge transfer absorption involving cysteine sulfur and Cu(II). The type 2 copper exhibits lower visible absorbance, and the type 3 site incorporates two copper centers and is responsible for a shoulder near 330 nm in the absorbance spectrum of native laccase. All these copper ions are apparently involved in the catalytic mechanism. The laccase reduces oxygen to water and simultaneously performs a one electron oxidation of many aromatic substrates (polyphenols, methoxy-substituted monophenols, aromatic amines, etc.). The enzyme is present in multiple isoforms in almost all fungal species, including *P. ostreatus* (9). Studies of the genes coding for these enzymes in *P. ostreatus* have led to the identification of two different genes and two corresponding cDNAs. One of these genes codes for the isoenzyme produced most abundantly under all the growth conditions examined (10).

In the present paper, we report the purification and the physico-chemical and catalytic properties of two different laccase isoenzymes isolated from *P. ostreatus*. One of these proteins shows peculiar differences with regard to copper content. This is the first laccase having 2 zinc, 1 iron, and only 1 copper atom/molecule.

EXPERIMENTAL PROCEDURES

Organism and Culture Conditions—White rot fungus *P. ostreatus* (strain Florida) was maintained through periodic transfer at 4 °C on potato dextrose agar plates (Difco) in the presence of 0.5% yeast extract (Difco).

Incubations were carried out at 25 °C in the dark by preinoculating 300 ml of potato dextrose broth (24 g/l) containing 0.5% yeast extract in 500 ml shaken flasks with the *P. ostreatus* mycelia. 50 ml of a 5-day-old culture were transferred in 1 liter flasks containing 450 ml of broth. The cultures were incubated in the dark at 25 °C on a rotary shaker (100 rev/min). At different incubation times, the medium was collected and filtered through gauze.

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Enzyme Purification—Proteins were precipitated from the filtered medium by the addition of $(NH_4)_2SO_4$ up to 80% saturation at 4 °C and centrifuged at 10,000 × *g* for 30 min. The precipitate was resuspended in 50 mM sodium phosphate buffer, pH 6.0, and extensively dialyzed against the same buffer. The sample was again centrifuged, and the supernatant, concentrated on an Amicon PM-10 membrane, was loaded on DEAE-Sepharose Fast Flow (Pharmacia Biotech Inc.) column (1,5 × 40 cm) equilibrated with the phosphate buffer. The column was washed at a flow rate of 30 ml/h with 400 ml of buffer, and a 0–0.5 M NaCl linear gradient (500 ml) was applied. Fractions containing phenol oxidase activity were pooled and concentrated on an Amicon PM-10 membrane.

The phenol oxidase POXA1 was then equilibrated in 0.1 M citrate buffer, pH 5 (buffer A), with a Centricon 30 microconcentrator and loaded onto an ion exchange Mono S HR 5/5 column in fast protein liquid chromatography (Pharmacia) equilibrated with the same buffer. The enzyme was eluted with a linear gradient (buffer B: 0.1 M citrate, pH 5, 0.3 M NaCl; gradient: $t = 0$, %B = 0; $t = 10$ min, %B = 0; $t = 30$ min, %B = 80; $t = 35$ min, %B = 80). The active fractions were pooled and desalted.

The phenol oxidase POXA2 was equilibrated in 0.02 M sodium phosphate buffer, pH 7 (buffer A), and loaded onto a cationic exchange Mono Q HR 5/5 column in a fast protein liquid chromatography system equilibrated with the same buffer. The enzyme was eluted with a linear gradient (buffer B, 0.02 M sodium phosphate buffer, pH 7, 0.5 M NaCl; gradient: $t = 0$, %B = 0; $t = 10$ min, %B = 0; $t = 60$ min, %B = 60; $t = 65$ min, %B = 80). The active fractions were pooled and desalted.

Enzyme Assays—Phenol oxidase activity was assayed at 25 °C using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)[1], guaiacol, 2,6-dimethoxyphenol (DMP), and syringaldazine as substrates as follows. (*a*) The assay mixture contained 2 mM ABTS and 0.1 M sodium citrate buffer, pH 3.0. Oxidation of ABTS was followed by absorbance increase at 420 nm (ϵ = 36,000 M^{-1} cm^{-1}). Enzyme activity was expressed in international units (IU). (*b*) The assay mixture contained 10 mM guaiacol, and the McIlvaine's citrate-phosphate buffer adjusted to pH 6.0. Oxidation was followed by the absorbance increase at 460 nm. One unit of activity is the amount of enzyme producing a 1.0 *A* increase/min. (*c*) The assay mixture contained 1 mM DMP and the McIlvaine's buffer adjusted to pH 5 for POXA1 and POXC isoenzyme and pH 6.5 for POXA2 isoenzyme. Oxidation of DMP was followed by an absorbance increase at 477 nm (ϵ = 14,800 M^{-1} cm^{-1}). Enzyme activity was expressed in IU. (*d*) The assay mixture contained 0.5 mM syringaldazine (dissolved in ethanol) and 50 mM phosphate buffer, pH 6. Oxidation of syringaldazine was followed by an absorbance increase at 525 nm (ϵ = 65,000 M^{-1} cm^{-1}). Enzyme activity was expressed in IU.

