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Shifting the optimal pH of activity for a laccase from the fungus *Trametes versicolor* by structure-based mutagenesis

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Abstract

Laccases are oxidizing enzymes of interest because of their potential environmental and industrial applications. We performed site-directed mutagenesis of a laccase produced by *Trametes versicolor* in order to improve its catalytic properties. Considering a strong interaction of the Asp residue in position 206 with the substrate xyloidine, we replaced it with Glu, Ala or Asn, expressed the mutant enzymes in the yeast *Yarrowia lipolytica* and assayed the transformation of phenolic and non-phenolic substrates. The transformation rates remain within the same range whatever the mutation of the laccase and the type of substrate: at most a 3-fold factor increase was obtained for k_{cat} between the wild-type and the most efficient mutant Asp206Ala with 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic) acid as a substrate. Nevertheless, the Asn mutation led to a significant shift of the pH ($\Delta pH = 1.4$) for optimal activity against 2,6-dimethoxyphenol. This study also provides a new insight into the binding of the reducing substrate into the active T1 site and induced modifications in catalytic properties of the enzyme.

Keywords: fungal laccase/pH-dependence/polyphenol oxidase/site directed mutagenesis/*Yarrowia lipolytica* expression system

Introduction

Laccases (EC 1.10.3.2) are polyphenoloxidases performing the reduction of oxygen to water while oxidizing organic substrates by a one-electron redox process. Laccase activity was first described in plants (*Rhus vernicifera*) by Yoshida in 1884 and more recently enzymes were evidenced in bacteria (Diamantidis et al., 2000). However, laccases are mainly produced by filamentous fungi (ascomycetes and mainly wood-decaying basidiomycetes) (Xu, 1999). In these organisms, they are involved in several biological processes such as lignin degradation (Eggert et al., 1997), morphogenesis and pathogenesis. In addition to lignocellulosic substrates, an extensive list of xenobiotics [pesticides, polycyclic aromatic hydrocarbons (PAHs), dyes, etc.] often containing phenolic or aromatic amino groups are transformed by laccases (Gianfreda et al., 1999; Jolivald et al., 1999) either by bond cleavage or by oxidative coupling. Owing to the great variety of reactions catalysed by laccase, this enzyme has attracted considerable interest in various fields of research, e.g. development of oxygen cathodes in biofuel cells, green biodegradation of xenobiotics, biosensors, organic synthesis, labelling immunoassays (Mayer and Staples, 2002). This ability to oxidize such a large range of substrates seems to be related to the non-specific free radical mechanisms which have evolved to degrade lignin and is greatly favoured by the high redox potential of the laccases, ranging from 430 mV for tree laccase from *Rhus vernicifera* to 780 mV for fungal laccase from *Polyporus versicolor* (Xu et al., 1999).

We demonstrated also that fungal laccases were efficient tools for bioprocesses leading to polluted water cleanup (Jolivald et al., 2000) and soil bioremediation (Rama et al., 2001). Nevertheless, laccase-mediated biotransformation of xenobiotics in natural media suffers from two main limitations of the enzyme: an acidic optimal pH for activity and the requirement in several cases (e.g. PAHs) for a redox mediator. The modification of these laccase properties should be achieved through a site-directed mutagenesis strategy. Based on sequence alignments without precise information concerning both the substrate cavity geometry and the interactions between amino acids and substrate, the pioneering work of Xu et al. (1998, 1999) suggested that a non-ligating tripeptide in the vicinity of the active site was involved in the redox potential level. Although the redox potentials were not significantly altered, the triple mutants had a phenoloxidase activity whose pH optimum shifted 1 unit lower or higher while the kinetic parameters were greatly changed. These results were interpreted as possible mutation-induced structural perturbations of the molecular recognition between the reducing substrate and the enzyme.

In recent years, crystal structures of free laccases from *Coprinus cinereus* (Ducros et al., 1998), *Trametes*

versicolor (Piontek et al., 2002), *Melanocarpus albomyces* (Hakulinen et al., 2002) and *Rigidoporus lignosus* (Garavaglia et al., 2004) have been published. They revealed a distorted trigonal planar Cu geometry of the T1 centre at the active site of the enzyme, with three strong and highly conserved ligands, two histidines and a cysteine and a relatively distant axial ligand, generally leucine or phenylalanine in fungal laccases. In addition, three-dimensional structures of laccases have been obtained in the presence of a reducing substrate (Bertrand et al., 2002; Enguita et al., 2004). Our group showed that the cavity of the laccase from *T. versicolor* was fairly wide and that it can accommodate a large variety of substrates which were not tightly buried in it (Bertrand et al., 2002). The presence of an arylamine (2,5-dimethylbenzeneamine or 2,5-xylydine) at the T1 active site of the enzyme revealed two important residues for the interaction between the amino group of the reducing substrate and the enzyme. The first is a histidine (458) that also coordinates the T1 copper and acts as the primary electron acceptor from the substrate. This histidine is highly conserved among laccases. The second, aspartate 206, is hydrogen bonded via the terminal oxygen of its side chain to the amino group of 2,5-xylydine. Asp206 appears relatively buried in the cavity, surrounded by a crown of hydrophobic residues which lie in a more external part (Figure 1). All these residues contribute to the positioning of the substrate. Moreover, it was found for phenolic substrates such as phenylurea derivatives that a pH increase from 3 to 5 generally induced a decrease in the K_m values, which can be interpreted as a better affinity between the substrate and the enzyme. As the protonation state of phenolic derivatives remains unchanged in this pH range, such an alteration in the interaction between the substrate and the enzyme could be a consequence of the deprotonation of one of the amino acid side chains interacting with the substrate, suggesting the involvement of an acidic amino acid, possibly Asp206.

