Environ Chem Lett (2003) 1:145–148
DOI 10.1007/s10311-003-0024-9

ORIGINAL PAPER

Christian Mougin · Claude Jolivalt · Pierre Briozzo · Catherine Madzak

Fungal laccases: from structure-activity studies to environmental applications

Accepted: 21 March 2003 / Published online: 20 May 2003

Abstract Laccases are multicopper oxidases mainly secreted by filamentous fungi. Producing radical forms from organic substrates, they are involved in numerous reactions leading to the degradation and polymerization of xenobiotics. Our studies have led to a better knowledge of the structural, catalytic and genetic properties of laccases and allowed to develop a strategy for their evolution through genetic engineering. Here, we show that fungal laccases, wild or engineered, may be potent tools to develop bioremediation processes of soils polluted by organic compounds, and assays to assess the ecotoxicological impact of these pollutants on soil fungi.

Keywords Fungal laccases · Xenobiotics · Biotransformation · Bioremediation · Ecotoxicological risk assessment · Genomics · Proteomics

Introduction

Laccases, EC 1.10.3.2, *p*-diphenol:dioxygen oxido-reductases, are a large group of multicopper oxidases produced by plants (*Rhus vernicifera*), insects (*Bombyx* sp.), bacteria (*Azospirillum lipoferum*). They also occur widely in several species of filamentous fungi, including the white-rot strain *Trametes versicolor*. The laccase of the lacquer tree was first described 120 years ago, then enzymes from plants and fungi have been extensively studied. Molecular weights, pH optima, substrate specificity and several other properties suggest that there is a great diversity of laccases. Their occurrence, characterization, functions and applications have been reviewed a number of times in recent years (Gianfreda et al. 1999; Mayer and Staples 2002).

Our objectives were to understand the biotransformation mechanisms involved in fungi using laccases, and to develop tools for environmental applications. These applications are (1) the bioremediation of polluted media and (2) the assessment of ecotoxicological impact of xenobiotics on terrestrial environments. Nevertheless, an efficient use of laccases for biotechnological and environmental applications requires additional studies to better understand the properties of the enzyme at a structural or genetic molecular level, and at a kinetic level, assessing the mechanism of action and reactions. We summarize in this minireview the results obtained by our research group during the last three years concerning the enzymes produced by the white-rot basidiomycete *T. versicolor*.

Structure and mechanism of action

Laccases are extracellular glycoproteins containing 4 atoms of copper, which are distributed into three sites (T1, T2, T3, Fig. 1) according to their spectroscopic properties. The T1 site contains the type 1 blue copper (Cu1), which coordinates a cysteine, and is responsible for the blue color of the enzyme. The T2 site contains a type 2 copper (Cu2). In the T3 site, Cu3a and Cu3b are strongly coupled. Laccases usually comprise 520–550 amino acids, with a molecular weight ranging from 60–80 kDa. They are produced in the cells, and they are secreted and mainly accumulate in the outside of the hyphal filaments.

Laccases occur widely in fungi as constitutive and inducible forms. We purified from *T. versicolor* cultures a

C. Mougin (✉)
Unité de Phytopharmacie et Médiateurs Chimiques,
INRA, Route de Saint-Cyr, 78026 Versailles Cedex, France
e-mail: mougin@versailles.inra.fr
Tel.: +33-1-30833102, Fax: +33-1-30833119

C. Jolivalt
Laboratoire de Synthèse Sélective et Produits Naturels,
UMR CNRS 7573, ENSCP,
11 rue P. et M. Curie, 75231 Paris Cedex 05, France

P. Briozzo
Laboratoire de Chimie Biologique,
UMR 206 INRA-INAPG, 78850 Thiverval-Grignon, France

C. Madzak
Laboratoire de Génétique Moléculaire et Cellulaire,
INRA-INAPG, 78850 Thiverval-Grignon, France

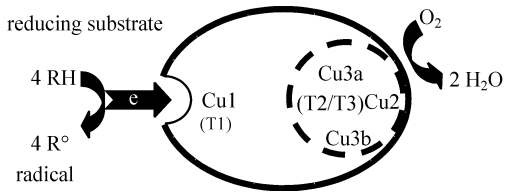

Fig. 1 Schematic mechanism of action of fungal laccases

laccase (LacIIIb) induced by the arylamine xylidine (2,5-dimethylbenzeneamine). Isoelectric focusing experiments on purified LacIIIb indicated five molecular isozymes of acidic pI, whose molecular weights were around 59 kDa for 494 amino acids. Last year, we crystallised the most abundant isozyme in its native form, and solved its crystal structure. The four expected copper atoms were present, and the enzyme was complexed with xylidine, a reducing substrate (Bertrand et al. 2002). This first high-resolution structure confirmed the four glycosylation sites of the protein, and, more interestingly, made it possible to study substrate recognition and correlation with kinetics. Several amino acid residues participate in hydrogen bonding with the aromatic ring of the ligand. The first one is a histidine (H458) which also coordinates the copper Cu1 that functions as the primary electron acceptor. The second one is an aspartate (D206) conserved among fungal laccases. Our results provide a model for engineering laccases that are either more efficient or with a wider substrate specificity. The structure of the active site of the enzyme appears conserved among all fungal laccases, but there seems to be a great divergence in the rest of the protein structure, and in the glycosylation rate. From one basic reaction, laccases catalyze a great number of oxidations affecting both natural and synthetic compounds.