Deoxygenase activity was assayed at 30 °C using the protocatechuic acid as substrate as follow. The reaction mixture contained 0.6 mM protocatechuic acid and 50 mM Tris/HCl buffer, pH 8.0. After 10 min of incubation at 30 °C, the reaction was stopped by adding 0.5 ml of 30% perchloric acid. In the control, perchloric acid was added at zero time. The deoxygenation of protocatechuic acid to β-carboxy-*cis*,*cis*-muconic acid was followed by an absorbance decrease at 290 nm (11).

Tyrosinase activity was assayed at 25 °C using 3,4-dihydroxy-DL-phenylalanine as substrate. The assay mixture contained 0.2 mM 4-dihydroxy-DL-phenylalanine and 0.1 sodium phosphate, pH 6. The oxidation of substrate was followed by an absorbance increase at 475 nm (ϵ = 3,600 M^{-1} cm^{-1}) (12).

A peroxidase-coupled assay was performed using diaminobenzidine as the peroxidase substrate. The reaction mixture contained 0.1 M sodium citrate buffer, pH 5.0, 0.3 milliunits of horse-radish peroxidase, 0.36 mM diaminobenzidine, 2 mM ABTS in a total volume of 1 ml. The oxidation of diaminobenzidine was followed by an absorbance increase at 460 nm (13).

Phenol oxidase activity as a function of pH was measured using a McIlvaine's citrate-phosphate buffer adjusted to different pH levels in the range 2.5–8.0. The same buffer was used to determine the pH stability of the three isoenzymes. The effect of various inhibitors were tested by using ABTS as a substrate and preincubating the isoenzymes for 5 min at room temperature before the addition of substrate.

The activity of phenol oxidases toward different substrates was assayed in 50 mM sodium phosphate buffer, pH 6.0, following the absorbance decrease at the indicated wavelengths and at 0.1 mM substrate concentrations: ferulic acid (318 nm), sinapic acid (317 nm), caffeic acid (318 nm), vanillic acid (261 nm), syringic acid (272 nm), 1,4-dihydroxybenzene (289 nm), 1,2-dihydroxybenzene (276 nm), *o*-diaminobenzene (250 nm), and *p*-diaminobenzene (250 nm) (14).

The absorption spectra of native isoenzymes were determined between 200 and 800 nm at room temperature in 50 mM sodium phosphate buffer, pH 6.0, using a Beckman DU 7500 spectrophotometer (Beckman Instruments).

Protein Determination—Protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin as a standard.

Determination of Molecular Mass—The molecular masses of native phenol oxidases were determined with a SMART system (Pharmacia) by using a Superdex 75 PC 3.2/30 gel filtration column (Pharmacia). The column was eluted with 50 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl, and the eluate was monitored at 280 and 220 nm (flow rate 0.05 ml/min). The calibration of the column was performed with bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa) as standards.

Matrix-assisted laser desorption ionization (MALDI) mass spectra were recorded with a Kratos Kompact MALDI III linear instrument (Kratos Analiticals, Manchster, U.K.). Protein samples (20 pmol) and 1 μl of sinapinic acid, as the matrix, were loaded onto the sample plate, and the spectra were acquired using bovine serum albumin and matrix peaks for external calibration.

Endoglycosidase Treatment—Digestion of POXA1 And POXA2 laccases was conducted as described by Salas *et al.* (14): 10 μg of each protein suspended in 30 μl of water were combined with 2 μl of 10% SDS and with 25 μl of a solution containing 50 mM Tris/HCl, pH 7.5, 4% β-mercaptoethanol, and 200 mM EDTA. The resulting mixture was boiled for 5 min, then 1 unit (5 μl) of endoglycosidase F (Boehringer Mannheim) was added, and the reaction mixture was incubated for 24 h at 30 °C.

Lectin Assay—POXA1, POXA2, and control standard glycoproteins (supplied with the Boehringer glycan differentiation kit) (1 μg) were directly spotted onto an Immobilon membrane and detected immunologically after binding to lectins conjugated with digoxigenin following the manufacturer's instructions (Boehringer Mannheim). Lectins used were the following: *Galanthus nivalia* agglutinin, specific for terminal mannose; *Sambucus nigra* agglutinin specific for sialic acid α-2,6-galactose, *Maackia amurenais* agglutinin, specific for sialic acid α-2,3-galactose; peanut agglutinin, specific for galactose β-1,3-*N*-acetylglucosamine; and *Datura stramonium* agglutinin, specific for galactose β-1,4-*N*-acetylglucosamine. The protein linked to the lectin was detected by a colorimetric reaction. The immunological detection was performed according to the manufacturer's instructions.

Electrophoresis and Isoelectrofocusing—Polyacrylamide (9%) gel slab electrophoresis in 0.1% SDS was carried out as described by Laemmli (15). For molecular mass determinations, the gel was calibrated with phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactoalbumin (14.4 kDa). Proteins were visualized by silver staining.

Analytical isoelectric focusing in the pH range 2.5–7 was performed on 5.0% acrylamide gel slab with a LKB Multiphor electrophoresis system (Pharmacia) following the manufacturer's instructions. Proteins were stained using the silver staining method. Phenol oxidase activity was revealed when incubating the gel at 25 °C in 0.1 M citrate buffer, pH 3, containing 2 mM ABTS or alternatively, using 10 mM guaiacol as substrates.