That new hypothesis led us to develop in the present work a mutagenesis approach performed on a *T.versicolor* laccase (AF414109) considering the Asp206 residue. Because different biological functions are generally attributed to laccases depending on their origin (white-rot fungal laccases are involved in the degradation of lignin whereas plant laccases participate in the initial stages of its synthesis), we decided to mutate Asp206 into three amino acids: (i) a non polar amino acid, alanine, (ii) glutamate, which can be found among ascomycetes whereas aspartate seems fully conserved in basidiomycetes, and (iii) asparagine, found in all plant laccase sequences (Figure 2).

Here we had three objectives: (i) to determine how the site-directed mutagenesis would impact the pH dependence of enzymatic activity, (ii) to investigate the possibility of increasing the efficiency of the catalytic activity of the enzyme towards selected substrates and (iii) to enlarge the permissivity of laccase

at the T1 site towards xenobiotics recalcitrant to laccase-catalysed oxidation.

Materials and methods

Chemicals

3,4,5-Trimethoxybenzaldehyde (3,4,5-TMB) was purchased from Aldrich, 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS) and 2,5-xylidine from Sigma and 2,6-dimethoxyphenol (2,6-DMP) from Acros. Coniferyl alcohol was synthesized according to the method of Ludley and Ralph (1996). Chemicals used as buffers were obtained from VWR (Prolabo, Rectapur grade) and solvents for HPLC and HPSEC from Carlo Erba (RS grade). The structures of the substrates of laccases used in this study are shown in Figure 3.

Bacteria and yeast strains used

Escherichia coli DH5 α F' strain (Life Technology) was used for the construction and routine propagation of vectors. *Yarrowia lipolytica* Po1g strain (Madzak et al., 2000) was used as the host for heterologous laccase expression. The isogenic *Y. lipolytica* Po1t prototrophic strain (Madzak, 2003) was used as a non-producing control.

Plasmid design and site-directed mutagenesis

The plasmids used in this study are listed in Table I. The previously described laccase-expressing vector p69TVLB is designed to integrate at the pBR322 docking platform of the *Y. lipolytica* Po1g recipient strain (Jolivalt et al., 2005). This vector uses the native signal peptide to direct the secretion of active recombinant laccase IIIb into the culture medium (Jolivalt et al., 2005). Our purpose was to introduce into *T. versicolor* laccase ORF a point mutation leading to the desired amino acid change at position 206. The ORF codon corresponding to this position is located within a 65 bp region delimited by XmaI and ClaI restriction sites. Our strategy for directed mutagenesis of laccase IIIb was to replace this DNA fragment from p69TVLB vector by a synthetic DNA fragment carrying the desired point mutation. The presence of a second ClaI restriction site in p69TVLB vector (located at 1400 bp from the former, between XPR2 terminator and LEU2 selection marker gene) obliged us to perform first a partial digest of p69TVLB vector DNA using ClaI restriction enzyme. One of the products of this first step was a linearized p69TVLB vector DNA, which was purified on agarose gel. We performed on this DNA a second restriction, using XmaI enzyme. Among the products of this second step was an 8.8 kbp XmaI–ClaI DNA fragment corresponding to p69TVLB vector deleted from its 65 bp XmaI–ClaI region. This deleted vector

was purified on agarose gel and ligated to one of three different synthetic sticky-ended (*Xma*I–*Cla*I) DNA fragments carrying the desired point mutation. Each point mutation was chosen so that the new codon fitted the peculiar *Y. lipolytica* codon bias. Each of the three synthetic fragments was obtained by hybridization of a couple of oligonucleotides (sequences of sticky ends in bold, codon 206 in bold underlined): (i) for p69TVLB- D206A plasmid, 50P-Ph-CCG GGT AAA CGC TAC CGT TTC CGC CTG GTG TCC CTG TCG TGC GCC CCC AAC TAC ACG TTC AGC AT-30 and 50-Ph-C GAT GCT GAA CGT GTA GTT GGG GGC GCA CGA CAG GGA CAC CAG GCG GAA ACG GTA GCG TTT AC-30; (ii) for p69TVLB-D206N plasmid, 50-Ph-CCG GGT AAA CGC TAC CGT TTC CGC CTG GTG TCC CTG TCG TGC AAC CCC AAC TAC ACG TTC AGC AT-30 and 50-Ph- C GAT GCT GAA CGT GTA GTT GGG GTT GCA CGA CAG GGA CAC CAG GCG GAA ACG GTA GCG TTT AC; (iii) for p69TVLB-D206E plasmid, 50-Ph-CCG GGT AAA CGC TAC CGT TTC CGC CTG GTG TCC CTG TCG TGC GAG CCC AAC TAC ACG TTC AGC AT-30 and 50-Ph-C GAT GCT GAA CGT GTA GTT GGG CTC GCA CGA CAG GGA CAC CAG GCG GAA ACG GTA GCG TTT AC-30. These constructions were checked by sequencing (Genaxis).

Prior to yeast transformation, p69TVLB-D206A, -D206N and -D206E plasmids were linearized with *Not*I restriction endonuclease, in order to direct their integration at the pBR322 docking platform of the recipient strain (see review by Madzak et al., 2004). Transformation of *Y. lipolytica* Po1g strain with mutated laccase-expressing vectors was performed according to Xuan et al. (1988). The transformants were selected on YNB minimal medium plates, as described previously (Madzak et al., 2005). For each construction, several transformants were checked for correct integration of a single copy of the vector at the pBR322 docking platform, using polymerase chain reaction (PCR) analysis (data not shown) and one of each was selected for further analysis.

Cultivation conditions and laccase isolation

The transformants obtained from Po1g strain using p69TVLB- D206A, -D206N or -D206E plasmids were checked for active laccase production on agar plates supplemented with CuSO₄ (0.1 mM) and ABTS (0.2 mM), as described previously (Jolivalt et al., 2005). Plates were incubated in the dark at 28°C for 3 days and checked for the development of a green colour.

