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Novel enzymatic oxidation of Mn^{2+} to Mn^{3+} catalyzed by a fungal laccase

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Abstract Fungal laccases are extracellular multinuclear copper-containing oxidases that have been proposed to be involved in ligninolysis and degradation of xenobiotics. Here, we show that an electrophoretically homogenous laccase preparation from the white rot fungus *Trametes versicolor* oxidized Mn^{2+} to Mn^{3+} in the presence of Na-pyrophosphate, with a K_m value of 186 μM and a V_{max} value of 0.11 μmol/min/mg protein at the optimal pH (5.0) and a Na-pyrophosphate concentration of 100 mM. The oxidation of Mn^{2+} involved concomitant reduction of the laccase type 1 copper site as usual for laccase reactions, thus providing the first evidence that laccase may directly utilize Mn^{2+} as a substrate.

Key words: Laccase; Manganese; Lignocellulose; Ligninolytic enzyme; Biodegradation; *Trametes versicolor*

1. Introduction

Laccases (EC 1.10.3.2) are multinuclear copper-containing oxidases that are widely distributed in fungi of different ecophysiological groups [1,2]. These enzymes seem to play a multi-functional role since they have been proposed to be involved in degradation of lignin and xenobiotics, humification, morphogenesis and plant pathogenesis [1–6]. Laccase catalyzes the reduction of molecular oxygen to water without the step of hydrogen peroxide formation. Thereby, four one-electron oxidations of a reducing substrate are coupled to the four-electron reduction of a single dioxygen molecule [2,7,8]. Due to its catalytical properties, laccase has gained considerably interest because of its potential biotechnological applicability [1–8].

The catalytic site of laccase from the white rot fungus *Trametes versicolor* consists of four copper ions per laccase molecule, which can be classified into three types, one type 1, one type 2 and two type 3 copper ions. Type 1 copper oxidizes laccase substrates by one-electron abstraction. Type 2 and type 3 copper form a trinuclear cluster site which is responsible for oxygen binding and reduction. Furthermore, type 2 and type 3 copper show high affinities for ,e.g., F^-, CN^- and N_3^- and may therefore reversibly be inhibited by such ions [2,7,8].

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Abbreviations: ABTS, 2,2′azino-bis(3-ethylbenzthiazoline-6-sulfonate); HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; *T. versicolor*, *Trametes versicolor*

Trivalent manganese (Mn^{3+}) is a powerful oxidant and is produced from divalent manganese (Mn^{2+}) by manganese peroxidases, which are part of the ligninolytic system of white rot fungi. Manganese peroxidases have been proposed to be involved in the degradation of lignin and lignin model compounds, as well as xenobiotics [9–12]. In the presence of Mn^{2+}, the oxidation of different phenols and hydroquinones to the corresponding phenoxy radicals and semiquinones, catalyzed by laccase, may also lead to the formation of Mn^{3+} [13,14]. The stable cation radical of the arteficial redox mediator 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), which is formed during ABTS oxidation by laccase, was shown to be reduced by Mn^{2+}, thereby leading to the formation of Mn^{3+} [15]. The existence of a natural redox mediator has been proposed in *T. versicolor* [5]. Here, we report on a novel enzymatic oxidation of Mn^{2+} to Mn^{3+}, catalyzed by purified laccase from *T. versicolor* in the absence of any redox-mediating compound and peroxidase activity.

2. Materials and methods

2.1. Organism and culture conditions

T. versicolor DSM 11269 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ GmbH, Braunschweig, Germany). Cultivation on a glucose-containing, nitrogen-limited medium was carried out as described previously [16]. After 4 days, 2,5-xylidine (200 μM final concentration) was aseptically added to stimulate laccase production.

2.2. Purification of laccase

Fungal cultures were harvested after 14 days and crude culture liquid was obtained as described earlier [17]. Concentration of the crude culture liquid was reached by two successive ultrafiltration steps, employing a tangential flow membrane filter (5 kDa cut-off, open channel, Pall Filtron, Dreieich, Germany), followed by a stirred cell (Amicon, Lexington, MA, USA) equipped with a 10 kDa cut-off polysulfone ultrafiltration membrane (Sartorius, Göttingen, Germany). For separation of proteins, the enzyme concentrate was applied to a Mono Q HR 5/5 anion exchange column (Amersham Pharmacia Biotech, Freiburg, Germany). Protein was eluted with a gradient of 0–0.25 M NaCl (Fig. 1) in 10 mM Na-acetate buffer (pH 5.5) at a flow rate of 1 ml/min, using a fast protein liquid chromatography system (Amersham Pharmacia Biotech). The laccase-containing fractions were collected by repeated separations, pooled, reconcentrated by ultrafiltration and stored at −20°C.