Sequence Analysis—The N-terminal amino acid sequence of purified phenol oxidases was determined by step-wise Edman degradation with an Perkin-Elmer Applied Biosystem 477A pulsed liquid protein sequencer equipped with a 120A high performance liquid chromatography apparatus for the on-line phenylthiohydantoin-amino acid identification. Proteins separated by SDS-PAGE were electroblotted on a polyvinylidene difluoride membrane, and the excised bands were directly analyzed.

Proteolytic digestion was performed by incubating 0.10 mg of the denatured proteins in 0.4% NH_4HCO_3, 1 M urea, pH 8.5, with trypsin (Sigma) at 37 °C overnight. Peptide separation was carried out on a Vydac C_{18} column (2.1 × 250 mm) eluted with linear gradient (2–70%) of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.2 ml/min.

Atomic Absorption—Zinc, copper, and iron contents were determined in triplicate and on different enzyme preparations by atomic absorption spectrometry using a Perkin-Elmer apparatus model 5100 equipped with Zeeman graphite furnace and autosampler.

Polarography—100 μl of the protein sample (100 μg) were calcined after the addition of 2 ml of an ultrapure mixture of HNO_3/H_2SO_4 1:1

[1] The abbreviations used are: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DMP, 2,6-dimethoxyphenol; PAGE, polyacrylamide gel electrophoresis.

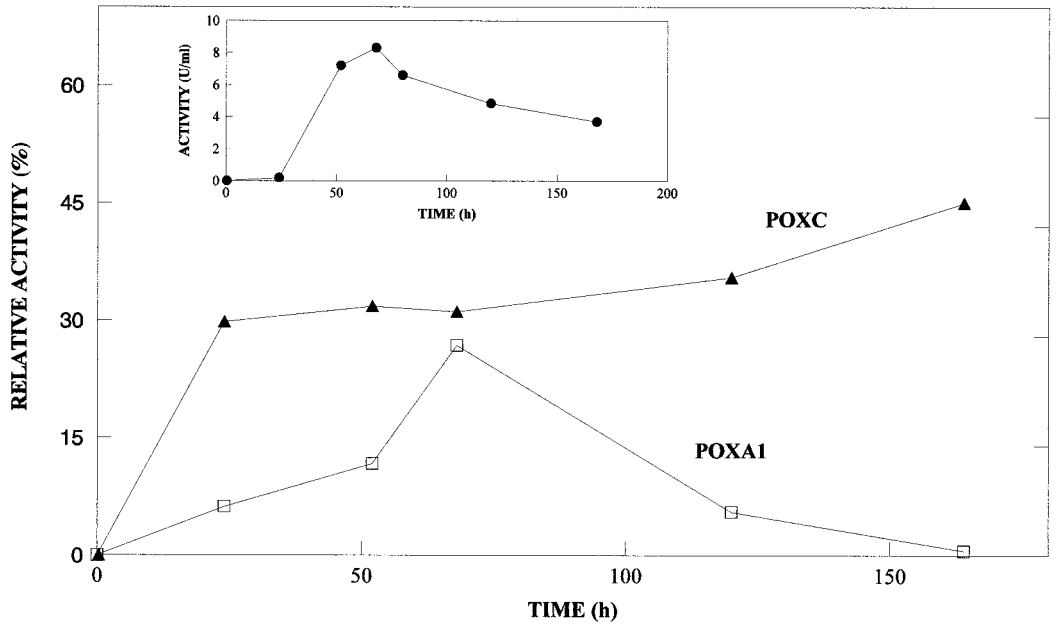

FIG. 1. **Laccase production in *P. ostreatus* cultures.** Time course of laccase POXA1 and POXC isoenzymes *versus* relative activity in cultures of *P. ostreatus* is shown. The *inset* reports total laccase activity production.

