Protein and gene structure of a blue laccase from *Pleurotus ostreatus*[1]

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A new laccase isoenzyme (POXA1b, where POX is phenol oxidase), produced by *Pleurotus ostreatus* in cultures supplemented with copper sulphate, has been purified and fully characterized. The main characteristics of this protein (molecular mass in native and denaturing conditions, pI and catalytic properties) are almost identical to the previously studied laccase POXA1w. However, POXA1b contains four copper atoms per molecule instead of one copper, two zinc and one iron atom per molecule of POXA1w. Furthermore, POXA1b shows an unusually high stability at alkaline pH. The gene and cDNA coding for POXA1b have been cloned and sequenced. The gene coding sequence contains 1599 bp, interrupted by 15 introns. Comparison of the structure of the *poxa1b* gene with the two previously studied *P. ostreatus* laccase genes (*pox1* and *poxc*) suggests that these genes belong to two different subfamilies. The amino acid sequence of POXA1b deduced from the cDNA sequence has been almost completely verified by means of matrix-assisted laser desorption ionization MS. It has been demonstrated that three out of six putative glycosylation sites are post-translationally modified and the structure of the bound glycosidic moieties has been determined, whereas two other putative glycosylation sites are unmodified.

Key words: copper, fungi, induction, lignin, phenol oxidase.

INTRODUCTION

Lignin is the second most abundant renewable organic compound in the biosphere after cellulose, and its biodegradation is a rate-limiting step in the carbon cycle [1]. White rot fungi secrete ligninolytic enzymes, which are able to generate radical species that allow the complete biodegradation of the lignin polymer [2]. Because of the complex structure of lignin, the biodegrading system is highly non-specific, and so ligninolytic enzymes can be employed in the degradation of structurally different environmental pollutants [3,4]. Among ligninolytic enzymes, laccases (EC 1.10.3.2) are phenol oxidases that catalyse one-electron oxidation of many aromatic substrates (polyphenols, methoxy-substituted monophenols, aromatic amines, etc.) with the concomitant reduction of O_2 to H_2O [5]. Moreover, the substrate range of these enzymes can be extended to include non-phenolic lignin subunits in the presence of readily oxidizable primary substrates, which can act as electron-transfer mediators [6].

Laccases belong to the class of blue oxidases and contain four copper atoms/molecule, distributed in three different types. The type-1 site is responsible for the intense blue colour of the enzyme due to a maximum absorbance at 605 nm; the type-2 site does not exhibit signals in the visible absorbance spectrum; and the type-3 site incorporates two copper centres and is responsible for a band near 330 nm [7]. These ligninolytic enzymes are secreted in multiple isoforms, depending on the fungal species and environmental growth conditions [8].

Laccase isoenzymes produced by *Pleurotus ostreatus*, a white rot basidiomycete fungus, have been studied extensively. One of these, POXC (where POX is phenol oxidase), is the most abundantly produced under all the growth conditions examined [9]. Two other isoenzymes, secreted by the mycelium, have also been purified and characterized (POXA1w and POXA2) [10]. POXA1w shows peculiar differences with regard to metal content. In fact, this enzyme contains two zinc, one iron and only one copper atom/molecule. Moreover, POXA1w shows greater stability with respect to temperature and pH than the other two isoenzymes [10].

Studies of the genes coding for laccase isoenzymes in *P. ostreatus* have so far led to the identification of two different genes and the corresponding cDNAs, *poxc* (previously named *pox2*) [9] and *pox1* (the latter coding for a laccase isoenzyme not yet identified) [11].

Analysing the effects of different substances as inducers of *P. ostreatus* laccase production, we found that the addition of $CuSO_4$ to the culture broth caused a strong increase in the total laccase activity and the production of a new isoenzyme. In this article, we report the purification of a copper-induced laccase, named POXA1b. This protein has been fully characterized, and the sequences of its gene and the corresponding cDNA have been determined. The amino acid sequence of POXA1b, deduced from the sequence of cDNA, has been verified almost completely by means of matrix-assisted laser desorption ionization (MALDI) MS. It has been demonstrated that three out of six putative glycosylation sites are post-translationally modified and the structure of the bound glycosidic moieties has been determined.

EXPERIMENTAL

Organism and culture conditions

White rot fungus, *P. ostreatus* (Jacq.:Fr.) Kummer (type, Florida), was maintained through periodic transfer at 4 °C on potato-dextrose agar plates (Difco, Detroit, MI, U.S.A.) in the

Abbreviations used: MALDI, matrix-assisted laser desorption ionization; ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid); DMP, 2,6-dimethoxyphenol; IEF, isoelectric focusing; PNGase F, peptide N-glycosidase F; POX, phenol oxidase.

[1] This article is dedicated to the memory of our friend and colleague, Giacomino Randazzo.

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The nucleotide sequences for *Pleurotus ostreatus poxa1b* gene and cDNA have been deposited in the GenBank database under accession numbers AJ005017 and AJ005018 respectively.

presence of 0.5 % yeast extract (Difco). Incubations were carried out as described previously [10]: 50 ml of a 5-day-old culture was transferred into 1-litre flasks containing 450 ml of broth supplemented with 150 μM $CuSO_4$. The enzyme purification was performed from a 5-day culture.

Enzyme purification

Secreted proteins were precipitated from the filtered medium by the addition of $(NH_4)_2SO_4$ up to 80 % saturation and loaded on to a DEAE-Sepharose Fast Flow column (Pharmacia Biotech) as described previously [10]. Fractions containing laccase activity recovered with the equilibrating buffer were pooled, concentrated on an Amicon PM-10 membrane and equilibrated in 0.1 M sodium citrate, pH 5.0. This pool was loaded on to an S-Sepharose Fast Flow column (Pharmacia Biotech) equilibrated with the citrate buffer. The column was washed at a flow rate of 2 ml/min with 40 ml of buffer and a 0–0.3 M NaCl linear gradient (50 ml) was applied. Two laccase active peaks were pooled separately and concentrated on an Amicon PM-10 membrane.

