Enhancement of minor laccases production in
the basidiomycete *Marasmius quercophilus* C30

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Abstract

The white-rot fungus *Marasmius quercophilus* C30 is able to produce several laccases. The proportion of the enzymes produced depends on culture conditions. On malt medium, LAC1 was produced continuously over the 14 days of the cultivation period and was the only activity detectable. Copper increased total laccase activity by a factor 10 and induced the transient expression of one or more extra laccases in the culture medium. A combination of copper and *p*-hydroxybenzoic acid made it possible to extend the expression of induced laccase activities over the cultivation period and to reach a maximum activity 30 times higher than in non-induced culture. Extracellular laccases produced in this last condition were eluted as four peaks on an anion exchange column and were partially characterized. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Laccases (*p*-diphenol oxidase, EC 1.10.3.2) are polyphenol oxidases that catalyze the oxidation of phenolic compounds with a concomitant reduction of oxygen to water. They are typically glycoproteins containing four atoms of copper per molecule and are widely distributed in plants, fungi, insects and bacteria [1]. In nature, laccases are involved in many functions including plant cell wall biosynthesis, lignin degradation, detoxification, sclerotization and virulence. Because of their remarkably high oxidative ability, laccases have received a lot of attention and can be potentially used in industrial processes such as the bleaching of paper pulp [2] or the purification of aromatic wastes in effluents [3].

Ligninolytic organisms like white-rot fungi are the best known laccases producers and the major source of these enzymes. Multiple laccase forms are frequently found in their culture supernatant. As shown recently in *Rhizoctonia solani* [4] for example, such a multiplicity results from the expression of gene families rather than a post-translational mechanism producing variants of the same gene. The regulation of laccase genes differs from organism to organism as the expression of some genes is probably constitutive whereas in other cases it is sensitive to culture conditions.

*Marasmius quercophilus* is a white-rot basidiomycete that colonizes the decaying litter of the evergreen oak (*Quercus ilex* L.) [5], a plant characteristic of Mediterranean forests. The C30 isolate produces several laccases among which LAC1, the major one, has been purified and fully characterized [6]. Low expression levels of other laccases, which could have a specific activity between one and two orders of magnitude higher than that of LAC1, have been mentioned in earlier studies [5,7]. Our objectives in the present study were to find appropriate conditions to reach a high laccase production and selectively enhance the synthesis of minor, potentially highly active forms. It has been shown here that a combination of CuSO4 and...
*p*-hydroxybenzoic acid stimulates the synthesis of multiple laccases in *M. quercophilus* C30 thus distinguishing at least three active fractions in addition to LAC1 on an anionic exchange column. Amino-terminal sequencing and electrophoretic behavior of laccases contained in these fractions confirm that *M. quercophilus* is able to produce at least four different laccases simultaneously under the conditions tested.

2. Materials and methods

*M. quercophilus* C30 was isolated and propagated as previously described [5,6]. Three 250-ml Erlenmeyer flasks containing either 50 ml of MET (2% malt extract+Tween 80, 0.05%), 50 ml of METCu (MET+CuSO₄ 5 mg l⁻¹) or 50 ml of METCu-pHB (MET+CuSO₄ 5 mg l⁻¹+*p*-hydroxybenzoic acid 250 mg l⁻¹) were inoculated with 2 ml of mycelial suspension and incubated at 28°C on a reciprocal shaker (50 rpm). After 8 days of incubation, mycelia were separated from the extracellular fluids by filtration and analyzed separately. Media were concentrated 10 times by ultrafiltration using YM30 membranes (Amicon, Millipore, Bedford, MA, USA) before electrophoretic analysis. To analyze intracellular laccases, the mycelium was frozen in liquid nitrogen and ground in a mortar and pestle. The powder was further homogenized with an Ultra Turrax T8 (IKA Labortechnik, Staufen, Germany) in cold phosphate buffer (20 mM pH 6.0) and cellular debris was then eliminated by centrifugation at 4°C for 5 min at 15,000 rpm.

The time course of laccase production was established using triplicate cultures. Three sets of 14 1-l Erlenmeyer flasks containing 200 ml of MET were inoculated with 2.5 ml of mycelial fragments and incubated at 28°C on a reciprocal shaker (50 rpm). After 72 h, CuSO₄ (5 mg l⁻¹) was added to two sets of flasks and, 24 h later, *p*-hydroxybenzoic acid was added to one of these sets. Flasks were harvested each day over a period of 14 days. Mycelium was separated from extracellular fluids, washed with distilled water, filtered on glass fibers filter (Whatman GF/D), dried for 4 days at 115°C and weighed. For each culture filtrate, the protein concentration and the laccase activity were determined. Subsequently, laccase composition of the daily harvest was analyzed on native gel electrophoresis for all three conditions tested.