For assaying laccase activity, transformants were cultivated on modified PPB liquid medium (glucose 20 g/l, yeast extract 1.32 g/l, NH₄Cl 1.32 g/l, MgSO₄·7H₂O 0.24 g/l, CuSO₄ 0.025 g/l, thiamine 0.33 mg/l) under vigorous shaking (180 r.p.m.), in baffled 2 l Erlenmeyer flasks containing 200 ml of medium, at

28°C. Modified PPB medium was buffered using 50 mM phosphate buffer (pH 6.3). Inoculation was performed using one-day cultures to give an initial OD₆₀₀ of 0.1. Yeasts were cultivated for 4–5 days. After centrifugation (6000 r.p.m. for 10 min), culture supernatants were filtered through glass-fibre filters of decreasing porosity (1.6, 0.7 and 0.22 µm) and then concentrated in an ultrafiltration cell (Amicon) equipped with a YM30 membrane (Millipore). The concentrate was dialysed in the same cell against 50 mM citrate–phosphate buffer (pH 6.8). The 4 ml of enzyme solution (11 U/ml) was stored at -20°C with 30% (w/v) glycerol.

Laccase activity measurements using spectrophotometric methods

Standard assays for laccase activity were performed by measurement of enzymatic oxidation of ABTS at 420 nm ($\epsilon = 3.6 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$ for the oxidation product). The reaction mixture contained 100 µL concentrated extracellular fluid (prepared as described above) and 900 µL ABTS (1 mM) in Na₂HPO₄/citric acid buffer (0.1 M, pH 3.0) at 30°C. The buffer solution was saturated with air by bubbling prior to the experiment. One unit of enzyme activity is defined as the amount of enzyme that oxidizes one mmole ABTS in one min. Kinetics analysis of the mutants were performed with ABTS 1 mM at pH 3 in citrate/phosphate buffer 0.1 M.

pH activity dependence of the mutants was tested with both ABTS and 2,6-DMP. Experiments were performed in the conditions described above for ABTS or with 2 mM 2,6-DMP in Na₂HPO₄/citric acid buffer (0.1 M, pH 3.4). 2,6-DMP oxidation product was detected at 468 nm ($\epsilon = 4.96 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$).

Enzymatic transformation of xenobiotics followed by HPLC and HPSEC analysis

2,5-Xylidine. Triplicate samples of 2,5-xylidine (0.08 mM) in 40 mM citrate–phosphate buffer (pH 5) were incubated at 30°C using 1 unit of laccase activity. After a 24 h incubation, 20% (v/v) acetonitrile was added to stop the enzymatic reaction and the reaction mixture was centrifuged (6000 r.p.m.) for 10 min at 14°C. Analysis of the supernatant was performed on a Varian HPLC system equipped with a Model 9010 solvent delivery system, a Rheodyne Model 7125 injection valve with a 20 µl sample loop and a Model 9050 absorbance detector operating at 254 nm. A TSK gel (250 × 4.6 mm i.d.) C18 column was used. The mobile phase, at a flow-rate of 1 ml min⁻¹, was composed of water (A) and acetonitrile (B), initially set at 15% A-85% B for 5 min, then ramped to 100% B in 13 min and maintained at 100% B for 7 min.

Coniferyl alcohol. Coniferyl alcohol transformation was assayed in the following reaction mixture: 0.6 ml of 25 mM citrate-phosphate buffer (pH 4.5) containing 0.67 mM coniferyl alcohol and 0.33 mM 3,4,5-

TMB, used as an internal standard for the subsequent analytical determination of the reaction extent. The solution was aerated for 15 min. The reaction was initiated by adding 0.0015 units of laccase activity, as measured following the protocol for laccase activity measurements described above, and incubated at 30°C for 4 h. The reaction mixture was acidified with 0.05 ml of 0.1 M HCl to enhance phenol solubilization in organic solvents and extracted with 1.5 ml of dichloromethane on an ice-bath. The organic phase was dried with Na₂SO₄ before solvent evaporation and dissolution of the solid residue in 400 µl of stabilized tetrahydrofuran prior to high-performance size-exclusion chromatography (HPSEC) (Baumberger et al., 2003). Samples were ultrafiltered on a 0.45 µm PTFE filter (Millipore) before injection of an aliquot (5 µl) on a styrene–divinylbenzene copolymer gel column (PL-gel, Polymer Laboratories, 100 Å, 600 x 7.5 mm i.d.) with THF as eluent (1 ml min⁻¹) and UV detection (280 nm). Calibration was performed using commercial polyethylene oxide standards (Igepal, Aldrich) and purified coniferyl alcohol dimers and tetramer. LC–MS analysis of the samples was performed after evaporation to dryness and dissolution in methanol with an ion trap mass spectrometer (ThermoFinnigan LCQ Deca, Finnigan MAT, San Jose, CA) equipped with an electrospray interface set in the negative mode (sprayer needle voltage 4 kV, temperature of the heated capillary 350°C).

Results

Expression of the Asp206 mutants

Ligninolytic enzymes are generally difficult to express heterologously in active form (Jönsson et al., 1997). No active recombinant laccase expression has been reported yet in prokaryotic systems such as *Escherichia coli*, probably owing to the lack of post-translational modifications such as glycosylation which are involved in the enzyme stabilization against proteolysis (Yoshitake et al., 1993). In the present work, the yeast *Y. lipolytica* was chosen as a host for laccase expression.

The plasmids used in this study (Table I) were obtained by inserting the wild-type or site-directed mutant sequence of *T. versicolor* laccase IIIb into the monocopy integrative pBR-based vector pINA1269, from the *Y. lipolytica* expression system, using the recombinant hp4d promoter (Madzak et al., 2000). As a host, *Y. lipolytica* is particularly suitable for the genetic engineering of heterologous genes. Indeed, pBR-based *Y. lipolytica* expression vectors offer (i) a very high transformation efficiency (10⁵ transformants per mg) and (ii) precise targeting of monocopy integration events at the pBR322 docking platform of recipient cells, leading to transformants similar in terms of copy number and integration locus [for reviews, see Madzak (2003) and Madzak et al. (2004)].