2.3. Gel electrophoresis and staining

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% polyacrylamide gels according to the method of Laemmli [18]. Samples (5 μg protein) were applied after boiling for 5 min. Protein concentrations were determined according to the method of Bradford [19]. Protein bands were stained with Coomassie brilliant blue R-250 (Serva Feinbiochemica, Heidelberg, Germany). The molecular weight of laccase was determined by comparison with commercial low range molecular weight markers (Bio-Rad, München, Germany).

Native PAGE was carried out in the absence of SDS on 12.5%

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polyacrylamide gels without prior boiling of the samples. The gels were stained for laccase activity with 3 mM ABTS.

2.4. Enzymatic determinations

Laccase activity was determined at pH 4.5 by following the oxidation of either ABTS or 2,6-dimethoxyphenol at 436 and 468 nm, respectively (ε_{436} = 29.3/mM/cm, ε_{468} = 30.5/mM/cm), as described before [16,17]. The assay for manganese-independent peroxidase (peroxidase, EC 1.11.1.7) was similar to that used for laccase determination with ABTS, containing in addition 100 μM H_2O_2 and 5 mM EDTA. Manganese peroxidase (EC 1.11.1.13) and lignin peroxidase (EC 1.11.1.14) were determined as described elsewhere [16].

Oxidation of Mn^{2+} to Mn^{3+} by purified laccase was monitored spectrophotometrically up to 4 h by following the formation of Mn^{3+} complexes, using Na-pyrophosphate as complexing agent. Mn^{3+}-pyrophosphate was identified by its absorbance spectrum at the wavelength ranges indicated in the text. This was confirmed by comparison with spectra obtained from synthetic Mn^{3+}-pyrophosphate, which was prepared immediately prior to use by dissolving Mn^{3+}-acetate (final concentration 1 mM) in 100 mM Na-pyrophosphate. Formation of Mn^{3+}-pyrophosphate was followed at 258 nm (ε_{258} = 6.2/mM/cm [20]). Mn^{2+} was applied as $MnSO_4$. The pH was always adjusted with phosphoric acid. All spectrophotometric measurements were carried out in air-saturated solutions at 35°C, using a double beam UV/VIS spectrophotometer (Lambda 2, Perkin-Elmer, Überlingen, Germany). If not otherwise stated, the reference cuvettes contained the respective complete assay, but enzyme that had been heat-inactivated by boiling for 1 h.

2.5. Absence of redox-mediating compounds in the purified laccase fraction

The absence of phenolic compounds, as well as any other low molecular weight compound was ensured by directly applying the purified laccase fraction to high performance liquid chromatography (HPLC) under conditions described before [17]. The absorbance between 220 and 450 nm was monitored with a diodearray detector [17].

2.6. Chemicals

All reagents were of analytical grade and were purchased from either Sigma-Aldrich Chemie GmbH (Steinheim, Germany) or Fluka Chemie (Neu Ulm, Germany), if not otherwise stated.

3. Results and discussion

3.1.. Production and purification of laccase

Under the culture conditions applied, the maximum activity of laccase was reached after 14 days. Laccase was purified to electrophoretic homogeneity according to the procedure summarized in Table 1. The elution profiles of laccase activity and protein after separation on a Mono Q HR 5/5 column are shown in Fig. 1. The protein fractions corresponding to an elution volume from 10 to 11 ml, which contained the major part of laccase activity, were pooled, re-concentrated and used for further experiments. This enzyme pool did not contain any activity of manganese peroxidase, manganese-independent peroxidase or lignin peroxidase. Purity determination by SDS-PAGE led to one predominant protein band with a molecular mass of approximately 68 kDa (Fig. 2). This is in the same range as molecular weights of laccase isoforms isolated from 2,5-xylidine-induced cultures of *T. versicolor*, as reported earlier [3,5]. Activity staining with ABTS after native PAGE visualized a single, green-colored spot (data not shown). Analysis of the purified laccase by HPLC did not lead to any indication for a low molecular weight compound, thus ruling out the presence of a hypothetical natural redox mediator.