Anionic chromatography was performed on an 8-day-old METCu-pHB liquid culture (2 l) as previously described [6]. Fractions containing laccase activity were concentrated and analyzed by electrophoresis. Determination of the protein concentration, syringaldazine oxidation tests and SDS-PAGE analysis were performed as described previously [6]. Proteins were stained with Coomassie brilliant blue G250 (Sigma, St. Louis, MO, USA). Laccases were separated by electrophoresis on 7.5% polyacrylamide gel in native conditions or by isoelectric focussing on a polyacrylamide gel (Servalytes Precote, Serva, Boehringer Ingelheim, Heidelberg, Germany) with a pH gradient from 3 to 6. Laccase activities were detected by incubating the gels at 25°C in 0.2 M acetate buffer containing 0.2% of *p*-phenylenediamine, at pH 3.6 or 5.2. After separation on SDS-PAGE and transfer on polyvinylidene fluoride (PVDF) membrane, Ponceau red-stained bands were excised and N-terminal peptide sequences were determined by stepwise Edman degradation. Dried gels were scanned with an Agfa Snapscan® 1236 piloted with Fotolook® 2.09.6 software. Legends were added with Canvas® 3.0.6 software.

3. Results

Total laccase activity from 8-day-old *M. quercophilus* C30 cultures increased from 40 U l⁻¹ in non-induced culture to 410 U l⁻¹ in METCu and to 1300 U l⁻¹ in MET-Cu-pHB. Intra- and extracellular laccases were separated on a native PAGE gel using purified LAC1 as standard and revealed by in-gel *p*-phenylenediamine oxidation.

When copper was omitted, LAC1 was the only laccase detected in the extracellular fluid (Fig. 1, lane 1) whereas no laccase activity was found in the corresponding mycelium extract (Fig. 1, lane 4). In the presence of copper, a second group of bands migrating closer to the cathode was detected in the lanes corresponding to the extracellular fluid (Fig. 1, lanes 2 and 3) and, to a lesser extent, in those corresponding to mycelium extracts (Fig. 1, lanes 5 and 6). Moreover, copper induced the synthesis of one or more laccases detected as a group of bands above LAC1 and found only in mycelium extracts (Fig. 1, lanes 5 and 6).

Patterns obtained in the absence or presence of *p*-hydroxybenzoic acid were very similar except that, in the latter

![Fig. 1. Native PAGE electrophoresis of laccases from *M. quercophilus* C30 grown on different media. 10 µg of either extracellular proteins (lanes 1–3) or mycelial extracts (lanes 4–6) were deposited per lane. Lanes 1 and 4: proteins from MET culture; lanes 2 and 5: proteins from METCu culture; lanes 3 and 6: proteins from METCu-pHB. Lane 7: 1 µg of purified LAC1 (double arrow). Activity staining was obtained with *p*-phenylenediamine.](image-url)
In the three culture conditions, the fungal dry weight increased during the first 6 days, reaching a maximum value of 3.34 ± 0.22 g L⁻¹, and was then stable until the end of the experiment. *M. quercophilus* C30 appeared to produce LAC1 continuously over the 2-week cultivation period in all conditions (Fig. 2). However, marked quantitative and qualitative differences were found regarding the other extracellular laccases produced in the three conditions tested. A moderate increase in activity level, up to a final value of 80 ± 10 U L⁻¹, was measured between day 3 and day 12 in MET (Fig. 2A). This activity correlates with the presence of LAC1 as the only laccase detectable in the extracellular fluid (photo in Fig. 2A). In METCu, laccase activity showed a steady increase to the end of the experiment, reaching a final value of 350 ± 70 U L⁻¹ (Fig. 2B) whereas in METCu-pHB, total activity increased dramatically after addition of the aromatic inducer (day 4) to reach a value of 860 ± 30 U L⁻¹ on day 9 and then decreased moderately towards the end of the experiment (Fig. 2C). Qualitatively, upon induction, a second group of bands appeared transiently between day 3 and day 8 with a peak on day 6 when copper only was present and between day 3 and day 14 with a peak on day 8 when both copper and *p*-hydroxybenzoic acid were present. Note that in the three cases, laccase production closely matched the protein secretion.

Laccases from a 8-day-old culture of *M. quercophilus* supplemented with copper and *p*-hydroxybenzoic acid eluted as four major peaks from an anion exchange column (Fig. 3). The protein and activity ratios between fractions named A, B, C and D were respectively 2:1:1:1 and 1:1:2:50. On native PAGE, laccase activity found in fraction A migrated as LAC1 (Fig. 4A, lane 2). In the condition of the test, activity in fraction B seemed associated with a group of five to seven bands located above the LAC1 migration mark (Fig. 4A, lane 3). Laccase(s) contained in fractions C migrated very close to the front of the gel and were detected as two active bands, the position of the lowest being identical to the single active band detected in fraction D (Fig. 4A, lanes 4 and 5). When subjected to a pH gradient, laccase from fraction A migrated with a pI of 3.6, identical to that of purified LAC1.