Enzyme assays

Phenol oxidase activity was assayed at 25 °C using 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), guaiacol, 2,6-dimethoxyphenol (DMP) and syringaldazine as substrates, as described previously [10]. The activity as a function of pH was measured using a McIlvaine's citrate/phosphate buffer adjusted to different pH values in the pH range 2.5–7.5. The same buffer was used to determine the stability at pH 3.0 and 5.0, whereas 50 mM Tris/HCl buffer was used at pH 8.0 and 9.0. The activity was measured using ABTS as substrate unless otherwise stated.

The absorption spectra 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 BioRad Protein Assay (BioRad), with BSA as standard.

Determination of molecular mass

The molecular mass of native phenol oxidase was determined with a SMART system (Pharmacia Biotech) by using a Superdex 75 PC 3.2/30 gel-filtration column. The column was eluted with 50 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl. The calibration was performed with BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa) as standards.

Electrophoresis and isoelectric focusing (IEF)

Polyacrylamide (12.5 %) gel slab electrophoresis in 0.1 % SDS was carried out as described by Laemmli [12]. Analytical IEF in the pH range 2.5–7.0 was performed on 5.0 % acrylamide gel slab with a Multiphor electrophoresis system (Pharmacia Biotech), used according to the manufacturer's instructions.

Lectin assay

Protein samples and control standard glycoproteins (1 μg; supplied with the Boehringer glycan-differentiation kit) were spotted directly on to an Immobilon membrane and detected immunologically after binding to lectins conjugated with digoxigenin, as per the manufacturer's instructions (Boehringer Mannheim). The protein linked to the lectin was detected by a colorimetric reaction. The immunological detection was performed according to the manufacturer's instructions. This experiment was performed in duplicate on two different enzymic preparations.

SH titration

The experiment was carried out with Ellman's reagent. Mixtures containing 0.33 μM enzyme, 0.4 mM Ellman's reagent, 5,5′-dithiobis-(2-nitrobenzoic acid), 6 M urea, 0.1 mM EDTA and 0.1 M Tris/HCl buffer (pH 8.0) were incubated for 30 min at 25 °C before the absorption at 412 nm was measured (ϵ_{412} = 11400 $M^{-1} \cdot cm^{-1}$).

Reduction and alkylation of cysteine residues

Laccase samples were reduced in 0.25 M Tris/HCl (pH 8.5)/1.25 mM EDTA (containing 6 M guanidinium chloride) by incubation with a 10:1 molar excess of dithiothreitol over the protein SH groups at 37 °C for 2 h under a nitrogen atmosphere. The free cysteine residues were alkylated by using a 10:1 molar excess of iodoacetamide over the total SH groups at room temperature for 1 h in the dark, under a nitrogen atmosphere. Protein samples were freed from salt and reagent excess by passing the reaction mixture through a PD10 pre-packed column (Pharmacia Biotech), equilibrated and eluted in 0.4 % ammonium bicarbonate, pH 8.5.

Enzymic hydrolysis and peptide purification

Trypsin, endoprotease Lys-C and endoprotease Glu-C digestions were performed on a carboxamidomethylated laccase sample (200 μg) in 0.4 % ammonium bicarbonate (pH 8.5) at 37 °C overnight using an enzyme/substrate ratio of 1:50. The tryptic peptide mixture (100 μg) was deglycosylated by 0.15 units of peptide N-glycosidase F (PNGase F) incubation overnight in 0.4 % ammonium bicarbonate (pH 8.5) at 37 °C. Aliquots of the digests were analysed directly by MALDI MS or separated on a narrow-bore Vydac C_{18} column (The Separation Group, Hesperia, CA, U.S.A.) using a linear gradient from 5 to 50 % of acetonitrile containing 0.1 % trifluoroacetic acid over a period of 60 min at a flow rate of 0.2 ml/min. Peptides were collected manually.

Protein-sequence analysis

Automated N-terminal degradation of the electroblotted protein or purified peptides was performed using a Perkin-Elmer Applied Biosystems 477A pulsed-liquid protein sequencer equipped with a model 120A phenylthiohydantoin analyser for the online identification and quantification of phenylthiohydantoin amino acids. Automated C-terminal degradation of the protein (three different samples) loaded on to Zitex membranes [13] was performed with a Hewlett Packard G1006A protein-sequencing system, according to the manufacturer's instructions.

MS analysis

MALDI mass spectra were recorded using a Voyager DE MALDI–time-of-flight mass spectrometer (Perkin-Elmer Perseptive Biosystem). A mixture of analyte solution, α-cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid as matrices, bovine insulin and horse heart myoglobin was applied to the sample

plate and dried *in vacuo*. Mass calibration was obtained using horse myoglobin (16952.50 m/z), bovine insulin (5734.59 m/z) and α-cyano-4-hydroxycinnamic acid (379.06 m/z) as internal standards. Raw data were analysed by using computer software provided by the manufacturer and are reported as average masses.

Atomic absorption

Zinc, copper and iron contents were determined by atomic-absorption spectrometry using a Perkin-Elmer apparatus model 5100, equipped with Zeeman graphite furnace and autosampler.

mRNA isolation, cDNA synthesis and primer extension

Total RNA was extracted from lyophilized mycelia as described by Lucas et al. [14]. Poly(A)$^+$-containing RNAs were purified by oligo(dT)-cellulose chromatography. mRNA reverse transcription was performed under the experimental conditions described by Frohman et al. [15] using the oligo(dT)–*Not*I-site fragment as primer. Amplification experiments of specific cDNAs were performed at 45 and 50 °C annealing temperature, using each of the following oligonucleotide couples: 5′-GCYAACAARGTBAT-CCARCC-3′ and 5′-ATRTCGTADATRCTRCCRGC-3′; and 5′-GACATTAAGCTGGGAGA-3′ and 5′-AATTCGCGGCC-GCTTTTTTTTTTTTTTT-3′ respectively. The two amplified fragments were cloned in the pUC18 plasmid and sequenced.