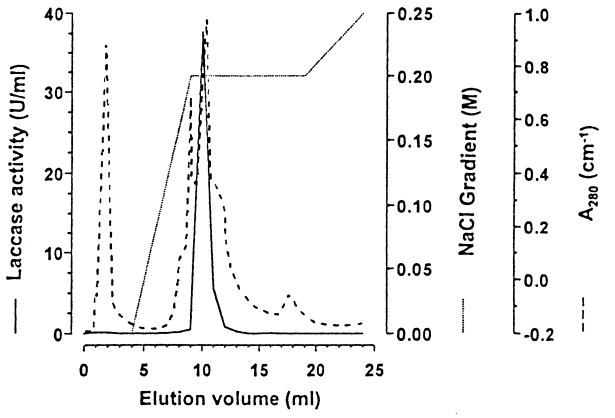

Fig. 1. Elution profiles of laccase activity and protein (absorbance at 280 nm), as obtained by separation of concentrated crude culture liquid on a Mono Q HR 5/5 column.

3.2. Oxidation of Mn^{2+} to Mn^{3+} catalyzed by laccase

Fig. 3 shows a typical time course of formation of Mn^{3+}-pyrophosphate catalyzed by purified *T. versicolor* laccase, as well as corresponding UV spectra recorded after different time intervals. The presence of Mn^{3+}-pyrophosphate is indicated by the specific absorbance maxima at 258 [20] and 478 nm [13] (not shown in Fig. 3). Spectra of synthetic Mn^{3+}-pyrophosphate complexes were identical to those obtained from the enzymatically generated Mn^{3+} complexes. No Mn^{3+}-pyrophosphate formation was observed in assays containing heat-inactivated enzyme.

The pH optimum of Mn^{3+}-pyrophosphate formation by purified *T. versicolor* laccase was found to be 5.0 (Fig. 4). With ABTS and 2,6-DMP as reference substrates, pH optima of 2.5 and 3.5–4.0, respectively, were obtained. For the oxidation of ABTS by laccases from different basidiomycetes, acidic pH optima have been well-documented [1,21,22]. For the oxidation of 2,6-DMP by *T. versicolor* laccase, a pH optimum of 3.8 was shown [23].

Catalytic properties of the purified laccase are shown in Table 2. At a Na-pyrophosphate concentration of 100 mM, oxidation of Mn^{2+} to Mn^{3+} by purified *T. versicolor* laccase followed typical Michaelis-Menten kinetics, as commonly known for laccase substrates such as ABTS and 2,6-DMP [25]. The K_m value for the oxidation of Mn^{2+} to Mn^{3+} was found to be approximately 5-fold and 13-fold higher, respec-

Table 1
Purification of *T. versicolor* laccase

Purification step	Volume (ml)	Protein (μg/ml)	Total activity[a] (μmol/min)	Specific activity[a] (μmol/min/mg protein)	Yield (%)	Purification (-fold)
Culture liquid	14 050	3	2 264	54	100	1.0
Concentrated ultrafiltrate	20	390	1 420	182	63	3.4
Mono Q eluate[b]	20	132	720	273	32	5.0

[a]The laccase activity was determined with ABTS at pH 4.5.
[b]The protein fraction corresponding to an elution volume from 10 to 11 ml (Fig. 1) is shown, prior to re-concentration.

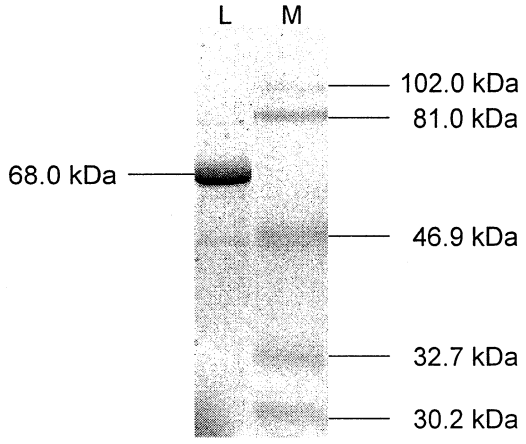

Fig. 2. Coomassie brilliant blue R-250-stained gel (SDS-PAGE), containing purified *T. versicolor* laccase (L) and molecular weight markers (M).

tively, as compared to the values obtained for ABTS and 2,6-DMP oxidation (Table 2). The V_{max} value was considerably lower, in comparison with the values obtained for ABTS and 2,6-DMP (Table 2). This low reaction rate is also obvious from the linear increase of the Mn^{3+}-pyrophosphate concentration shown in Fig. 3, which indicates that there was no substrate limitation during the whole time of reaction. At a $MnSO_4$ concentration of 1 mM, the initial rate of Mn^{2+} oxidation was found to be proportional to the amount of laccase over a tested range of 0.37–1.48 μg/ml (data not shown).