The constructs were successfully expressed in *Y. lipolytica*. The mutant proteins were secreted in an active form, as checked by growing transformants on plates supplemented with ABTS which was oxidized into a green radical cation by excreted laccase activity (data not shown).

Owing to the targeted monocopy integration of the constructs, the effect of different site-directed mutations can be compared directly in culture supernatants, provided that yeasts are grown under similar conditions and that the laccase secretion level is the same for all of the mutants. In our experiments, transformants were grown simultaneously following the same protocol and we checked, using optical density (OD₆₀₀) measurements of cultures, that growth variations between transformants did not exceed 10% (data not shown). In parallel, activity measurements were performed using ABTS as a substrate during the time course of the culture. No activity was detected during the first 48 h, which was consistent with the growth-phase dependence of hp4d promoter, which began to exert its full activity at the end of the growing phase (Nicaud et al., 2002). On the second day, significant levels of activity were detected for each of the transformants, but different levels were observed depending on the mutation, in the decreasing order Asp206Ala > Asp206Asn > Asp206Glu (Figure 4). The activity levels increased during the subsequent days while their respective order remained the same during the whole culture time course. The culture was stopped after 5 days. The laccase production level remained rather low (around 1 mg/l) and the quantity of enzyme available was not sufficient to undergo an extended purification process. The crude supernatant was only filtered, concentrated and dialysed against pH 6.8 phosphate buffer. This neutral pH was chosen to preserve catalytic activity.

Kinetic parameters determination

Kinetic parameters were determined using a spectrophotometric method for two substrates, ABTS at pH 3 and 2,6-DMP at pH 3.4, both in 0.1 M citrate–phosphate buffer. As the culture supernatants were only concentrated by ultrafiltration and dialysed without any further purification step, k_{cat} values were calculated using the total protein concentration in the enzyme solution studied. This method leads to an under-estimation of the k_{cat} values. However, the calculated k_{cat} values obtained for ABTS as a substrate (Table II) led to the same ranking of the mutants as obtained for the time course profile of activity measurements in the different mutant culture supernatants (Figure 4). It can therefore be considered that, even if they are underestimated, the k_{cat} values provide an accurate comparison of the relative mutant catalytic rates.

For ABTS as a reducing substrate, the data given in Table II clearly show that the Asp to Ala mutation

induced the most significant changes in the enzymatic properties of laccase. Indeed, the substitution of Asp206 with Ala led to a 5-fold decrease in the K_m value (10 mM for the mutant compared with 50 mM for wild-type enzyme). This mutation also resulted in a significant increase in the k_{cat} value: 4.3 s^{-1} for the Ala mutant versus 1.3 s^{-1} for the wild-type recombinant laccase. The lowest change in the enzymatic properties was observed for the substitution with Glu: K_m decreased 2-fold compared with the wild-type enzyme, whereas there was no significant change in the reaction rate. The Asn mutant exhibited intermediate values between wild-type laccase and Ala mutant for both kinetic parameters.

The kinetic parameters were determined for 2,6-DMP at pH 3.4, i.e. the pH with maximum activity for the recombinant wild-type laccase. Under such conditions, the efficiency of all the laccases tested was lower (by a factor of at least 10) for 2,6-DMP than for ABTS, mostly due to higher values of the Michaelis constant K_m (Table II), whereas only marginal changes were observed for k_{cat} values between the mutants and the recombinant wild-type laccase. Compared with its value for the wild-type laccase, a significant enhancement of K_m was found for both Asp206Asn and Asp206Ala mutants. with 17- and 8-fold increases, respectively.

Transformation of 2,5-xylydine, a non-phenolic substrate

After a rough purification of the culture supernatant consisting of centrifugation and dialysis against citrate-phosphate buffer (pH 6.8), followed by sample concentration, the extent of transformation of 2,5-xylydine by wild-type and mutated laccases was measured. For each laccase, the amount of enzyme used in the experiment was normalized with respect to its activity towards ABTS. Experiments were performed at pH 5. Table III provides details of the 2,5-xylydine transformation efficiency for wild-type and mutated laccases. Both the native laccase expressed by *T. versicolor* and the recombinant wild-type laccase expressed by *Y. lipolytica* exhibited a similar transformation extent of around 55%. Experimental conditions were chosen to maximize the sensitivity of the experiment while allowing the comparison between all the mutants tested: the most efficient mutant, Asp206Glu, almost fully oxidized 2,5-xylydine (94%). 2,5-Xylydine is therefore almost 2-fold more efficiently transformed by the Asp206Glu mutant than by the wild-type enzyme. In contrast, replacement of an aspartic amino acid by a non-charged amino acid leads to a lowering of the oxidation rate of the substrate: the loss remains limited for the Asn mutant (45%, instead of 58% for the wild-type) whereas the Ala mutant is 2-fold less active than wild-type laccase. To summarize the results, the order of transformation rates is Asp206Glu > wild-type > Asp206Asn > Asp206Ala.

Transformation of coniferyl alcohol, a phenolic substrate

Wild-type and mutated laccases were assayed for their efficiency in oxidizing coniferyl alcohol, which is a precursor in lignin formation (Brunow et al., 1998). Incubation conditions similar to these used for 2,5-xylidine, but with a pH of 4.5, were used. The *T. versicolor* laccase expressed in *Y. lipolytica* exhibited the highest transformation rate compared with the mutants, whose reaction rates decreased in the order wild-type > Asp206Glu > Asp206Ala > Asp206Asn (Table III).

HPSEC analysis of the reaction mixture showed that incubation of the enzymes with coniferyl alcohol led in all cases to the formation of dimeric species (Figure 5) and LC-MS revealed that the oxidation products exhibited similar compositions. The main dimers were identified by LC-MS according to their retention time and mass spectra compared with purified standards. They consist in three dehydrodimers resulting from the radical coupling of oxidized coniferyl alcohol through pinoresinol β - β , phenylcoumaran β -5 and arylglycerol β -0-4 bonds (Brunow et al., 1998). Together with the dimers, traces of trimers showing molar masses of 538 were detected in all cases. They were tentatively assigned to a guaiacylglycerol- β , α -bisconiferyl ether structure (De Angelis et al., 1999).