Two primer-extension experiments were performed, according to Sambrook et al. [16], on 150 μg of RNA using the following oligonucleotides: oligo 1, 5′-GGCCTCAACGCGTACGAGT-GCTAATGCGAGTG-3′, and oligo 2, 5′-GACTTTGTTCGC-GATGTTCAGCGTTCCGCG-3′, as primers.

Isolation and sequencing of the gene

Screening of the *P. ostreatus* genomic library [11] was performed using the two cDNA fragments (see previous section) as probes, labelled by random-priming method. Colony-hybridization experiments were carried out in 5 × SSC at 65 °C [16] (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate).

DNA preparation, subcloning and restriction analysis were performed by standard methods according to Sambrook et al. [16]. Sequencing by the dideoxy chain-termination method was performed using Sequenase version 2.0 (Amersham) and universal and specific oligonucleotide primers.

RESULTS

Induction and purification of the laccase isoenzyme POXA1b

The addition of $CuSO_4$ to *P. ostreatus* culture broth gives rise to an increase of total phenol oxidase activity. This increase is proportional to the amount of copper added until it reaches the maximal laccase activity (about 30 units/ml) at 150 μM $CuSO_4$. On the other hand, the laccase activity detected in the basal medium, not supplemented with $CuSO_4$, is not strictly reproducible (0.5–4 units/ml), probably because of the variability of trace copper content (0.05–0.2 μM). The presence of copper in the culture medium does not affect the fungal growth since the biomass dry weight, examined at different growth times, was the same either in the presence or in the absence of copper.

P. ostreatus laccase isoenzymes can be separated by DEAE-Sepharose chromatography at pH 6.0 [10]. Comparison of the elution profiles of the proteins secreted in the presence or in the absence of Cu^{2+} showed an increase of all active peaks when Cu^{2+} was added to the culture broth. Our attention has been focused on neutral laccases secreted in the presence of copper.

The protein fractions recovered from DEAE-Sepharose with the equilibrating buffer were further purified on S-Sepharose chromatography at pH 5.0, and two active peaks were eluted by the saline gradient. The first one corresponded to the same protein, POXA1w, produced under normal conditions, and the N-terminal sequence and the metal content confirmed its identity. The second active peak, eluted at a slightly higher salt concentration (0.24 M NaCl), corresponded to a new protein, POXA1b (Figure 1). Subsequent analysis (SDS/PAGE, IEF, N-terminal sequencing and gel filtration chromatography) showed that the protein was homogeneous. The purified protein had a specific activity of 1800 units/mg and represented 15 % of the total laccase activity.

Metal-content analysis

The absorption spectrum of POXA1b displayed the characteristic peak at 600 nm, due to the presence of type-I copper. The ratio A_{280}/A_{605} was 17. This value is similar to those reported for laccases from other sources [17,18]. The copper content was determined by atomic absorption and the analysis showed a value of 3.7 mol/mol of protein.

Structural analysis of the enzyme

The molecular mass was 62 kDa in denaturing conditions (SDS/PAGE) and 57 kDa in native conditions (gel-filtration chromatography), thus indicating the monomeric nature of the protein. The pI value of 6.9 is similar to that of POXA1w but different from other laccases, which are usually characterized by more acidic pI values [5].

The N-terminal sequence of POXA1b was determined as: Ser-Ile-Gly-Pro-**Arg**-Gly-Thr-Leu-Asn-Ile-Ala-Asn-Lys-Val-Ile-Gln-Pro-Asp-Gly-Phe-Tyr-Arg-Ser-Thr-Val-Leu-Ala-Gly-Gly-Ser-Tyr-Pro-Gly-Pro-Leu…7 The analysis of this sequence showed that the first 19 amino acids were identical to the POXA1w N-terminus [10], except for the fifth residue, which was Arg instead of Asn (shown in bold).

Moreover, the sequence of six tryptic peptides of POXA1b was determined. Three of these were identical to the known peptides of POXA1w [10], whereas the sequences of the other three peptides were as follows: -Ala-Asp-Thr-Xaa-Met-Pro-Val-Asp-Thr-Ser-Ile-; -Gly-Xaa-Val-Val-Glu-Ile-Thr-Met-; and -Asn-Tyr-Glu-Asn-Pro-.

When subjected to automatic C-terminal-sequence analysis, the purified POXA1b was shown to be heterogeneous. Samples of the purified protein were analysed by automated degradation, obtaining in each run more than one amino acid residue identified at each cycle. By comparison of the results obtained, three overlapping C-terminal sequences were identified easily, as follows: -Leu-Pro-Ala-Pro-Leu-Lys (relative abundance, 40–45 %); -Leu-Pro-Ala-Pro-Leu (40–45 %); and -Leu-Pro-Ala-Pro (10–20 %). The three C-terminal sequences appeared to be generated from the same polypeptide chain. It is worth noting that not one of the above sequences terminates with Ala, the C-terminal residue deduced in the cDNA sequence. The heterogeneity observed could be explained by the existence of C-terminal processing of the protein, occurring either *in vivo* and/or during the purification procedure.

Only one free SH group was titratable by Ellman's reagent, as determined for POXA1w and POXC, possibly the SH group involved in type-I copper binding, as demonstrated for other known laccases [19].