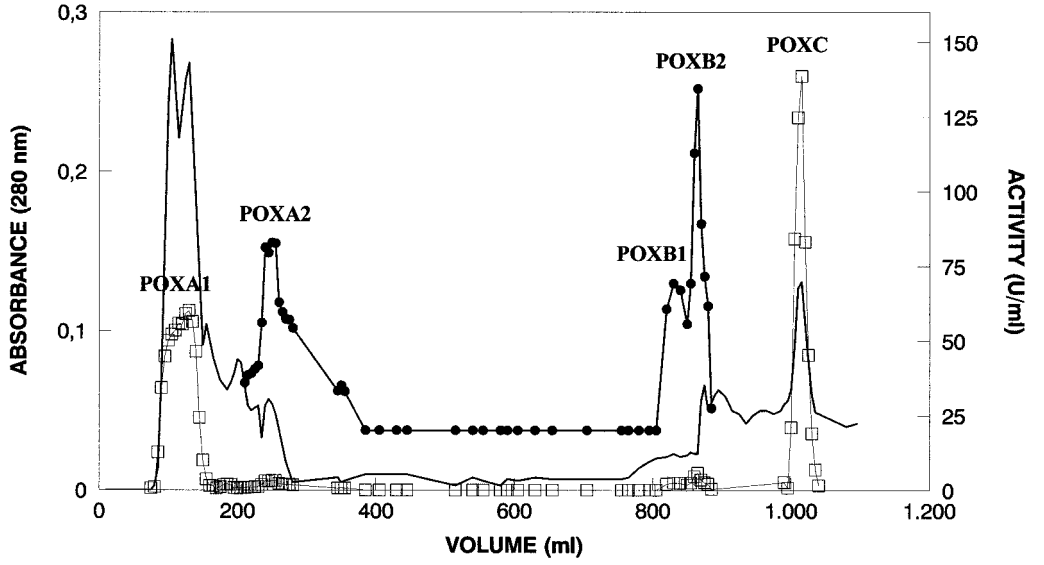

FIG. 2. **DEAE-Sepharose chromatography of *P. ostreatus*-secreted proteins.** DEAE-Sepharose fast flow elution profile of proteins and laccase isoenzymes secreted by *P. ostreatus* is shown with five different active protein fractions (POXA1, POXA2, POXB1, POXB2, and POXC): —, absorbance at 280 nm; □, laccase activity (units/ml); ●, laccase activity (units/ml ×10).

(v/v). The calx was resuspended with 1 ml of 37% HCl and then diluted to 25 ml with ultrapure H_2O (Merck, Darmstadt, Germany). 20 ml were utilized for polarographic analysis using a Metrohm apparatus model 645 equipped with an mercury electrode. Measures have been performed by the anodic stripping method with a sweep time of 60 s at −1.2 volt potential.

RESULTS

Production of the Isoenzymes—A time course of phenol oxidase activity production in *P. ostreatus* culture broth is shown in Fig. 1 *inset*. The activity reached a maximum at about 70 h after inoculation and decreased slowly thereafter. Analysis of samples withdrawn from the media at different growth times indicated that the activity was associated mainly with the production of two isoenzymes (named POXA1 and POXC, see below). Moreover, the highest levels of POXA1 production were found to correspond to the maximum of the total phenol oxidase activity in the culture broth. In contrast no significant differences in the relative amount of the POXC isoenzyme were detected at different growth times (Fig. 1).

Purification of the Isoenzymes—*P. ostreatus* culture broth, after 70 h of growth, was fractionated by ammonium sulfate precipitation followed by anionic exchange chromatography. As shown in Fig. 2, five different phenol oxidase fractions named POXA1, POXA2, POXB1, POXB2, and POXC were separated. A major peak (POXA1) of phenol oxidase activity and a fraction (POXA2) containing a minor phenol oxidase isoenzyme were recovered with the equilibrating buffer, whereas the other three isoenzymes, POXB1, POXB2, and POXC, were eluted with a saline gradient at approximately 0.17, 0.18, and 0.32 M NaCl, respectively.

Fractions corresponding to the POXA1 isoenzyme were collected and further purified by cationic exchange chromatography (Mono S) in a 0.1 M citrate buffer at pH 5.0. The enzyme was eluted as a single sharp peak with a saline gradient at

TABLE I
Purification of laccases from Pleurotus ostreatus cultures

Purification step	Total activity	Total protein	Specific activity	Recovery
	katal	*mg*	*katal/mg*	*%*
Broth	1.6×10^{-4}	105.8	0.15×10^{-5}	100
$NH_4(SO_4)_2$ precipitate	1.1×10^{-4}	22	0.51×10^{-5}	70
DEAE:				
POXA1	4.3×10^{-5}	3.05	1.4×10^{-5}	26.7
POXA2	1.3×10^{-6}	0.11	1.2×10^{-5}	0.8
POXB1 + B2	0.2×10^{-5}	0.21	1.0×10^{-5}	1.3
POXC	3.4×10^{-5}	0.37	9.1×10^{-5}	21
Mono S: POXA1	3.7×10^{-5}	0.29	1.3×10^{-4}	23.2
Mono Q: POXA2	0.9×10^{-6}	0.07	1.2×10^{-5}	0.5

about 0.1 M NaCl. The POXA2 isoenzyme was chromatographed on a Mono Q column equilibrated in 20 mM sodium phosphate buffer, pH 7.0, and eluted in the fractions corresponding to 0.075 M NaCl. The two purified proteins, POXA1 and POXA2, appeared to be homogeneous when analyzed by SDS-PAGE, isoelectric focusing, and gel filtration chromatography. The two more acidic phenol oxidases, POXB1 and POXB2, were not further purified, whereas the POXC isoenzyme was found to correspond to the previously fully characterized enzyme (10, 16).

A summary of the purification procedure is shown in Table I; 85-fold purification was achieved for POXA1 isoenzyme with a final yield of 23%, whereas a lower yield for POXA2 isoenzyme was obtained, probably due to the loss of activity during the purification procedure. The specific activities of POXA1 and POXA2 are 1.3×10^{-4} katal/mg and $1.2\ 10^{-5}$ katal/mg, respectively.