Effect of pH on laccase activity

Experiments conducted with ABTS showed that the pH-activity profile of the mutants was very similar to that observed for the recombinant wild-type laccase (Figure 6A). Assayed solutions were prepared from concentrated stock solutions in order to exhibit a similar activity at pH 3 (12-13 U/ml). The activity of all wild-type and mutated laccases decreased in a monotonic manner on varying the pH from 3 to 6 in citrate-phosphate buffer, with very similar profiles. Only slight differences could be noticed. In particular, Glu mutant activity remained systematically lower than all others until pH 5, whereas wild-type laccase exhibited the highest activity, especially at pH higher than 4.5 where the difference became significant. Ala and Asn mutants showed very similar activity over the whole pH range studied.

The effect of the mutations appeared in a much more marked way when we examined the influence of pH on the oxidation of a phenolic substrate, 2,6-DMP. In that case, we observed a bell-shaped profile of the activity as a function of pH for all the laccases tested, whether mutated or not (Figure 6B). Both wild-type recombinant and Asp206Glu mutant laccases showed very similar profiles, with maximum activity at pH 3.4. The only observed slight difference was that, at pH above 3.4, the activity loss of the Glu mutant was more rapid than for the wild-type laccase. When laccase was mutated with an uncharged amino acid at position 206, a shift of the maximum activity towards higher pH values was observed. Compared with the

wild-type laccase behaviour, the most affected mutant was that of Asn, which exhibited a shift of the maximum from 3.4 to 4.8, whereas the Ala mutant showed maximum activity at an intermediate pH value of ~4.

Discussion

The catalytic properties of laccases mutated in position 206 were assayed for their transformation efficiency towards different substrates and for the nature of the transformed products of a ligninolytic compound. The laccase sequence alignment emphasized that in fungal laccases an Asp or Glu residue is found in position 206 whereas Asn is highly conserved in this position in plant laccases. One of the objectives of this work was to test whether this difference could determine the biological function of the enzyme. Indeed, fungal laccases are involved in lignin depolymerization whereas plant laccases would rather participate in the early stages of lignin polymerization. However, preliminary tests with synthetic dimers or tetramers did not show any significant degradation with *T. versicolor* laccase. It is worth mentioning that in vitro assays with fungal laccases give contrasting results (Solomon et al., 1996). As far as the opposite reaction, e.g. the polymerization stage, is concerned, coniferyl alcohol was chosen as a model precursor of lignin in order to test the efficiency of both wild-type and mutated laccases towards the lignin formation process. Accordingly, all the laccases investigated here led to the same dimers and trimers in similar proportions. The only observed difference between the variants studied concerns the conversion rate of the phenolic substrate, which is lowered by the mutations. These results were obtained under conditions very different from natural ones (in the presence of lignin and in synergy with other fungal enzymes and mediators), which do not allow any extrapolation on the in vivo biological function of fungus or plant laccases.

From a more quantitative point of view, the study of the catalytic properties of Asn206-mutated laccases with ABTS as a substrate clearly showed that the replacement of an acidic residue by a non-polar Ala significantly enhanced the efficiency (kcat/Km) of the enzyme by a factor of 15. Enguita et al. (2004) reported the crystallographic structure of an endospore coat laccase from *Bacillus subtilis* in the presence of ABTS. Because of the low occupation factor of the ABTS atoms, the substrate binding site could not be precisely described. However, it was suggested that ABTS binds in a large pocket mainly formed by apolar residues. In such an environment, where interactions between aromatic groups dominate, it is therefore consistent to note that the substitution of Asp206 leads to a catalytic efficiency increase inversely proportional to the substituted residue polarity. This increase in efficiency is due to both an

increase in k_{cat} and to a decrease in K_m , indicating a concomitant increase in the affinity for the substrate and in the kinetics of electron transfer, all the more marked as the amino acid in position 206 is less polar. Further, the contribution of the 206 residue to the pH–activity profiles seems to be very weak since the curves corresponding to the different mutants are very similar. We would nevertheless expect that the replacement of an amino acid carrying a negatively charged lateral chain at a pH above its pK_a (~5 for carboxylic acids) could contribute to reducing the unfavourable electrostatic interactions with a negatively charged substrate (due to the presence of two sulfonate groups in ABTS). However, pH variations could not evidence such an interaction.

The study of the pH dependence of the activity profile of the mutants with 2,6-DMP as a substrate indicated that the nature of the amino acid at position 206 significantly impacts the value of the observed maximum (pH_{opt}). For the Asn mutant, a pH_{opt} shift towards higher pH (of 1.4 units) for the oxidation of 2,6-DMP was observed. For all mutants studied, the activity curve exhibits a bell-shaped profile, as observed previously for phenolic substrates. Xu (1997) postulated that this bell-shaped profile is a consequence of two opposite effects: the ascending part of the curve at acidic pH is generated by the redox potential difference between the reducing substrate and the type 1 copper of laccase and is favoured, for a phenolic substrate, by higher pH. The descending part is generated by the binding of a hydroxide anion to the type 2/type 3 coppers of laccase, which inhibits the activity at higher pH, combined with a decrease in the oxygen reduction potential with increase in pH. The resulting bell-shaped profile of the pH dependence of laccase activity is almost identical for both non-mutated laccase and Asp206Glu mutant, showing that the replacement of the aspartic residue by another carboxylic acid has a negligible influence on the enzyme catalytic properties. However, a significant shift of the maximum activity towards higher pH is observed for the neutral mutants, the shift being greater for Asp206Asn than for Asp206Ala. Interestingly, a pH shift similar to that observed for Asp206Asn was also obtained with a recombinant laccase carrying a double mutation (Asp206Asn and Ser212Cys; data not shown), which reinforces the causal link between this shift and the Asp206Asn mutation. Assuming that the descending part of the bell-shaped pH profile of the pH dependence of activity results from the inhibition of laccase activity by hydroxide binding at the type 2/type 3 site and that this interaction is not affected by the mutations at the 206 position because of its distance from the mutation site, the observed pH_{opt} shift must be attributed to a corresponding shift of the ascending part of the curve. According to Xu's conclusions, a shift towards more acidic pH can be induced by a favoured deprotonation of the reducing substrate. As the pK_a of 2,6-DMP is 9.9, deprotonation of 2,6-DMP does not occur spontaneously at acidic pH. However, the vicinity of a carboxylic residue interacting with the reducing substrate in the active site, such as an