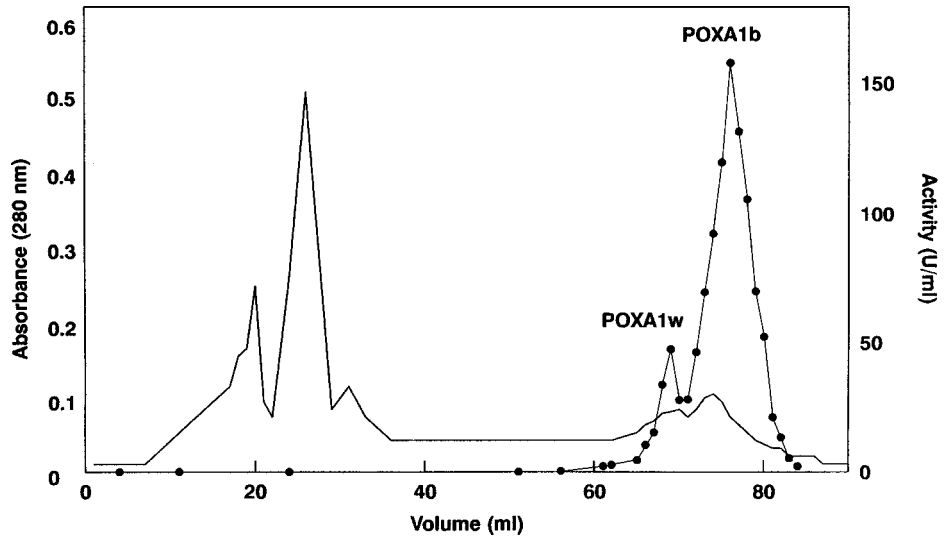

Figure 1 Elution profile of S-Sepharose fast-flow chromatography at pH 5.0

Fractions containing laccase activity eluted from DEAE-Sepharose with the equilibrating buffer were chromatographed on S-Sepharose. Two different active peaks (POXA1w and POXA1b) are shown. (—) Absorbance at 280 nm; (●) laccase activity [units(U)/ml].

Table 1 Kinetic constants of *P. ostreatus* POXA1b isoenzyme

	Optimum pH	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1} \cdot min^{-1}$)
ABTS	3	0.37	9.0×10^4	2.4×10^5
Syringaldazine	6	0.22	2.0×10^4	9.1×10^4
DMP	4–5	0.26	3.6×10^5	1.4×10^6

Table 2 Stability of POXA1b at 60 °C and at different pHs compared with POXA1w

	Stability ($t_{1/2}$)	
Condition	POXA1b	POXA1w
60 °C	3.0 h	3.2 h
pH 3.0	20 h	24 h
pH 5.0	6 days	10 days
pH 8.0	7 days	20 h
pH 9.0	30 days	Not determined

Catalytic properties

POXA1b was able to oxidize ABTS, syringaldazine and DMP, but no activity against guaiacol was detected at any of the pHs tested, as observed for POXA1w [10]. Table 1 shows the catalytic parameters and the optimum pH of POXA1b towards these substrates.

The stability of POXA1b compared with that of POXA1w at different pHs (room temperature) and at 60 °C (pH 7.0) was examined. As shown in Table 2, POXA1b exhibited a much higher stability than POXA1w at alkaline pH, whereas the situation was reversed at acidic pH.

When the activity of the enzyme was studied as a function of temperature, POXA1b showed maximal activity in a broad temperature range (20–50 °C). On the other hand, the activity decreased between 50 and 60 °C, the range for maximal activity of POXC and POXA1w [10].

Cloning and sequencing of *poxa1b* gene and cDNA

The knowledge of the sequence of the N-terminus and internal peptides allowed us to design oligonucleotide-primer mixtures for reverse transcriptase-PCR experiments. A cDNA fragment of 1200 bp was amplified, cloned and sequenced.

To obtain the complete coding sequence, another reverse transcriptase-PCR experiment was performed using a specific primer, localized at about 100 bp upstream from the 3′ end of the 1200-bp fragment, in combination with oligo(dT). Three different amplified fragments were cloned and sequenced. They differed only in the lengths of the non-coding region between the stop codon and the poly(A)$^+$ tail (101, 140 and 144 bp). This was probably due to the presence of other polyadenylation signals beyond that indicated in Figure 2.

A *P. ostreatus* genomic library [11] was screened using the amplified 1200-bp cDNA fragment. Two positive clones (2300 and 2000 bp) were isolated. The analysis of the restriction maps showed that the two clones partially overlapped. The first one covered almost all the coding sequence (amino acid residues 24–513), whereas the other one overlapped for about 400 bp and extended to the 5′-non-coding region.

The complete sequence of the first clone and part of the second one gave account of the whole coding sequence, together with