Oxidation of Mn^{2+} to Mn^{3+}, as well as oxidation of ABTS and 2,6-DMP, was found to be similarly inhibited by different concentrations of F^-, which was applied as NaF (Fig. 5). The NaF concentration causing 50% inhibition of enzyme activity was found to be approximately 30 μM for Mn^{2+} and 2,6-DMP and approximately 50 μM for ABTS. This is in the same range with values reported for inhibition of ABTS oxidation by recombinant laccases from the fungi *Polyporus pinsitus*, *Rhizoctonia solani* and *Myceliophtora thermophila* [24,25]. At 1 mM NaF, approximately 15% remaining activity

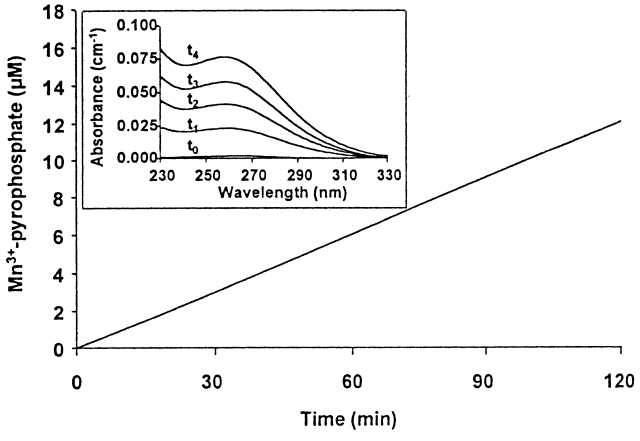

Fig. 3. Typical time course of Mn^{3+}-pyrophosphate formation, catalyzed by purified *T. versicolor* laccase. The assay contained 1 mM $MnSO_4$, 0.73 μg laccase and 100 mM Na-pyrophosphate in a final volume of 1 ml (pH 5.0). The inset shows spectrum scans after the following times of reaction: $t_0 = 0$ min, $t_1 = 30$ min, $t_2 = 60$ min, $t_3 = 90$ min, $t_4 = 120$ min.

Table 2
K_m and V_{max} values obtained with purified *T. versicolor* laccase for Mn^{2+}, ABTS and 2,6-DMP as substrates[a]

Substrate	K_m (μM)	V_{max} (μmol/min/mg protein)
Mn^{2+}	186.3	0.11
ABTS	37.3	310.0
2,6-DMP	14.5	182.2

Assays for determination of V_{max} and K_m values contained 0.73 μg laccase, substrates at different concentrations of up to 5 mM and 100 mM Na-pyrophosphate in a final volume of 1 ml (pH 5.0 for Mn^{2+} and 4.0 for ABTS and 2,6-DMP, respectively).
[a]All values were calculated by linear regression (correlation coefficients ≥0.98) from double-reciprocal plots of V versus the substrate concentration (Lineweaver-Burk). All data points used for this represented means of triplicate measurements (sample mean deviations ≤10%).

was detected for oxidation of Mn^{2+} and approximately 5% for oxidation of both ABTS and 2,6-DMP (Fig. 5). F^- strongly interacts with laccase type 2 copper and notably influences the redox potentials of type 2 and type 3 copper. In contrast, the redox potential of type 1 copper was found to remain essentially unaffected [7]. Furthermore, F^- was shown to inhibit the intramolecular electron transfer from type 1 copper to the trinuclear cluster formed from type 2 and type 3 copper, which are assumed to be cooperative in the reduction of oxygen to water [7,26]. F^- was also used to study the substrate-dependent reduction of type 1 copper [7,26,27].