Asp or a Glu residue at the 206 position, is likely to favour its deprotonation. Such a protic event cannot take place when mutating the Asp 206 residue into Ala or Asn, as confirmed by the weak docking of 2,6-DMP with these neutral mutants, compared with Asp 206 and Asp206Glu, as deduced from their respective K_m values (Table II). For coniferyl alcohol, also a phenolic substrate, the transformation rates at pH 4.5 follow the order Asp > Glu > Ala > Asn, which is similar to that observed in the ascending part of the pH activity profile of 2,6-DMP, suggesting that the same behaviour could be generalized to all phenolic substrates.

For 2,5-xylidine, a slightly different transformation efficiency order was observed: Glu > Asp > Asn > Ala. In this case, we suggest that the stabilization of the radical formed after electron transfer to the type 1 copper could account for this result. Indeed, the carboxylate lateral chain at position 206 is likely to provide an electron-rich environment. The amide group of Asn could also provide a stabilization of the radical due to the presence of a negative charge on oxygen atom in its mesomeric form, but is less favourable owing to the concomitant presence of the positive charge on the nitrogen, whereas the Ala mutant lacks electrons able to enhance radical stabilization.

Conclusion

The present work emphasizes the influence of the residue at position 206 on the catalytic properties of fungal laccases. Substrates with different structures and functional groups were tested. For all the substrates, mutation-induced modification of their transformation rate with ABTS remains within the same range, around 3-fold. Ranking the wild-type and the mutated laccases according to their transformation efficiencies depends on the substrate and the results could be explained on the basis of interactions between the substrate and the mutated residue at the catalytic site of the enzyme. The influence of the mutations on the pH-activity profile of laccase with 2,6-DMP showed that the Asn mutation led to a significant shift ($\Delta\text{pH} = 1.4$) of the optimum towards higher pH. The same tendency is expected for other phenolic substrates such as pesticide derivatives and nonylphenol, which are known to be environmental pollutants and so could contribute to an optimized degradation of these molecules in situ in soils at neutral pH.

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Table I. Laccase-expressing integrative vectors used for studying the effects of different mutations in *T. versicolor* laccase codon 206

Plasmid	Codon 206	Amino acid	Characteristics	Genotype	Reference
p69TVLB	GAC	Asp (D)	Acidic residue, negative charge	Wild-type	Jolivalt <i>et al.</i> (2005)
p69TVLB-D206A	GCC	Ala (A)	Small, hydrophobic	Mutant	This work
p69TVLB-D206N	AAC	Asn (N)	Polar, no charge	Mutant	This work
p69TVLB-D206E	GAG	Glu (E)	Larger, acidic residue, negative charge	Mutant	This work

Table II. Kinetic parameters of the *T. versicolor* laccase expressed in *Y. lipolytica* (YL4) and of the mutants at the 206 position^a

Laccase	ABTS as substrate			2,6-DMP as substrate		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \text{mM}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \text{mM}$)
YL4	50	1.3	26	190	0.51	2.7
Asp206Glu	26	1.6	61	140	0.42	3
Asp206Asn	16	3.3	209	3280	0.29	0.09
Asp206Ala	10	4.3	426	1630	0.66	0.4

^aAssays were performed with ABTS and with 2,6-DMP as substrates in 0.1 M phosphate buffer, pH 3 and 3.4, respectively. k_{cat} was calculated versus the total protein concentration in solution.

Table III. Comparison of the extent of transformation of 2,5-xylidine and coniferyl alcohol by native or recombinant laccase from *T. versicolor* and its mutated derivatives

Incubation conditions	Transformation (%)	
	2,5-Xylidine (pH 5)	Coniferyl alcohol (pH 4.5)
Negative control (Po1t strain)	2 ± 1.5	2.9
Native <i>T.versicolor</i> laccase + culture medium	51 ± 3	n.d.
YL4 ^a	58 ± 2	77.5
Asp206Ala	27 ± 2	46
Asp 206Asn	45 ± 2	27
Asp 206Glu	96 ± 4	65

^aYL4: wild-type recombinant laccase expressed in *Y. lipolytica*.

Fig. 1. Orthogonal views of the binding site for xyloidine. Left: view from the entrance of the cavity. Right: orthogonal view obtained by a 90° rotation around a vertical axis. The enzyme residues and the substrate are shown in ball-and-stick form and the copper from the type 1 site (Cu1) as a sphere. Carbon atoms are grey, nitrogen atoms light grey and oxygen atoms dark grey. The 2,5-xyloidine substrate (Xyl) backbone is black. The surface of those residues that contribute to hydrophobic interactions with the substrate is shown. Hydrogen bonds with the nitrogen atom from xyloidine are shown by dotted lines and the coordination of Cu1 with His458 by a black line. The figure was drawn with PyMOL (DeLano, 2002).