In 1997 Messerschmidt published a book focusing on the enzymology, kinetics and mechanisms of action of copper oxidases. In a first step, a reducing substrate binds to the active site and it is transformed into radical forms. Then, the mononuclear T1 site extracts electrons from the reducing substrate and mediates their transfer to the trinuclear T2/T3 site where molecular dioxygen is reduced to water (Fig. 1). Finally, the radical compounds formed from the substrate are released. They are very reactive and able to interact with themselves or with other compounds present in the vicinity, thus inducing numerous reactions. An interesting property of most of fungal laccases concerns their high redox potential, amounting to 0.7 V (versus normal hydrogen electrode).

Xenobiotic transformation by fungal laccases

According to the species and to the great number of reactions they could catalyze, laccases are involved in numerous physiological processes or applications. Fungal enzymes are present as key proteins responsible for the biodegradation of lignocellulosic materials, the pigmentation process of spores, as well as morphogenesis and pathogenesis. They play also an important role in soil humus synthesis. In higher plants, laccases are involved in lignification of cell walls and regeneration of protoplasts. According to their biochemical mode of action, laccases have been studied for many years for applications in the textile, dye and food industry. Recent research has also dealt with pulp paper bleaching and environmental applications.

It has been known for two decades that wild laccases catalyze the direct oxidation of phenols and amines such as chlorophenols and dyes to produce oligomers. Our objective was to show the involvement of these oxidases in the transformation of other xenobiotics of actual environmental concern, e.g. pesticides, industrial and domestic pollutants. In this context, we demonstrated the involvement of laccases purified from *T. versicolor* or *Pycnoporus cinnabarinus* cultures to transform oxidized forms of phenylurea herbicides. According to the pH of the incubation medium, the hydroxylated fenuron is converted to oxidized products: *p*-benzoquinone (pH 3) and *N*′,*N*′-dimethyl-*N*-[(2,5-cyclo-hexadiene-1-one)-4-ylidene] urea (pH 6) (Jolivalt et al. 1999).

Laccases can catalyze the direct oxidative coupling of other xenobiotics. For example, the surfactant nonylphenol, which is an endocrine disrupter found widely in aquatic ecosystems, is efficiently converted by the enzyme into polymeric compounds in liquid media (Dubroca et al. 2003).

Beside these direct reactions, laccases catalyze the indirect oxidation of chemicals that are not phenols or amines. In that case, they need the presence of a redox mediator, which can be of natural or synthetic origin. The mediator is intended to promote or facilitate enzyme action by increasing its oxidation potential. A famous exogenous mediator is 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS). During the catalytic cycle, the mediator is activated by the enzyme to species reacting with the xenobiotic. This mode of action is observed with the pesticide isoxaflutole (Mougin et al. 2000) or polycyclic aromatic hydrocarbons (Rama et al. 1998; Mougin et al. 2002a). In the first case, the herbicide undergoes an oxidation leading to the cleavage of the parent compound. In the case of hydrocarbons, transformation products are mostly diones.

Genetic insights into fungal laccases

Wild laccases are not always suitable for industrial purposes. For example, they suffer from several limits: (1) in some cases, their activity needs the presence of a redox mediator, (2) their pH of activity is acidic and does not correspond to environmental conditions. Genetic engineering technology may provide recombinant enzymes with wider substrate specificity, higher oxidation potential, and neutral pH of activity.

The first step in genetic manipulation requires the cloning of the genes encoding laccases. Numerous fungal

laccases genes have been cloned today and the sequences deposited in gene banks. The gene for our laccase form (accession number AF414109) comprises 1563 bases (manuscript in preparation). After amplification in *Escherichia coli*, the gene was inserted into a vector and expressed in the heterologous host *Yarrowia lipolytica*. This yeast appeared to be a potent tool to produce high amounts of laccase, further characterized by mass spectrometry (manuscript in preparation).

Structural data presented above concerning the active site of the laccase allowed the development of a strategy for laccase improvement. Several mutants have been designed and expressed in *Y. lipolytica*. Their characterization is currently in progress. This first generation of mutated enzymes will allow us to better understand enzyme/substrate interactions. In the next stage, a second generation of enzymes could provide laccases with a high affinity for selected environmental pollutants. Then, according to the structure of the pollutants to be transformed and to the media to be remediated, we will have the choice between natural or improved laccases.

