Phylogenetic and biochemical characterisation of a recombinant laccase from *Trametes versicolor*

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Abstract

Laccases are important enzymes for bioremediation and the best-characterised are from the fungus *Trametes versicolor*. Here, we describe the cloning and characterisation of a new variant of laccase from *T. versicolor* and its expression in *Saccharomyces cerevisiae*. We have performed a sequence-based analysis of *Trametes* laccases that leads to a proposal for a new nomenclature of this fungus laccases according to their phylogenetic relationships since their nomenclature based on IPs is ambiguous. We also describe the kinetic properties of the recombinant enzyme.

Keywords: Laccase; Phylogenetic analysis; *Trametes*; Yeast heterologous expression

1. Introduction

Laccases are multicopper oxidoreductases (benzene-diol:oxygen oxidoreductases, E.C. 1.10.3.2) capable of oxidising several phenolic and non-phenolic compounds, including industrial dyes, polycyclic aromatic hydrocarbons (PAHs), pesticides and alquenes [1–3]. Laccases have been identified in all groups of organisms and their natural roles embraces lignin biosynthesis in plants, melanin biosynthesis in bacterial spores, lignin degradation and pathogenesis in fungi, and esclerotisation in insects [3–7]. The reactions performed by laccases include ruptures of alkyl-aryl bonds, benzylic alcohol oxidation, and rupture of aromatic rings generating a wide variety of oxidised phenolic compounds [8]. In addition, *in vitro* studies have also shown that laccases are capable of polymerisation, depolimerisation, methylation and demethylation reactions as well as oxidation of α- and p-diphenols, aminophenols, polyphenols, polyamines, aryl-amines and several other phenolic compounds [9,10].

In particular, fungal laccases comprise a monophyletic branch of copper-binding oxidoreductases [11] and have been thoroughly studied, including their three-dimensional structure by means of X-ray crystallography [12–15]. A comparison among fungal laccases has shown conserved regions in which histidine residues are abundant and important to bind four copper atoms that are essential for the enzymatic activity. The copper...
atoms are involved in the one-electron transfer from a reducing substrate towards molecular oxygen with its eventual reduction to water [16,17].

The enormous potential of fungal laccases for various environmental and industrial applications has been recognised [1,3,18]. Among the best-characterised laccases are those from the white-rot fungus *Trametes versicolor*. Earlier works have shown that the *T. versicolor* laccases are capable of degrading PAHs, polychlorophenols, anthracene, benzo[a]pyrene and other phenolic compounds [19–24]. Usually the degradation studies with *Trametes* laccases employ a mixture of at least two isoforms making it difficult to analyse the individual contribution of each one of them to a certain specificity or activity.

Analysis of individual recombinant laccase isoforms from *Trametes* has been achieved elsewhere. Larson et al. [25] expressed the *lcc2* gene in *Saccharomyces cerevisiae* in order to construct a strain resistant to phenolic fermentation inhibitors present in lignocellulose hydrolysates. Other attempts include expression of *lcc2* at low temperatures [26] and expression of *lcc1* in *Pichia pastoris* [27]. In this work, we present the isolation of a variant of a laccase gene from *T. versicolor* strain UAMH 8272. This laccase was expressed in *S. cerevisiae* in order to characterise its biochemical parameters. Furthermore, we performed a phylogenetic analysis of the published *T. versicolor* laccase sequences and, based on our findings, we propose a new nomenclature and classification system for laccases from *T. versicolor*.

2. Materials and methods

2.1. *Trametes versicolor lcc1* gene amplification and cloning

In order to amplify the *T. versicolor* laccase gene, two oligonucleotides with sequences: 5'-ATGCCATGGG-TCTGCAGCGAT-3', (LCC5') and 5'-GACTCGTCC-GATTGGTCACTTCGAGTTCGAACC-3' (LCC3') were designed based on the homologous sequence reported under the gi number 1172163 [28]. Total RNA was isolated using Triazol (Gibco) from liquid cultures of *T. versicolor* UAMH 8272 grown for 10 days at 28 °C and 180 rpm in a glucose, malt and yeast extract medium (as described by Pickard et al. [2]), with the 1% addition of a stock solution containing: 30 mM CuSO4, 13.2 mM MnSO4, 4.8 mM ZnSO4 and 12.1 mM CoCl2. Single-stranded cDNA was synthesised with total RNA preparations using the Super Script II enzyme (Invitrogen) and a polydT oligonucleotide. In order to amplify the target sequence, samples of the cDNA preparation were used as a template under the following reaction conditions: a 3 min “hot-start” at 94 °C then 40 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and a final extension time of 10 min at 72 °C. The amplification reaction was prepared in a volume of 50 μl and included Expand PCR buffer 1× (Roche), 0.2 mM dNTPs, 2 mM MgSO4, 1 μg of each primer, 10% glycerol as reported by Lu and Negre [29], and 2 μl of the Expand polymerase (Roche).