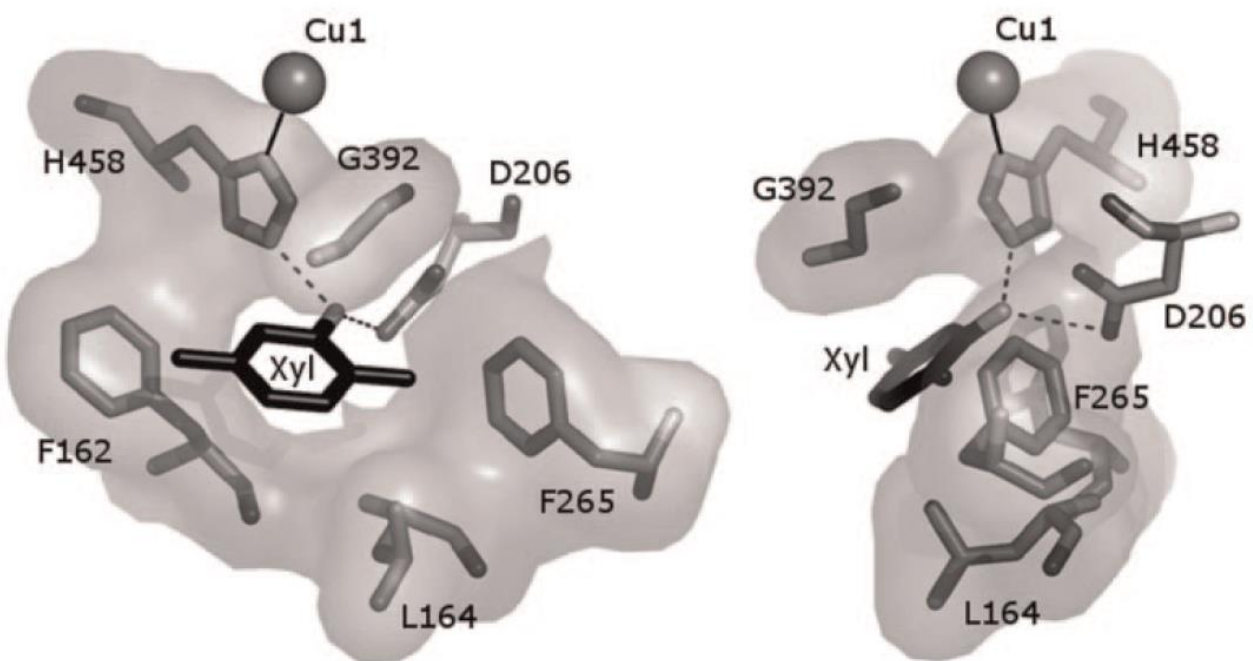


Fig. 2. Multiple sequence alignments of fungus and plant laccases in the vicinity of position 206.

			199				206					216											
Basidiomycetes	<i>Trametes versicolor</i>	R	F	R	L	V	S	L	S	C	D	P	N	Y	T	F	S	I	D	G	H	N	M
	<i>Pycnoporus cinnabarinus</i>	R	F	R	L	V	S	L	S	C	D	P	N	H	T	F	S	I	D	G	H	T	M
	<i>Polyporus ciliatus</i>	R	F	R	L	I	S	I	S	C	D	P	N	F	T	F	T	I	Q	G	H	T	M
	<i>Phlebia radiata</i>	R	F	R	L	V	S	I	S	C	D	S	F	F	T	F	S	I	D	G	H	S	L
	<i>Coprinus cinereus</i>	R	M	R	L	I	S	L	S	C	D	P	N	W	Q	F	S	I	D	G	H	E	L
Ascomycetes	<i>Botryotinia fuckeliana</i>	R	L	R	L	I	N	T	S	V	D	N	H	F	M	V	S	L	D	N	H	A	F
	<i>Fusarium proliferatum</i>	L	L	R	I	I	N	T	S	V	D	T	T	F	V	F	G	I	D	N	H	Y	L
	<i>Gaeumannomyces graminis</i>	R	F	R	I	V	N	T	A	I	D	T	H	F	K	F	G	I	D	N	H	T	L
	<i>Melanocarpus albomyces</i>	R	L	R	I	L	N	T	S	T	E	N	H	F	Q	V	S	L	V	N	H	T	M
	<i>Neurospora crassa</i>	R	L	R	L	I	N	T	S	V	E	N	H	F	Q	L	L	L	V	N	H	S	M
Plants	<i>Nicotiana tabacum</i>	M	L	R	V	I	N	A	A	L	N	E	E	L	F	F	K	I	A	G	H	K	M
	<i>Populus balsamifera</i>	M	L	R	L	I	N	A	A	L	N	E	E	L	F	F	K	I	A	G	H	K	L
	<i>Arabidopsis thaliana</i>	L	L	R	L	V	N	A	A	L	N	E	E	L	F	F	K	V	A	G	H	I	F
	<i>Oryza sativa</i>	M	L	R	L	I	N	A	A	L	N	E	E	L	F	F	A	V	A	N	H	T	L
	<i>Rhus vernicifera</i>	L	L	R	I	V	N	A	V	L	N	E	E	K	F	F	G	I	A	K	H	T	L
	<i>Acer pseudoplatanus</i>	L	L	R	I	I	N	A	I	L	N	E	E	M	F	F	G	I	A	Q	H	N	L

Fig. 3. Structures of laccase substrates used in this work.