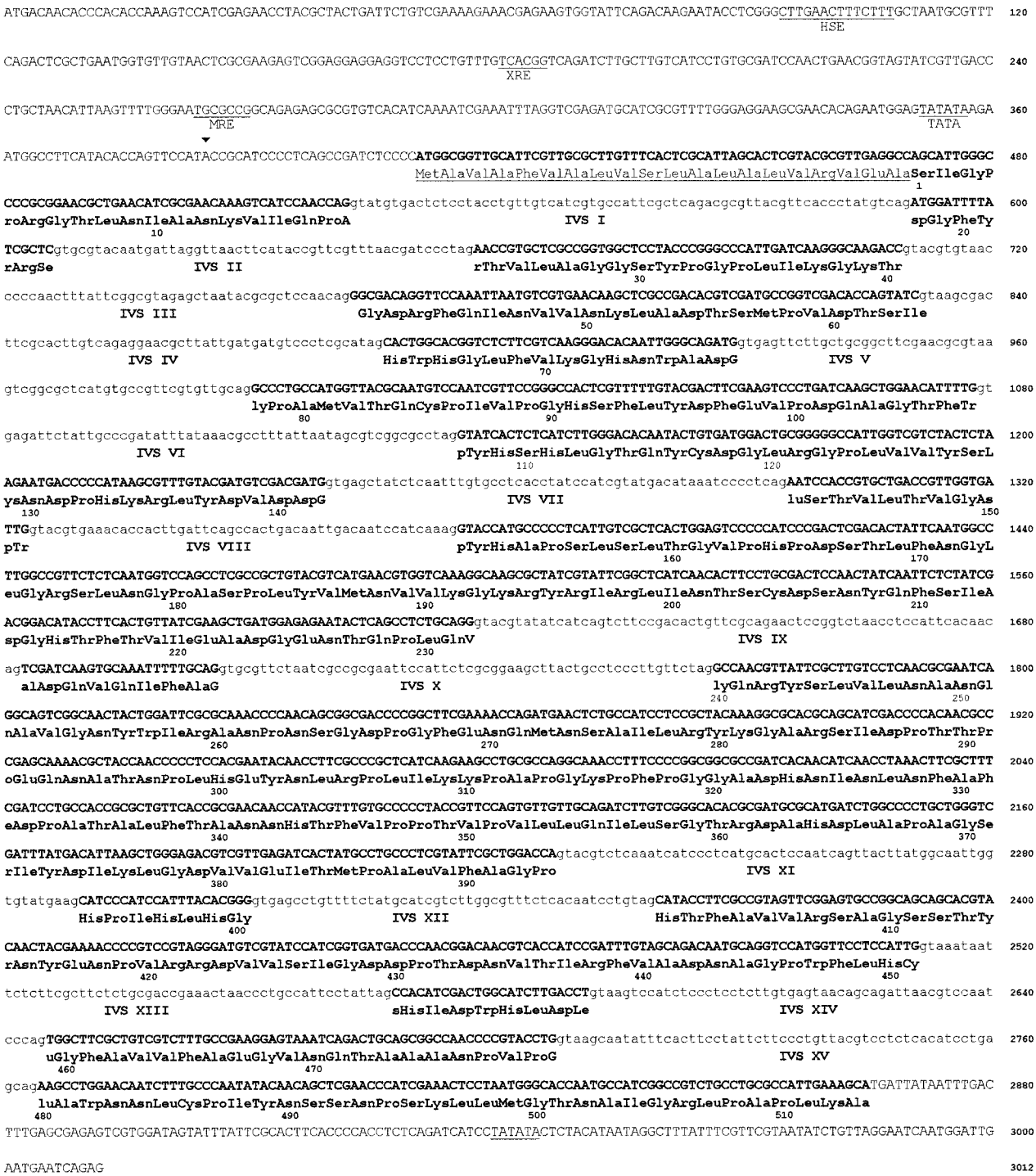

Figure 2 Nucleotide sequence of *P. ostreatus poxa1b* gene and deduced amino acid sequence of POXA1b

Introns are shown in lower-case type and indicated by IVS. The transcription-initiation site is shown by an arrowhead. The putative signal peptide is underlined. The heat-shock element (HSE), xenobiotic-responsive element (XRE), metal-responsive element (MRE), TATA box and polyadenylation consensus signal (second-to-last line) are underlined.

410 bp of the 5′-non-coding region (Figure 2). Thus the entire structure of the *poxa1b* gene was determined: the coding sequence contained 1599 bp interrupted by 15 introns. It is worth noting that the other two known laccase genes of *P. ostreatus*, *pox1* and *poxc* [9,11], contain a higher number of introns; a comparison of the gene structures is shown in Figure 3.

On the basis of the N-terminal sequence of the mature protein, a unique signal-peptide sequence of 20 amino acids could be singled out, which satisfied criteria for a 'minimal' signal sequence [20]. Two transcription start sites were mapped to 26 and 27 bp upstream of the ATG initiation codon when oligo 1, corresponding to the C-terminal portion of the signal peptide,

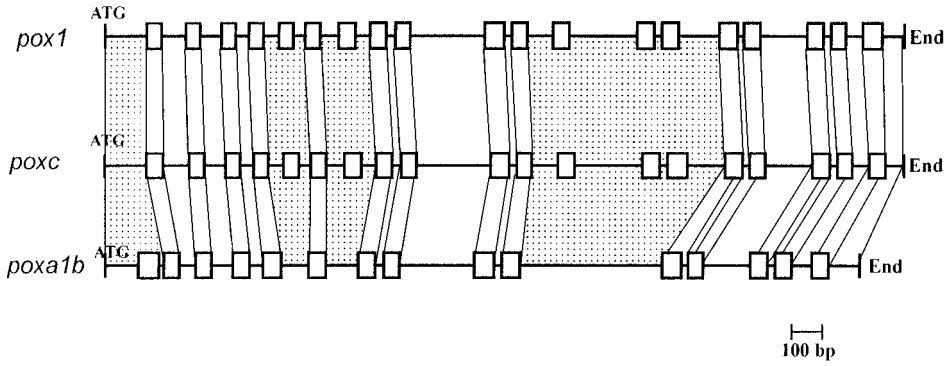

Figure 3 Comparison of the gene structures of *P. ostreatus* laccase genes *pox1*, *poxc* and *poxa1b*

Open boxes represent introns. Stippled areas represent regions of differing structure between *pox1*, *poxc* and *poxa1b*.