Physical and Chemical Properties—The molecular masses determined by SDS-PAGE analysis were 61 kDa and 67 kDa for POXA1 and POXA2, respectively. A more accurate determination of the POXA1 molecular mass was performed by MALDI mass spectrometry; a broad peak centered at 61,373 Da was obtained. Gel filtration chromatography of the native enzymes allowed the determination of molecular mass of 54 and 59 kDa for POXA1 and POXA2, respectively. These results confirmed the monomeric structure of these proteins. The isoelectric points of POXA1 and POXA2 are 6.7 and 4.0, respectively.

POXA1 and POXA2 samples were treated with endoglycosidase F and analyzed by SDS-PAGE. Proteins migrated in the gel to positions corresponding to molecular masses of 59 and 61 kDa thus indicating a 3 and 9% carbohydrate content, respectively.

POXA1 and POXA2 samples were analyzed for the presence of specific oligosaccharides by lectin binding assays. Both proteins were specifically recognized by *G. nivalia* agglutinin lectin, which binds to terminal mannose residues, thus suggesting the presence of high mannose-type glycans. Experiments with *D. stramonium*, *S. nigra*, *M. amurenais*, and peanut agglutinin lectins gave negative results, thus indicating the absence of both sialylated complex-type glycans and *O*-linked oligosaccharide chains containing the structure galactose β-1,3-*N*-acetylgalactosamine at their nonreducing end.

In Fig. 3, the N terminus and three tryptic peptide sequences of POXA1 isoenzyme are shown and aligned with the sequences of other known laccases (9, 10, 17–21). Directly sequencing the N terminus of POXA2 from samples either in solution or after blotting on polyvinylidene difluoride membrane were unsuccessful, thus suggesting a blocked N terminus for this isoenzyme.

When the UV-visible spectra of the purified enzymes were analyzed and compared with that of the already characterized POXC isoenzyme, a different behavior was observed. In fact,

N-TERM. POXA1		**SIGPNGTLNIANKVIQPDG**	
PO	POXC	A**I**GPAGNMYI**V**NEDVS**P**DG	1-19
PO	POX1	A**I**GPTGDMYI**V**NEDVS**P**DG	1-19
CH	LACCASE	A**I**GPTADL**T**IS**N**AEVS**P**DG	1-19
PM1	LACCASE	**SIGP**VADL**T**IS**N**GAVS**P**DG	1-19
TV	LACCASE	A**I**GPVTDL**T**IS**N**ADVS**P**DG	1-19
PR	LACCASE	**SIGP**VTDFHI**V**NAAVS**P**DG	1-19
AB	LACCASE	-DTKTFNFDLV**N**TRLA**P**DG	1-18
1st	PEPTIDE POXA1	**NPNSG-DPGFENQMNSAIL**	
PO	POXC	**NPN**L**G**-ST**GF**VGGI**NSAIL**	272-289
PO	POX1	D**PN**L**G**-ST**GF**DGGI**NSAIL**	270-287
CH	LACCASE	**NPN**F**G**-NV**GF**AGGI**NSAIL**	262-279
PM1	LACCASE	L**PNSG**-TRN**F**DGGV**NSAIL**	261-278
TV	LACCASE	L**P**SA**G**-TTS**F**DGGI**NSAIL**	262-279
PR	LACCASE	**NP**GIGITT**GF**AGGI**NSAIL**	263-281
AB	LACCASE	GG**N**PDRN**P**NLNISLTL**AIL**	270-288
2nd	PEPTIDE POXA1	**APAGSIYDI**	
PO	POXC	L**PSGSIY**EL	377-385
PO	POX1	L**PSGSIY**SL	375-383
CH	LACCASE	L**PSGSVY**SL	366-374
PM1	LACCASE	L**PSGSVY**SL	365-373
TV	LACCASE	L**PSGS**LFAL	366-374
PR	LACCASE	L**PSGSVY**AL	370-374
AB	LACCASE	L**P**SEQ**I**FFV	374-382
3rd	PEPTIDE POXA1	**DVVSIGD-DPT**	
PO	POXC	**DVV**NTG**T**-GAN	433-442
PO	POX1	**DVV**NTG**T**-**D**AN	431-440
CH	LACCASE	**DVVSTG**TPAAG	424-434
PM1	LACCASE	**DVVSTG**SP--G	444-452
TV	LACCASE	**DVV**NTG**T**A--G	424-432
PR	LACCASE	**DVVSIG**N--TG	426-434
AB	LACCASE	**DVY**----PING	427-433

FIG. 3. **N terminus (*N-TERM.*) and three tryptic peptides sequences from *P. ostreatus* POXA1.** POXA1 sequences have been aligned with those of *P. ostreatus* POXC (*PO POXC*), *P. ostreatus* POX1 (*PO POX1*), *Coriolus hirsutus* laccase (*CH LACCASE*), PM1 laccase (*PM1 LACCASE*), *Trametes versicolor* laccase (*TV LACCASE*), *Phlebia radiata* laccase (*PR LACCASE*), and *Agaricus bisporus* laccase (*AB LACCASE*).

the UV-visible spectrum of POXC showed a peak at 605 nm and a shoulder at approximately 330 nm (Fig. 4); these signals are typical of the "blue" oxidase enzymes corresponding to type 1 or blue copper atom and to type 3 binuclear copper atoms. The ratio of A_{280} to A_{605} is 20, which is similar to those of laccases from other sources (14, 22, 23). In contrast, in the POXA1 absorption spectrum, the 605-nm signal was absent, as confirmed also by the colorless concentrated solutions of the protein, whereas a broad peak at about 400 nm was detected (Fig. 4). The spectrum of the POXA2 isoenzyme showed a shoulder at 400 nm and a less intense absorption at 605 nm (Fig. 4) with a A_{280}/A_{605} of 50.