Here, we used F^- to prevent re-oxidation of type 1 copper by electron transfer to the trinuclear cluster site, in order to demonstrate the Mn^{2+}-dependent reduction of type 1 copper of purified laccase (Fig. 6). Oxidation of Mn^{2+} to Mn^{3+} by laccase led to gradual disappearance of the absorbance peak at nearly 600 nm, which is characteristic for type 1 copper in its oxidized state [2,7]. No such disappearance was observed in control assays in the absence of Mn^{2+}. It seems therefore likely that the formation of Mn^{3+} by laccase occurs via an electron transfer from Mn^{2+} to type 1 copper, as generally assumed for laccase substrates [2,7,25–28].

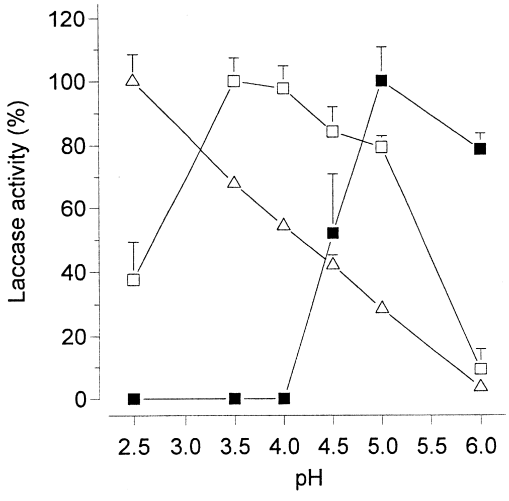

Fig. 4. Influence of the pH on oxidation of Mn^{2+} to Mn^{3+} (■), ABTS (Δ) and 2,6-DMP (□) by purified *T. versicolor* laccase. The assays contained 2 mM substrate, respectively, 0.73 μg laccase and 100 mM Na-pyrophosphate in a final volume of 1 ml. 100% refers to the V_{max} values shown in Table 2. Symbols represent means and sample mean deviations for triplicate measurements.

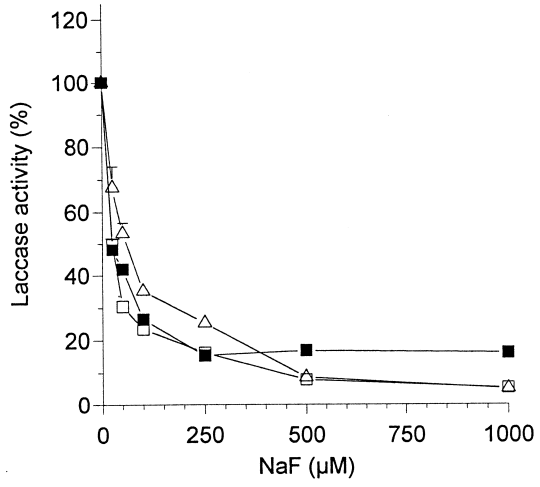

Fig. 5. The influence of NaF on oxidation of Mn^{2+} to Mn^{3+} (■), ABTS (Δ) and 2,6-DMP (□) by purified *T. versicolor* laccase. The assays contained 2 mM substrate, 0.73 μg laccase and 100 mM Na-pyrophosphate in a final volume of 1 ml (pH 5.0 for Mn^{2+} and 4.0 for ABTS and 2,6-DMP). 100% refers to the V_{max} values shown in Table 2. Symbols represent means and sample mean deviations for triplicate measurements.

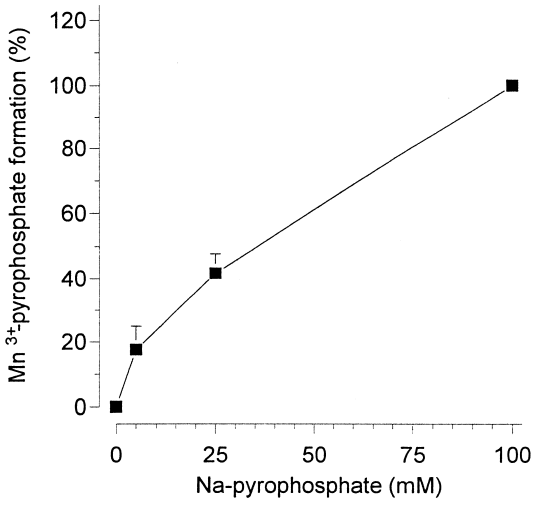

Fig. 7. The Influence of Na-pyrophosphate on oxidation of Mn^{2+} to Mn^{3+} by purified *T. versicolor* laccase. The assays contained 2 mM Mn^{2+}, 0.73 μg laccase and Na-pyrophosphate as indicated (pH 5.0). At concentrations of 0, 5 and 25 mM Na-pyrophosphate, respectively, 100, 95 and 75 mM Na_2HPO_4 were employed in addition. 100% refers to the V_{max} value for Mn^{3+}-pyrophosphate formation shown in Table 2. Symbols represent means and sample mean deviations for triplicate measurements.