Table 3 MALDI-MS analysis of the reduced and carboxamidomethylated (CAM) laccase POXA1b digested with trypsin and PNGase F

Experimental mass value	Peptide sequence	Modification
1341.4	1–13	
830.9	6–13	
1095.4	14–22	
2537.3	14–37	
1460.2	23–37	
1391.7	40–51	
961.6	44–51	
2255.8	52–71	
5738.2	72–121	2 × CAM
6581.7	72–129	2 × CAM
863.3	122–129	
1609.3	122–135	
4346.8	136–175	
4503.2	135–175	
6174.4	135–191	
1689.8	176–191	
5889.9	192–242	CAM
4959.7	199–242	CAM
1981.8	243–259	
2132.9	260–279	
3148.7	282–309*	
2864.4	285–309*	
–	311–315	
–	316–361	
1586.9	362–376	
3333.4	377–407	
1546.0	408–421	
1702.3	408–422	
1716.6	423–438	
5768.2	439–489	2 CAM
6484.5	439–496*	2 CAM
1046.6	497–506	

* Asn-294, Asn-470 and Asn-490 were converted into Asp by the PNGase F deglycosylation step, thus increasing the molecular masses of peptides 282–309, 439–489 and 285–309 by 1 Da, and that of 439–496 by 2 Da.

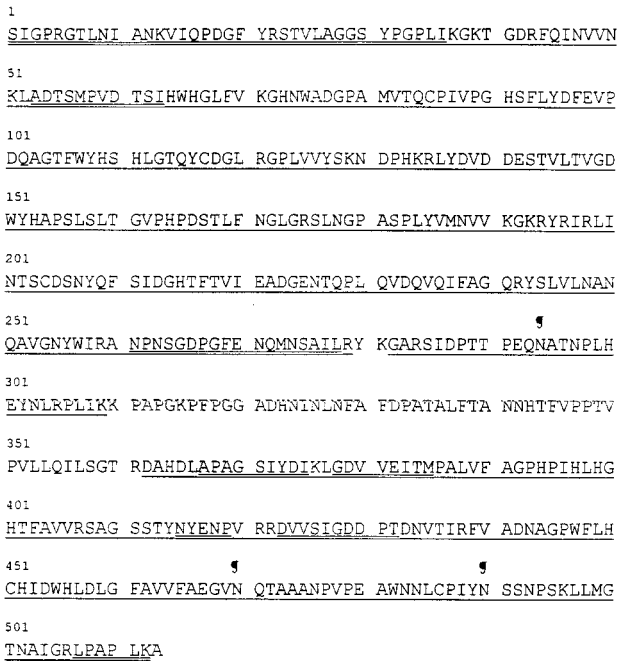

Figure 4 Amino acid sequence of POXA1b laccase from *P. ostreatus*

The underlined stretches have been verified by MALDI MS. The double-underlined stretches have been also verified by peptide-sequence analysis. The N-glycosylation sites are indicated by ¶.

was used as the primer. On the other hand, when the same experiment was performed using oligo 2 as the primer (the 3′ terminus of which corresponded to the codon for the fifth amino acid of the mature POXA1b), a unique transcription start site was mapped to 26 bp upstream of the ATG initiation codon. The promoter region sequenced thus far contained putative metal-responsive-element, xenobiotic-responsive-element and heat-shock-element consensus sequences (Figure 2).

Verification of the primary structure of the enzyme

Reduced and carboxamidomethylated POXA1b was analysed by MS in order to verify the protein primary structure deduced from the nucleotide sequence and to ascertain the presence of possible post-translational modifications. A portion of the POXA1b tryptic peptide mixture obtained after PNGase F digestion was submitted directly to MALDI-MS analysis. The results obtained are shown in Table 3. Each signal recorded in the spectrum was