The copper content was determined by atomic absorption. The POXA2 and POXC isoenzymes showed values of 3.3 ± 0.1 and 3.7 ± 0.5 mol/mol copper/protein ratio respectively, whereas only 0.7 ± 0.2 mol of copper/mol of protein was determined for POXA1. Moreover, when different preparations of POXA1 isoenzyme were examined for metal content (cadmium, mercury, nickel, iron, zinc) by atomic absorption, the presence of two other metals, iron and zinc, was revealed; a quantitative analysis resulted in a 0.7 ± 0.2 mol/mol iron/protein ratio and 2.0 ± 0.2 mol/mol zinc/protein ratio. These values suggest a

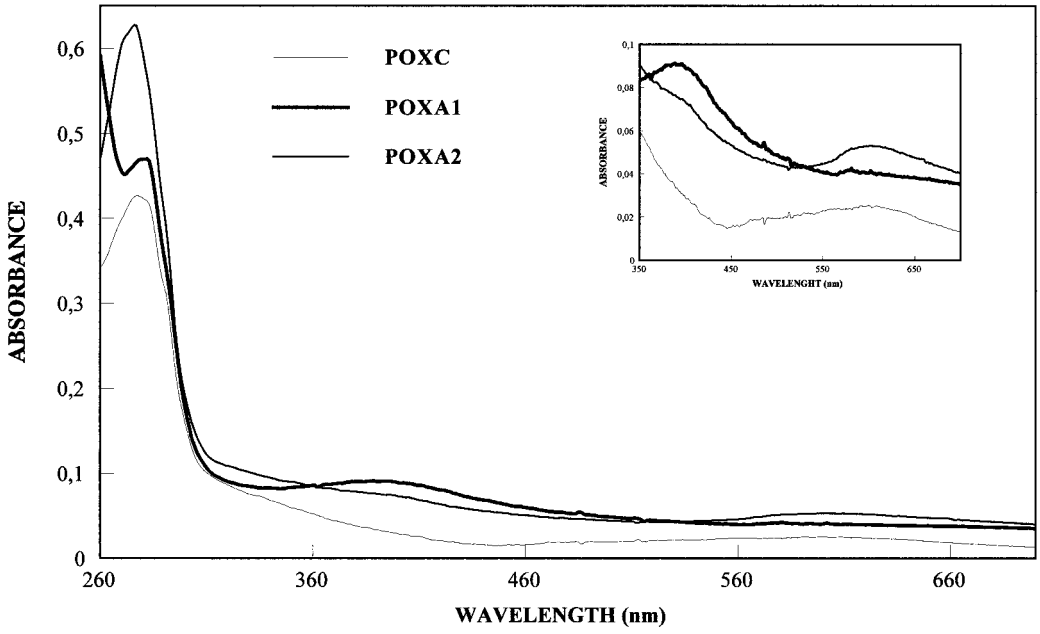

FIG. 4. **UV/visible absorption spectra of POXA1, POXA2, and POXC laccase isoenzymes from *P. ostreatus*.**

copper/iron/zinc stoichiometry of 1:1:2 for POXA1 isoenzyme. Polarographic analysis of POXA1 and POXC confirmed the presence of copper and zinc (iron cannot be detected by this technique) for the POXA1 isoenzyme and only the presence of copper for the POXC isoenzyme. Western blot analysis using anti POXC antibodies revealed that the three isoenzymes are not immunologically related.

Catalytic Properties—The activities of POXA1 and POXA2 at different pH values was examined over different incubation times and compared with that of POXC. POXA1 was the most stable enzyme at all pH values (3.0, 4.0, 5.0, 6.0, 7.0) investigated; the stability of this enzyme is almost unaffected by acidic pH ($t_{1/2}$ = 24 h at pH 3.0) in comparison with the other two isoenzymes, which proved to be very sensitive to pH decrease ($t_{1/2}$ = 2 h for POXA2 and 30 min for POXC at pH 3.0) (Fig. 5, *upper* and *lower panels*).

Thermal stabilities of all isoenzymes were investigated at pH 7.0 where all of them showed their maximum stability. Plots of the residual activity after incubation at 60 °C *versus* time indicated a $t_{1/2}$ of 200 min for POXA1, 30 min for POXC, and 10 min for POXA2 (Fig. 6).

When the activity of these enzymes was studied as a function of the temperature, POXA1 showed maximal activity in the range 45–65 °C, whereas POXC showed maximal activity in a narrower range (50–60 °C) and POXA2, at a lower temperature (25–35 °C).

The catalytic parameters of POXA1 and POXA2 with respect to four substrates (ABTS, guaiacol, DMP, and syringaldazine) were determined and compared with those of POXC (Table II). The K_{cat} values of POXA2 have not been included because of the high instability of this enzyme, which did not allow a correct determination of the concentration of the active form of the enzyme.