The amplification product (1580 bp) was cloned into plasmid pBluescript KS (Stratagene) after digestion with *XbaI* and *SstI* generating plasmid pRN001. *Escherichia coli* DH5α competent cells were transformed with the ligation reaction and positive clones were grown on selective LB media containing 50 μg ampicillin/ml. This construction was sequenced to confirm the identity of the amplification product using the universal KS and SK primers as well as two internal primers: 5'-GCAGACCGGCACAGGAACT-3' (LCC1INT1) and 5'-CCCCAGGTTCACCTCGGC-3' (LCC1INT2). The sequence of the laccase gene isolated for this project was deposited in Genebank database under Accession No. AY693776.

2.2. Cloning of the laccase cDNA in a yeast expression vector

Once the identity of the cloned amplification product was confirmed by sequencing, the laccase cDNA was subcloned from the pKS vector into the pSAL6 yeast expression vector [30] using the *NcoI* (5') and *SstI* (3') restriction sites. This procedure gave rise to an expression vector in which the laccase cDNA was expected to be under the control of the copper-induced *CUP1* promoter (plasmid pRN002). *S. cerevisiae* W303-1a [31] cells were transformed using a lithium acetate method [32] and positive clones were selected by growth in minimal media without histidine. Laccase production was induced by adding CuSO4 to the growth medium at different concentrations, from 100 μM to 1 mM. The ability of the recombinant *S. cerevisiae* cultures supernatant to oxidise ABTS [2,2' -azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt] was determined spectrophotometrically at 436 nm (ε436nm = 29,300 mM⁻¹ cm⁻¹) using 0.5 mM of the substrate in 100 mM acetate buffer pH 4.5. The typical reaction mixture contained 100 μl of supernatant in a 1 ml reaction.

2.3. Laccase activity determination

*Saccharomyces cerevisiae* W303-1a cells harbouring either plasmids pSAL6 or pRN002 were cultured for three days in a 10 L-stirred tank using SD medium plus the appropriate supplements [33] at 19 °C, 260 rpm and 0.3vvm. Laccase activity was induced by the addition of 400 μM CuSO4. Approximately 8 l of the culture supernatant were recovered after elimination of the cellular material by centrifugation, precipitated with 70% ammonium sulphate at 4 °C and centrifuged. Precipi-
tated protein material was dissolved in 10 mM phosphate buffer pH 5 and dialysed overnight against 40 volumes of the same buffer. A final concentration step was performed by ultrafiltration using a 3 kDa membrane (Amicon) until a 3 ml volume was reached. Laccase activity was determined spectrophotometrically by monitoring the oxidation of 0.5 mM 2,6-dimethoxyphenol ($\epsilon_{468\text{ nm}} = 14,800 \text{ M}^{-1}\text{ cm}^{-1}$), 0.14 mM ABTS ($\epsilon_{436\text{ nm}} = 29,300 \text{ M}^{-1}\text{ cm}^{-1}$) or 1 mM tetramethylbenzidine ($\epsilon_{655\text{ nm}} = 39,000 \text{ M}^{-1}\text{ cm}^{-1}$) at room temperature in air saturated buffer. One unit was defined as the amount of enzyme that catalyses the appearance of 1 μmol of product per minute. Catalytic constants were derived by fitting the experimental data onto the Michaelis–Menten model using the EnzFitter Software (BIOSOFT).

3. Results and discussion

3.1. Isolation and cloning of the T. versicolor laccase gene

Total RNA from cultures of T. versicolor UAMH 8272 was purified and used to synthesise single-stranded cDNA as described in Section 2. These cDNA preparations were used to amplify the laccase gene using specific primers designed to recognise the two ends of the gene reported by Ong et al. [28]. The amplification product was of the expected size of 1580 base pairs, and gave the expected pattern after digestion with restriction enzymes. The laccase protein product predicted from the sequence obtained from construction pRN001 was compared with other T. versicolor laccase sequences available using the Clustal_X program [34] (Fig. 1). As can be observed, sequenceAY693776 presented significant similarity to known laccases, including the catalytically important histidine residues.

The phylogenetic relationship among the aligned sequences was inferred using the Neighbour joining option of the Clustal_X program (Fig. 2). All T. versicolor sequences clustered into a single group close to sequence 2833237 from Pleurotus ostreatus [35], and are only distantly related to the outgroup sequence 1346406 from Neurospora crassa [36]. This is consistent with the relative relationship previously observed for these taxa [11]. The internal arrangement within the T. versicolor group consisted of four different branches: while the sequence isolated in the present work (AY693776) clustered with the sequence used for the primer design (1172163), and with sequences 15617226 [37] and 23200086 [28], the other T. versicolor sequences formed independent branches. While the sequences within each subgroup shared at least 97% identity and might represent allelic variants, sequence identity among groups was approximately 70%. The biological significance of this arrangement is confirmed by their isoelectric point (IP) prediction, since all laccases with higher IP values (5.9–6.7) clustered within a single group while the rest of the sequences with IP values ranging from 4.1 to 4.7 distributed among the other three groups. The IP of Trametes laccases has been commonly used as a criterion by which two groups of isoenzymes are recognised based on their chromatographic elution profiles [26,38]. However, this classification system is inconsistent because sequences which are not so closely related share similar IP values (Fig. 2 and Table 1). Furthermore, the classification system has been complicated by a confusing assignment of individual laccase isoform and group names (see Table 1).