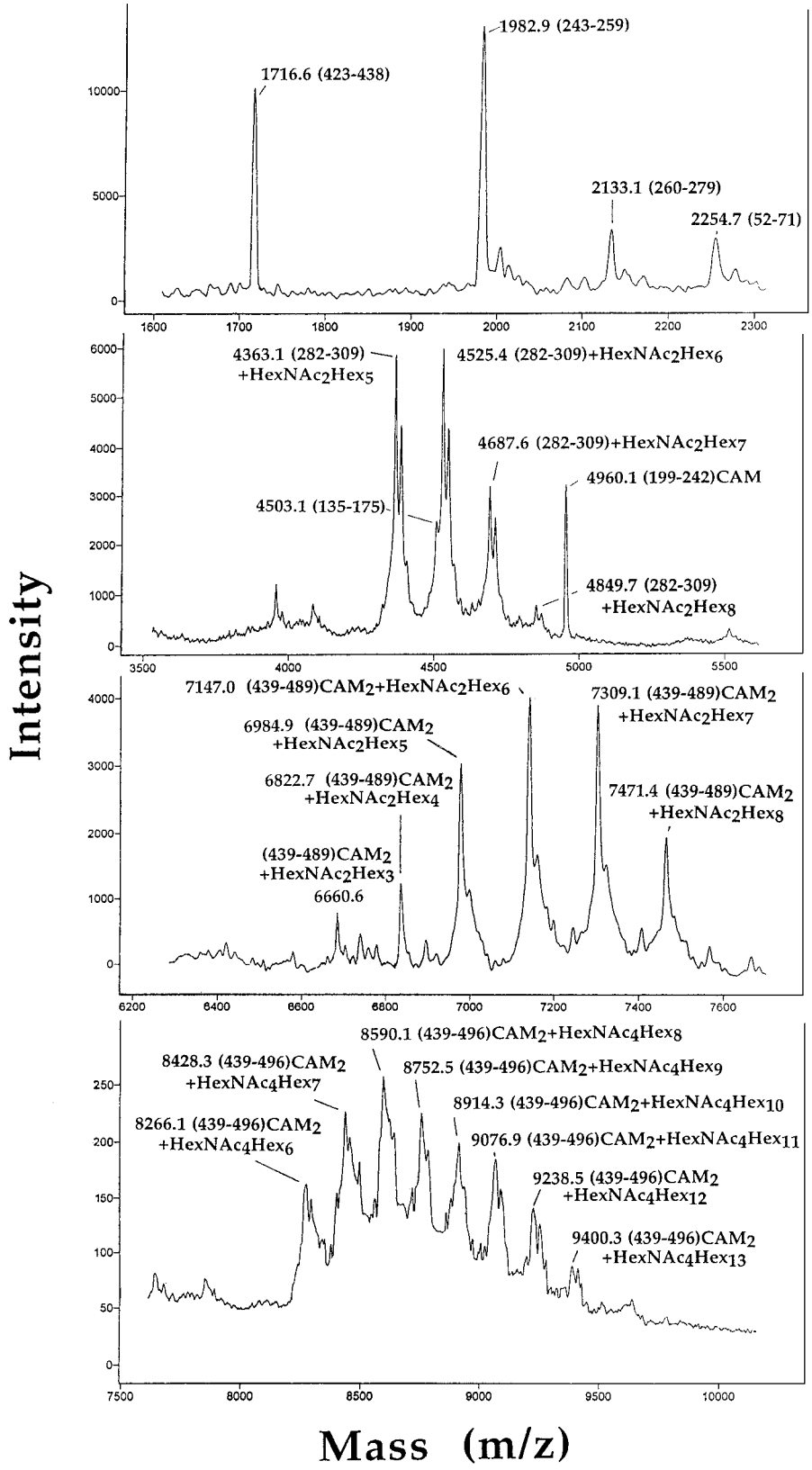

Figure 5 Partial MALDI-MS spectrum of the reduced and carboxamidomethylated (CAM) POXA1b following digestion with trypsin

The signals recorded in the spectrum were associated with peptides containing putative N-glycosylation sites within the protein sequence. The sugar composition of the N-linked glycopeptides is also reported. Hex, hexose; HexNAc, *N*-acetylhexosamine.

associated with the corresponding peptide along the amino acid sequence on the basis of its mass value and trypsin specificity. All the assignments were confirmed by submitting a portion of the digest to a single step of Edman degradation followed by mass analysis of the truncated peptide mixture. Complementary mass-mapping experiments carried out using endoprotease Lys-C and endoprotease Glu-C as proteolytic agents confirmed earlier results but did not provide additional structural information.

Results obtained from the combined Edman degradation, C-terminal sequencing and MALDI-MS analyses allowed the verification of 89.3% of the primary structure of POXA1b, as deduced from the nucleotide sequence (Figure 4). The sequence analysis of POXA1b revealed the presence of six putative N-glycosylation sites at Asn-201, Asn-294, Asn-342, Asn-434, Asn-470 and Asn-490.

Structural characterization of the POXA1b glycopeptide moiety

POXA1b samples were analysed for the presence of oligosaccharides using the lectin-binding assays. The protein was recognized specifically by *Galanthus nivalia* agglutinin lectin, which binds to terminal mannose residues, and *Datura stramonium* agglutinin, specific for galactose β(1-4)-*N*-acetylglucosamine. On the basis of lectin specificity, the presence of both high-mannose- and hybrid- or complex-type N-linked glycans was suggested.

A sample of the laccase tryptic digest before PNGase F treatment was submitted directly to MALDI-MS analysis using different matrices. Figure 5 reports a portion of the spectrum obtained using α-cyano-4-hydroxycinnamic acid, where the presence of adjacent peaks differing by 162 mass units made the glycopeptides immediately recognizable.

The signals at m/z 4363.1, 4525.4, 4687.6 and 4849.7 were associated with glycosylated forms of the peptide 282–309. On the basis of the known biosynthetic pathway of N-linked oligosaccharides and the molecular mass of the peptide moiety, these signals were interpreted as the fragment 282–309 bearing high-mannose-type N-linked glycans, containing between two and five mannose residues linked to the pentasaccharide core present on Asn-294. The satellite peaks at $+22$ m/z were due to sodium-adduct ions. No signal corresponding to the unglycosylated peptide was present in the spectrum.

Similarly, the nature of the post-translational modifications present on Asn-470 was determined from the presence in the spectrum of the peaks at m/z 6660.6, 6822.7, 6984.9, 7147.0, 7309.1 and 7471.4. These signals were associated with glycosylated forms of the carboxamidomethylated peptide 439–489, resulting from the unspecific hydrolysis of the polypeptide chain at Tyr-489. The occurrence of aspecific hydrolysis at this site was confirmed by the presence of the signal at m/z 5768.2, observed during the analysis of the tryptic digest after PNGase F treatment. The oligosaccharide chains linked to Asn-470 were identified as high-mannose-type glycans containing between zero and five mannose residues.

Moreover, the cluster of adjacent peaks differing by 162 mass units observed in the range between m/z 8000 and 9500 allowed us to confirm the glycosylation status of Asn-470 and to determine the nature of the oligosaccharides linked to Asn-490. On the basis of the core structure and attached building blocks determined for Asn-470 and the molecular mass of the peptide moiety, these peaks were attributed to different high-mannose-type N-linked forms of the carboxamidomethylated glycopeptide 439–496, presenting two pentasaccharide cores at Asn-470 and Asn-490, each containing a various number of mannose residues. No signal corresponding to the unglycosylated carboxamidomethylated peptides 439–496 and 439–489 was present in the spectrum reported in Figure 5, thus indicating that these peptides were modified totally.

Furthermore, the intense signals at m/z 1716.6 and 4960.1 were attributed to the fragments 423–438 and 199–242. No peak relative to glycosylated forms of these peptides was observed in the spectrum. These findings suggested that Asn-201 and Asn-434 did not exhibit any post-translational modifications.