No activity of POXA1 against guaiacol was observed at any of the pH levels tested. As shown in Table II, POXA2 oxidized 2,6-dimethoxyphenol optimally at pH 6.5, in this respect behaving quite differently from the other two isoenzymes.

To investigate the oxidative reaction catalyzed by the POXA1 isoenzyme, a number of different substrates were tested (see "Enzyme Assays" under "Experimental Procedures"). The enzyme oxidized *o*- and *p*-dihydroxybenzene and *o*- and *p*-diaminobenzene, which are also substrates of all laccases. In particular, POXA1 more efficiently oxidizes *o*-dimethoxy-substituted phenols (DMP, syringic, and sinapic acids) compared with *o*-monomethoxy-substituted phenols (guaiacol, ferulic, and vanillic acids).

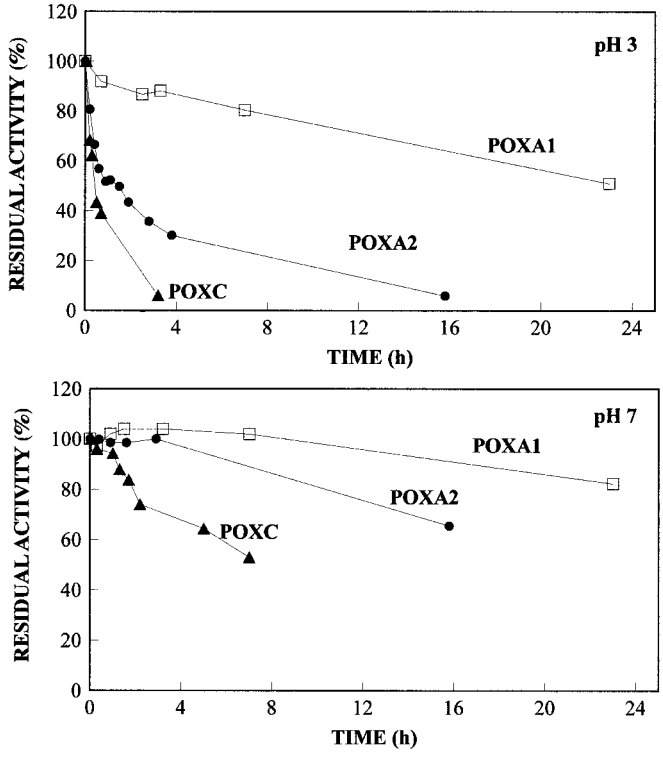

FIG. 5. **Stability of POXA1, POXA2, and POXC laccase isoenzymes from *P. ostreatus* incubated at pH 3 and 7.**

Moreover, POXA1 did not exhibit any tyrosinase and protocatechuate deoxygenase activities. It has also been proved that both POXA1 and POXA2 reduce O_2 during the reaction that they catalyze and that no H_2O_2 formation could be observed.

The effects of several known laccase inhibitors on the activity of all the studied isoenzymes were examined using ABTS as substrate at pH 3.0. All enzymes were totally inhibited by 0.02 mM sodium azide and by thioglycolic acid at 0.05 mM. In the

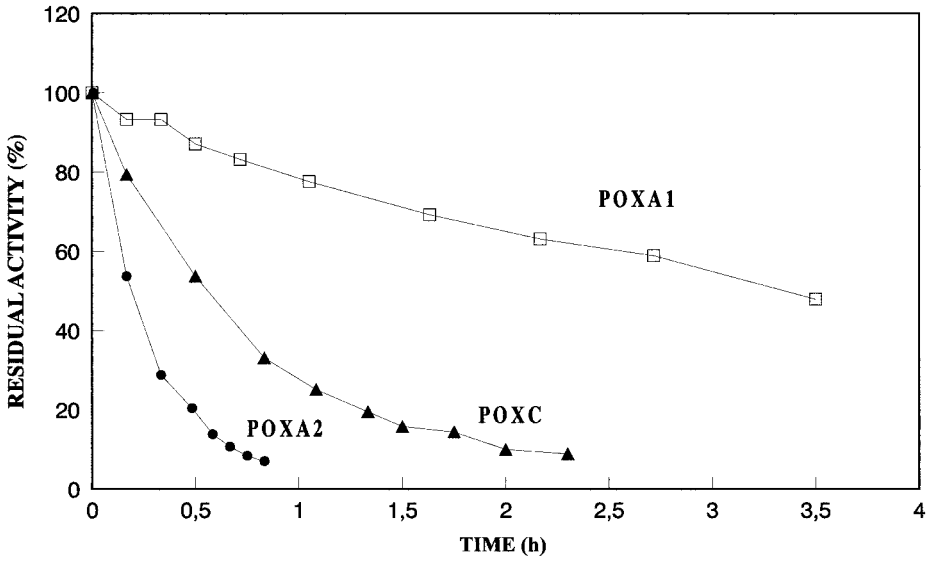

FIG. 6. **Stability of POXA1, POXA2, and POXC laccase isoenzymes from *P. ostreatus* incubated at 60 °C.**

TABLE II
Kinetic constants of Pleurotus ostreatus laccase isoenzymes

NA, Not active; ND, Not determined.