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC1</td>
<td>SIGPVA</td>
<td>[10]</td>
</tr>
<tr>
<td>A</td>
<td>SIGPVA</td>
<td>this work</td>
</tr>
<tr>
<td>B1</td>
<td>SIGPVA</td>
<td>this work</td>
</tr>
<tr>
<td></td>
<td>A(KT)</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>AIGPKT</td>
<td>this work</td>
</tr>
<tr>
<td>C1</td>
<td>AIGPKA</td>
<td>this work</td>
</tr>
<tr>
<td>C2</td>
<td>AIGPKA</td>
<td>this work</td>
</tr>
<tr>
<td>D</td>
<td>AIGPKA</td>
<td>this work</td>
</tr>
</tbody>
</table>

*More than one protein was detected in this sample.
whereas values for laccases in fractions C and D were found below 3.5. Interestingly, the pI of the two most intense bands of fraction B are much less acidic as they were found to be close to 5 (Fig. 4B).

SDS-PAGE analysis revealed that laccases in fractions A and D migrated as a single band with an apparent molecular mass identical to that of purified LAC1 used as standard whereas those in fractions B and C migrated as a doublet (data not shown). Proteins contained in each band were recovered separately after transfer on PVDF and subjected to Edman degradation. The sequences of the first six residues at the amino-terminus from each protein are shown in Table 1. Comparison of these sequences with those contained in the database confirmed that all the samples contained a laccase. The amino-terminus of the protein contained in fraction A was identical to that of LAC1. This, together with its general behavior, strongly suggests that the laccase contained in fraction A is identical to LAC1. More than one protein was present in the upper band of the doublet found after SDS-PAGE separation of fraction B (Table 1, sample B1). Among the eight possible combinations, the sequence SIGVPA could reflect the presence of LAC1 as a contamination of the laccase(s) contained in fraction B. In the lower band of fraction B, there was a single sequence AIGPKT different from that of LAC1 (Table 1, sample B2). The result obtained for C1, C2 and D revealed that laccases contained in these samples share the same amino-terminus.

4. Discussion

*M. quercophilus* is able to secrete more than one laccase according to the growth conditions used [5,8]. In a previous study, we purified and characterized LAC1, the most
abundant enzyme produced by *M. quercophilus* C30 [6]. On the other hand, a preliminary work had revealed that minor forms secreted by the fungus could be more active than LAC1 [7]. In this study we investigated the effect of different growth conditions on laccase production in *M. quercophilus* C30 and succeeded in specifically enhancing the production of three new inducible laccases.

Although laccase activity is apparently constitutive in most basidiomycetes [1,9], it can be enhanced by several inducers such as aromatic substances [10] or copper [11,12]. Total laccase activity in *M. quercophilus* C30 culture is increased up to 10-fold when the malt extract medium is amended with copper sulfate and up to 30-fold when both copper and p-hydroxybenzoic acid are used. Moreover, as expected, the laccase composition changes upon induction revealing the synthesis of new enzyme forms in addition to LAC1. Intracellular activities appear in mycelia from both Cu- and Cu-pHB-induced cultures. Whether these activities reflect the presence of intracellular laccase(s) or precursors of secreted forms has not yet been established. The time course experiment reveals that copper induces a new extracellular activity, or perhaps activities, which is transient since the production of these enzymes is also dependent on the growth phase [13]. Specific laccase activities of MET and METCu cultures are almost superimposable (not shown). This underlines that, although LAC1 is always synthesized, its level is sensitive to copper as was already suggested by the discovery of a laccase in *M. quercophilus* C30 [6,14]. With a combination of both copper and p-hydroxybenzoic acid, induced enzymes are present in the extracellular fluid until the end of the experiment. However, again, the expression of inducible laccase genes in *M. quercophilus* C30 could depend on the growth phase as is the case for the veratryl alcohol-induced expression of the *lec1* and *lec2* genes of the basidiomycete *I.-62* [13]. Therefore, it should be noted that the enzymes present at the end of the experiment are not necessarily the same as those present at the beginning. Concerning its global effect, p-hydroxybenzoic acid, like other aromatic acids, is a known fungal toxin and it is therefore possible that the strong induction of laccase activity it provokes is a response developed by the fungus to reduce its toxicity. As the deep brown coloration of the medium observed at the end of the experiment suggests, the fungus could dispose of the aromatic acid through polymerization.

Some of the induced laccases should be very active forms as judged by the increase in specific activity in METCu-pHB relative to MET culture. Indeed, after one step of chromatographic separation of the enzymes produced on METCu-pHB, one of the fractions (D) is at least 100 times more active on syringaldazine than LAC1. Among the four laccase activities separated and characterized by electrophoresis and amino-terminal sequencing, fractions B and D contain proteins different from LAC1 (fraction A) and from each other. An identical amino-terminus does not allow us to distinguish laccase present in fraction C from that in D yet, but, as suggested by its electrophoretic behavior, it is highly probable that fraction C contains a fourth different laccase.

Laccases are generally produced as a number of isoenzymes that are encoded by gene families. In response to inducers, laccase gene expression varies from fungus to fungus. For example, 2,5-xylidine induces laccase in *Trametes versicolor* [15] whereas it is apparently inefficient in the basidiomycete *PM1* [16]. Our study shows that, under appropriate culture conditions, the production of minor laccase forms in *M. quercophilus* C30 can be enhanced and that this fungus produces at least four different laccases.

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**References**