Bioremediation of polluted media

Wild or engineered laccases can be used to develop bioremediation processes of media polluted by organic compounds. These processes may imply either whole fungi producing enzymes of interest or purified enzymes immobilized onto various types of carriers.

We developed solid carriers to inoculate *T. versicolor* in a soil from a manufactured gas plant site, which was heavily polluted by polycyclic aromatic hydrocarbons (PAHs) and heavy metals (Rama et al. 2001). Pelleted wheat bran carriers were very efficient in stimulating the growth of the fungus, which secreted high amounts of laccases during the first weeks of soil treatment. The enzymes were used as markers of metabolic activity. Forty-three percent of initial extractable PAHs were degraded during the 50-week soil treatment in the presence of neutral surfactant. Laccases immobilized onto montmorillonite have also been recently described as potent tools to transform and stabilize pollutants in soils.

We also studied the application of laccase to the removal of the herbicide derivative, *N′,N′*-dimethyl-*N*-(2-hydroxyphenyl) urea from waste water in a microfiltration module (Jolivalt et al. 2000; Fig. 2). For that purpose, laccase purified from *T. versicolor* was covalently immobilized onto a hydrophilic PVDF microfiltration membrane. The hebicide derivative was transformed via an oxidation reaction catalyzed by the enzyme into an insoluble compound, which was simultaneously separated by filtration through the membrane.

The resulting compound was an insoluble dark purple precipitate, resulting from the polymerization of the phenylurea. The kinetics of the reaction depended on the grafting rate of the membrane.

Development of engineered laccases may greatly improve the efficiency of bioremediation processes.

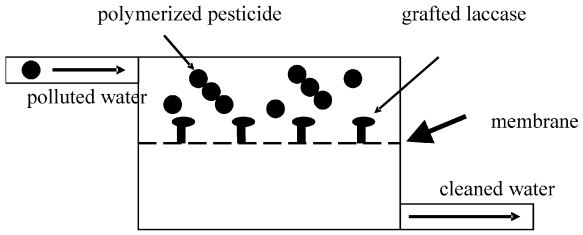

Fig. 2 Scheme of the module used to remove pesticide derivatives from water

Nevertheless, components from heavily polluted soils, such as metals, are able in some cases to interfere with the laccase-mediated oxidation of organic pollutants (Mougin et al. 2002a). A additional studies are needed to minimize such interference.

Assessment of ecotoxicological impact

Assays available today for ecotoxicological risk assessment underline the need to develop new methods specific to soil micro-organisms. Toxicogenomics attempt to define the relationships between regulation and expression of genes with exposure to a chemical. As fungi make up most of the biomass in the soil and play an important role in biogeochemical cycles, they constitute pertinent models for this purpose. Fungal functional genomics can be applied to risk assessment by providing high-throughput tools spotting sequences of genes that can be induced by specific soil contamination. Then, expression profiles of genes can be used to detect and classify the contamination.

As large amounts of laccases are required for industrial use, methods to enhance their production have been studied. The production of enzymes often depends on the growth conditions of the fungus, including nutrient availability, but also presence of inducers of natural or synthetic origin. In this context, the inducing effect of xylidine was established several decades ago and we routinely use that chemical to produce high amounts of enzyme. Other known inducers include mainly phenolic compounds related to lignin or lignin derivatives (Gianfreda et al. 1999).

We showed for the first time that laccase production from *T. versicolor* could be induced following treatment of fungal cultures with xenobiotics of environmental interest, including agrochemicals, industrial compounds or their derivatives (Mougin et al. 2002b). The most efficient compounds were 4-*n*-nonylphenol, aniline, transformation products of the herbicides diquat, *N′,N*-dimethyl-*N*-(5-chloro,4-hydroxyphenyl)urea and 9-fluorenone.

DNA microarrays become one of the tools of choice for gene expression profiling, by giving a snapshot of the gene transcriptional responses at a particular time and within a particular tissue. Laccases and other exocellular enzymes secreted by fungi may provide a basis for DNA

microarray applications. The most common application of these techniques is to determine patterns of differential gene expression or to compare differences in mRNA expression levels between cells subjected to different treatments. Nevertheless, the environmental relevance of fungal mRNA responses needs to be completed by a proteomics approach, intended to clearly identify the highly or newly expressed proteins.

We established for the first time that laccases are induced by transformation products formed from pollutants being their substrates. For that reason, an assay based on laccase production may be useful to assess the impact of compounds formed through bioremediation processes.

Conclusion

Fungal laccases are very useful enzymes able to catalyze many reactions leading to numerous functions and applications. The most recent results obtained by our group and others in the world will allow the efficient improvement of these oxidases for environmental applications.

Acknowledgments The authors thank Annick Lacombe (INRA, Jouy en Josas) for attentive reading of the manuscript.

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