In fact, a review of the literature on the laccases from T. versicolor reveals incongruence in the nomenclature used to describe the laccase(s) characterised by each report. The establishment of a common nomenclature for these enzymes would, among other things, facilitate the interchange of information, and the comparison of results in distinct reports. Currently, the lack of concordance is perhaps due to the fact that there does not appear to be clarity as to the exact number of laccase isoenzymes found in this fungus. This can be further complicated by the appearance of several isoforms of a particular isoenzyme due to differences in glycosylation patterns [13]. An alignment of the ten T. versicolor laccase sequences reported in the Genebank database to date reveals that they share between 68% and 99% identity (Fig. 1) but makes it difficult to determine whether they are distinct enzymes or if the differences are due to the natural variation of single alleles. It is only after a phylogenetic analysis that one can clearly see that these ten laccase sequences cluster into four independent branches (Fig. 2). This is consistent with previously reported data from southern genomic analysis showing that laccases from T. versicolor are encoded by at least four genes [39]. On the basis of experimental evidence and our phylogenetic analysis, we propose a standardisation of the current nomenclature, with the unambiguous assignment as isoenzymes groups $\alpha$, $\beta$, $\delta$ and $\gamma$. This would provide a reference point upon which “new” laccases, isolated from T. versicolor in future experiments, could be classified and named accordingly (Table 1).

Therefore, the sequence-based classification of T. versicolor laccases is far more robust than the arbitrary assignment based on relative chromatographic elution methods, and may be used to distinguish different laccase isoenzymes despite similarities in IP values.

3.2. Heterologous expression of T. versicolor lcc$\alpha$ in S. cerevisiae

After the laccase cloned in the present work was confirmed by sequence comparison (see Figs. 1 and 2), this gene was cloned into the yeast expression vector pSAL6.
under the transcriptional control of the copper-inducible promoter CUP1 and a selection marker that complements a histidine auxotrophy [30]. S. cerevisiae strain W303-1a was transformed with the recombinant pRN002 expression vector and selected in medium without histidine.

Fig. 1. Multiple alignment of Trametes versicolor laccase sequences. Catalytically important residues are shown in bold. Shading denotes sequence similarity. Number AY693776 corresponds to the sequence reported in this manuscript. Number 1346406 corresponds to the N. crassa laccase sequence while 2833237 is the P. ostreatus sequence. The rest of the accession numbers are T. versicolor sequences and its equivalence is shown in Table 1.
The induction of the laccase activity in the recombinant S. cerevisiae cultures was enhanced using different amounts of CuSO₄, resulting 400 μM to be the optimal concentration (data not shown). At higher CuSO₄ concentrations, the growth of S. cerevisiae was adversely affected, apparently by copper intoxication. As shown in Fig. 3, once cultivation temperatures were adjusted to 19 °C, as recommended by Cassland and Jönsson [26], it was possible to detect low but significant activity levels of the recombinant laccase secreted to the medium by the S. cerevisiae transformants. In contrast, the supernatant from cultures transformed with the null vector (pSAL6) did not present measurable activity levels. Since the highest levels of laccase activity obtained were measured between the third the fourth days after inoculation, these conditions were followed for the medium-scale production of recombinant laccase.

The recombinant laccase α was processed from the supernatant of medium-scale cultures as described in Section 2, yielding preparations with total protein contents of 200 and 40 μg ml⁻¹ for the recombinant and control S. cerevisiae strains, respectively. The enzyme preparation was used for the estimation of the recombinant laccase affinity (K_M) values towards different...
substrates, as shown in Table 2. As far as we know, our study reports for the first time the catalytic parameters of a heterologously expressed laccase. Comparison with $K_m$ values reported for native *Trametes* laccase isoenzymes [40] shows that the recombinant laccase had five times more affinity for ABTS.

The processed enzyme preparation was used for the laccase specific activity determination using three different substrates. The oxidative activity of the concentrated supernatant from the recombinant strain was significantly higher than the endogenous activity found in the control strain supernatant (Table 3). Using ABTS as a substrate, the specific activity of laccase expressing transformants was 57 times higher than that detected in the control transformants. Interestingly, we found a discrepancy of almost 6 orders of magnitude (864,000 ×) between the measurements from untreated and concentrated extracts. This cannot be entirely explained by the ~3000× concentration of the supernatant (from 10 L to 3 ml), and must be attributed to another variable, perhaps the presence of strong laccase inhibitors in the supernatant. This may be one of the reasons that several authors report difficulties in obtaining high levels of heterologous laccase activity [41–44]. Altering culture conditions such as pH [44], temperature [26,43], and aeration [25] resulted in improved heterologous laccase activity, however, not greater than one order of magnitude. In contrast, our study reports an increment of almost six orders of magnitude and shows promising results for the use of the *Trametes* laccase α reported in the present study in future bioremediation applications.

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