Substrate	POXA1	POXA2	POXC	K_m			k_{cat}			k_{cat}/K_m		
				POXA1	POXA2	POXC	POXA1	POXA2	POXC	POXA1	POXA2	POXC
	Optimum pH			*mM*			min^{-1}			$mM^{-1}\ min^{-1}$		
ABTS	3.0	3.0	3.0	$(9.0 \pm 0.8)10^{-2}$	$(1.2 \pm 0.2)10^{-1}$	$(2.8 \pm 0.4)10^{-1}$	$(3.5 \pm 0.3)10^{5}$	ND	$(1.6 \pm 0.2)10^{4}$	3.9×10^{6}	ND	5.7×10^{4}
Guaiacol	NA	6.0	6.0	NA	3.1 ± 0.6	1.2 ± 0.2	NA	ND	$(1.5 \pm 0.1)10^{2}$	NA	ND	1.3×10^{2}
DMP	3–5	6.5	3–5	2.1 ± 0.3	$(7.4 \pm 0.9)10^{-1}$	$(2.3 \pm 0.2)10^{-1}$	$(2.5 \pm 0.4)10^{2}$	ND	$(1.0 \pm 0.1)10^{2}$	1.2×10^{2}	ND	4.3×10^{2}
Syringaldazine	6.0	6.0	6.0	$(1.3 \pm 0.2)10^{-1}$	$(1.4 \pm 0.5)10^{-1}$	$(2.0 \pm 0.4)10^{-2}$	$(2.8 \pm 0.6)10^{4}$	ND	$(2.3 \pm 0.1)10^{4}$	2.1×10^{5}	ND	1.1×10^{6}

presence of EDTA, no inhibition was observed up to 50 mM concentration for all the three isoenzymes. Furthermore, 5 mM hydroxylamine caused 50% inhibition of POXA1 and POXC, whereas it was necessary to use a concentration of 50 mM to obtain the same inhibition for POXA2. Differences among the enzymes were also revealed when kojic acid was used; in fact, 50% inhibition was obtained at 200, 60, and 40 mM for POXA1, POXA2, and POXC respectively.

DISCUSSION

This work complements and extends recent reports (9, 10, 16) that demonstrated the production of multiple laccase isoforms in the Basidiomycete white rot fungus *P. ostreatus*.

In earlier studies we identified in *P. ostreatus* two genes and the corresponding cDNA coding for two laccase isoenzymes; the product of one of these genes, (*pox2*) POXC, is the isoenzyme most abundantly produced in all growth conditions examined (10, 16), whereas the protein coded by the other gene (*pox1*) has not been identified so far (9). To further investigate the organization of the laccase isoenzymes produced by *P. ostreatus*, we isolated and characterized two other phenol oxidases (POXA1 and POXA2) from this fungus.

The time course of POXA1 production showed significant differences with respect to that of POXC laccase. The maximum POXA1 activity was reached later during the fungal growth with respect to that of POXC; thereafter, a fast decrease of the POXA1 activity was observed, probably due to the presence in the old culture medium of a low molecular weight inhibitor (data not shown). This behavior could suggest a different physiological role for the two isoenzymes. The other isoenzyme, POXA2, was produced in lower amounts at all growth times analyzed.

POXA2 exhibits characteristics similar to those of known laccases from other fungi; in fact, molecular mass, pI, metal content, and kinetic constants lie well within the range determined for other laccases (24). However, the stability of POXA2 as a function of temperature was particularly low. This characteristic leads to a considerable loss of the activity during the purification procedure, providing a very low yield of the homogeneous protein. Otherwise, POXA1 isoenzyme shows a remarkable high stability with respect to both pH and temperature and if compared with that of POXC and of other known laccases.

Another peculiar characteristic of POXA1 is its neutral pI (6.7); to the best of our knowledge, all the laccases so far purified and characterized showed a pI in a pH range from 3 to 5. However, the unusual pI value is not the most striking characteristic of this protein; in fact, concentrated solutions of POXA1 lack the typical blue color that characterizes all the blue oxidases. This fact was confirmed by the analysis of the UV/visible spectrum of the protein, thus indicating the absence of the type I copper moiety. When the metal content of the protein was analyzed both by atomic absorption and by polarography, a more noticeable feature was observed. The protein was revealed to contain only 1 copper atom/molecule instead of the usual 4, and furthermore, 2 zinc atoms and 1 iron atom were present/each protein molecule.

Moreover, the fact that the enzyme belongs to the laccase family is confirmed by (i) the high degree of identity of the determined primary structure with the corresponding sequences of known laccases, and (ii) the use of O_2 as oxidative substrate and the lack of formation of H_2O_2 as a product in the catalyzed reaction, (iii) the almost standard pattern of substrate specificity displayed by this enzyme if compared with that of other known laccases.

The reported data give clear evidence that the POXA1 laccase from *P. ostreatus* belongs to the laccase family but displays structural characteristics that render it unique and allow its classification as a white laccase. Further investigation is

needed to clarify the mechanism of the oxidative reaction catalyzed by this enzyme and the role of the metal ions present in this protein.

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A Novel White Laccase from *Pleurotus ostreatus*

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