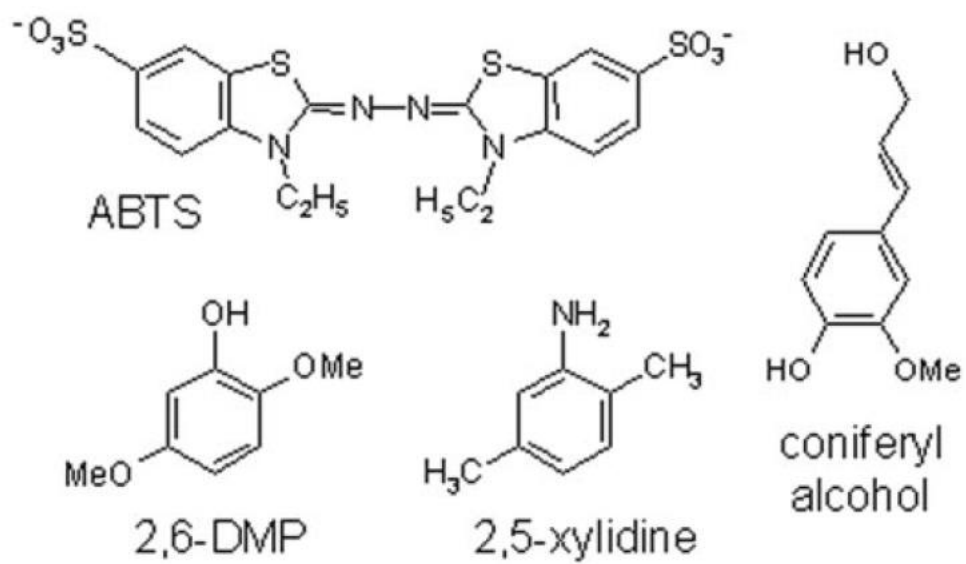


Fig. 4. Time course of laccase activity in the extracellular medium of *Y.lipolytica* cultures producing mutated enzymes. Filled diamond, Asp206Ala; filled square, Asp206Asn; filled triangle, Asp206Glu.

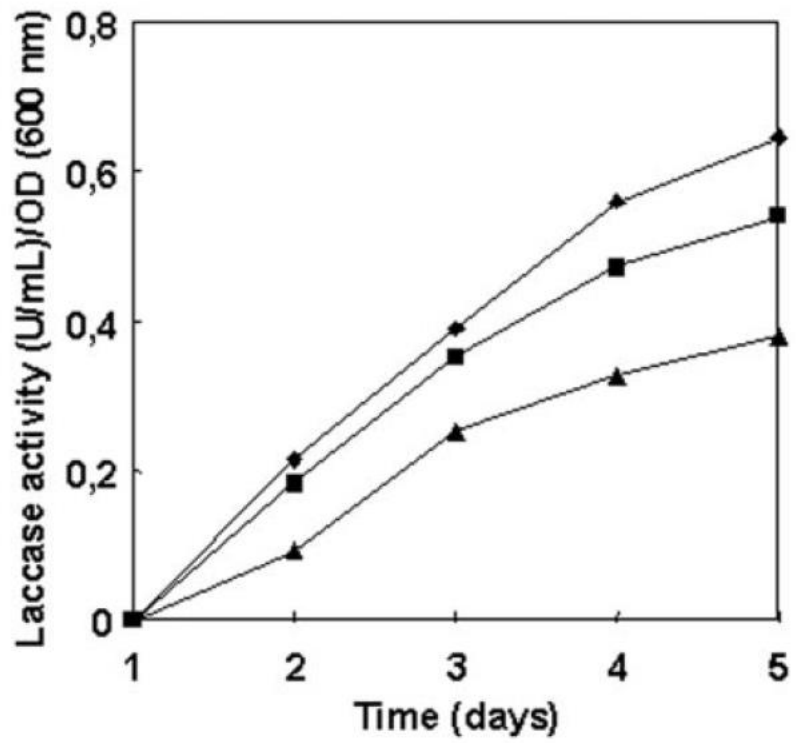


Fig. 5. Chromatographic analysis of coniferyl alcohol transformation by wild-type and mutated laccases expressed in *Y. lipolytica*. YL4, wild-type recombinant laccase expressed in *Y. lipolytica*.

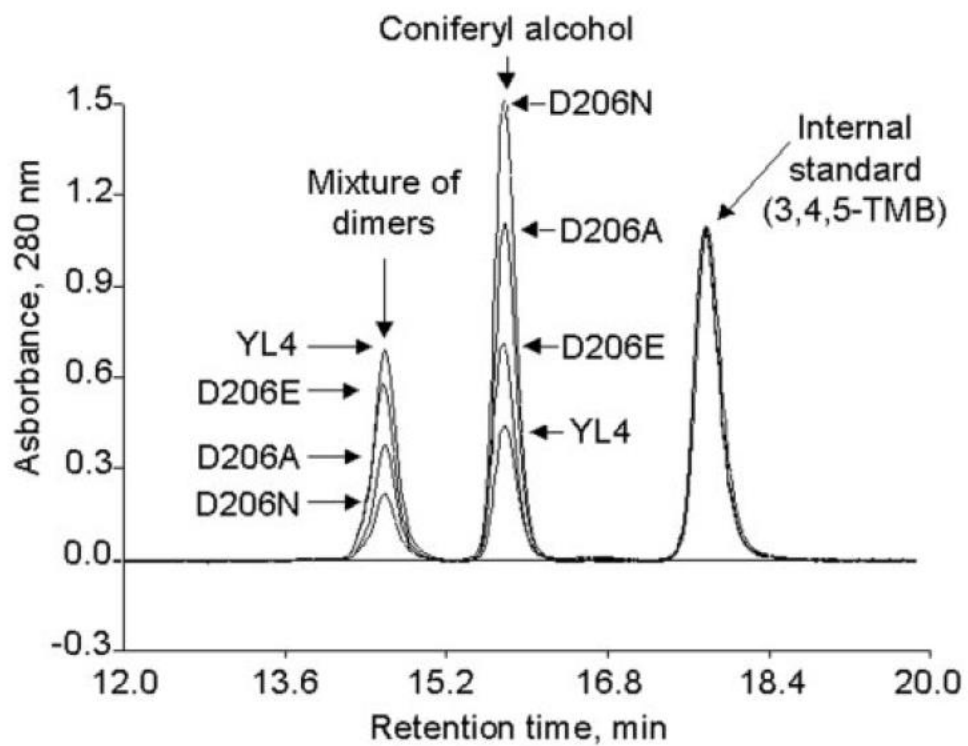


Fig. 6. Activity of wild-type and mutated laccases produced in *Y. lipolytica* as a function of pH, with ABTS (A) or 2,6-DMP (B) as substrates. Open diamond, wild-type recombinant laccase expressed in *Y. lipolytica* (YL4); filled diamond, Asp206Ala; filled square, Asp206Asn; filled triangle, Asp206Glu.