No signal relative to the region 310–361 was observed in any spectrum recorded. Therefore, no information regarding the N-linked glycans eventually present on Asn-342 could be obtained. Independent experiments, performed using different proteases (endoprotease Lys-C and endoprotease Glu-C) and a different matrix (2,5-dihydroxybenzoic acid), confirmed the post-translational modifications at Asn-294, Asn-470 and Asn-490 but did not give information on Asn-342.

DISCUSSION

It is known that laccase production in different white rot fungi is influenced by a number of environmental factors. It has been reported that almost all fungal species studied secrete more than one laccase isoenzyme and different patterns of isoenzymes are obtained according to the growth conditions used [8,21]. As far as *Pleurotus* species are concerned, Leonowicz and Trojanowski [22] studied the effect of ferulic acid as an inducer of a specific laccase isoenzyme in *P. ostreatus*. Moreover, Munoz et al. [23] demonstrated the induction of a laccase isoenzyme by wheat straw alkalilignin and vanillic and veratric acids in *P. eryngii*.

We observed that copper is a strong laccase inducer in *P. ostreatus*, as is known for other fungal species [24,25]. In particular, when the neutral laccases were analysed, production of the previously characterized isoenzyme POXA1w was substantially unaffected by the presence of copper but, interestingly, a new neutral laccase isoenzyme POXA1b was produced under these conditions. This behaviour supports the hypothesis of distinct regulation mechanisms of laccase isoenzyme expression in *P. ostreatus* and suggests a different physiological role for these isoenzymes.

The main structural characteristics (molecular mass in native and denaturating conditions and pI) of purified POXA1b are almost identical to those of POXA1w, but, in contrast, POXA1b displays the classical laccase UV/visible spectrum and contains the characteristic four copper atoms per molecule. The catalytic constants of POXA1b, using the most common laccase substrates, ABTS, syringaldazine and DMP, reveal that the enzyme has a higher catalytic efficiency towards DMP than POXA1w. On the other hand, both POXA1b and POXA1w are unable to oxidize guaiacol at any of the pHs tested here. Since guaiacol is a common laccase substrate, this characteristic renders these isoenzymes peculiar with respect to the substrate specificity of other characterized laccases [5].

Taking into consideration the thermal stability of the two isoenzymes and the stability at various pHs, it is worth noting that both isoenzymes are more stable than the other three *P. ostreatus* isoenzymes characterized already [10,26]. Above all, POXA1b shows an increase of $t_{1/2}$ from pH 5.0 to 9.0 and, surprisingly, displays a $t_{1/2}$ value at pH 9.0 of about 30 days. This characteristic should be a very appreciable property for biotechnological applications.

The gene and cDNA coding for POXA1b have been isolated and sequenced. All the introns have a GTRNGY consensus sequence at the 5′-splicing site and a YAG consensus sequence at the 3′-splicing site, both similar to the canonical splicing sequences in other eukaryotes [27]. In Figure 3 the comparison

among the gene structures of *poxa1b*, *pox1* and *poxc*, performed by aligning the corresponding encoded amino acids, is shown. The gene structures of *pox1* and *poxc* [9,11] are fully conserved whereas the intron/exon organization of *poxa1b* gene differs in some regions. In particular, a DNA region of *pox1* and *poxc* that includes three introns corresponds to a unique exon in *poxa1b* (Figure 3). Therefore, on the basis of the number and position of the introns, these genes can be classified into two subfamilies [28].

In the 5′-flanking region of *poxa1b*, several sequences have been identified that match closely the consensus of regulatory elements (Figure 2), in particular a metal-responsive element [29] and a xenobiotic-responsive element [30]. Similar consensus sequences can also be observed in *pox1* and *poxc* promoters. Moreover, a putative heat-shock element [31] is present only in the *poxa1b* promoter. More investigations are needed to verify if these regulatory elements are functional.

The amino acid sequence deduced from the cDNA sequence was almost completely verified by means of MALDI-MS mapping. All the putative copper-binding residues are present in the sequence, as well as the five Cys residues found in almost all the known laccase sequences [32]. Four of them should be involved in the formation of two disulphide bridges, because only one Cys residue, which is putatively involved in the binding of type-I copper [19], is titratable in non-reducing conditions.

The analysis of the glycosidic moiety of POXA1b showed the presence of high-mannose glycan structure containing between zero and five mannose residues linked to the pentasaccharide core, on three out of six putative N-glycosylation sites. The absence in the spectra of the signals corresponding to the unglycosylated peptides demonstrated that Asn-294, Asn-470 and Asn-490 were totally modified, whereas no post-translational modification of Asn-201 and Asn-434 was observed. Moreover, two different lectins specifically recognized POXA1b, also indicating the presence of other N-linked glycans in addition to the high-mannose type. These glycans could be linked to the unique putative N-glycosylation site (Asn-342) that was not mapped by MALDI-MS analysis.

The amino acid sequences of POXA1b and POXC show an identity of 59 %, whereas the identity between POX1 and POXC is much higher (90 %). On the basis of the limited structural data available for POXA1w, this enzyme seems to display a high degree of similarity to POXA1b (three identical tryptic peptides [10] with only one difference in the N-terminus out of 19 amino acid residues). In order to investigate the structural differences between POXA1w and POXA1b, the MALDI-MS tryptic maps were compared (results not shown). The analysis of the spectra revealed that only a few signals with the same m/z values occurred in both spectra, indicating that the two proteins are not strictly correlated. On the other hand, the occurrence of two transcription start sites, when using non-specific primers, suggests the presence of two different transcripts, arising from two different genes, with similar N-terminal coding sequences. Knowledge of the primary structure of POXA1w will throw light upon the observed differences between POXA1b and POXA1w and the structure-function relationships of these laccase isoenzymes.

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