The redox potential difference between type 1 copper of laccase and substrate has been implicated to govern the first electron transfer from various phenols, anilines and other aryl analogs to type 1 copper, which has been considered as the rate-limiting step in the oxidation of these compounds [24]. The oxidation of certain monoaromatic compounds by fungal laccases has been demonstrated to be even possible at redox potential differences of approximately −700 mV [24]. From a thermodynamic point of view, under conditions where laccase oxidizes a substrate whose redox potential is higher than that of laccase type 1 copper, the driving force of the reaction should be the coupled reduction of oxygen to water.

The one-electron redox potential of *T. versicolor* laccase type 1 copper has been reported to be 785 mV (versus normal hydrogen electrode [7]), while the redox potential of the Mn^{2+}/Mn^{3+} couple (aqua complexes) is 1510 mV [2]. The well-known stabilization of the Mn^{3+} state by complexation with pyrophosphate may well lower this potential, which may be either the reason or an additional support for the oxidation of Mn^{2+} by laccase. Both possibilities are supported by the fact that the rate of Mn^{3+}-pyrophosphate formation was found to be enhanced upon increasing the Na-pyrophosphate concentration over a tested range of 0–100 mM (Fig. 7). Since hypothetical aqua Mn^{3+} complexes possibly produced in the absence of pyrophosphate would not be stable [13], from these experiments, it cannot be concluded that the presence of a sufficient chelator is essential for the oxidation of Mn^{2+} to Mn^{3+} by laccase. Further experiments should therefore involve the influence of both Mn^{2+} and chelators on oxygen consumption, as well as on transient state kinetics of type 1 copper reduction.

Although the novel oxidation described in this study showed a rather slow reaction rate, it further expands the scope of compounds that can serve as substrate for laccase and emphasizes the potential applicability of laccase for biotechnological purposes. Regarding the physiological and ecological relevance, further investigations should clarify if the capability of oxidizing Mn^{2+} to Mn^{3+} enables laccase to fulfil such functions in degradation of lignin and xenobiotics, that until now are attributed to manganese peroxidases [9–12,28].

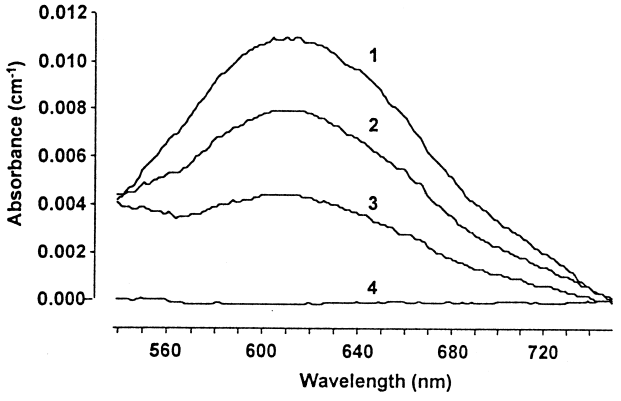

Fig. 6. Absorbance spectrum of type 1 copper of purified *T. versicolor* laccase, as observed during oxidation of Mn^{2+} to Mn^{3+}, at the beginning (1), after 10 (2) and after 20 min (3) of reaction. (4) Shows the spectrum of a complete assay that was totally reduced by the addition of some crystals of Na-dithionite, serving as reference. The assay contained 257 μg laccase, 2 mM $MnSO_4$, 1 mM NaF and 100 mM Na-pyrophosphate in a final volume of 1 ml (pH 5.0).

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References

[1] Thurston, C.F. (1994) Microbiology 140, 19–26.
[2] Call, H.P. and Mücke, I. (1997) J. Biotechnol. 53, 163–202.
[3] Bourbonnais, R., Paice, M.G., Reid, I.D., Lanthier, P. and Yaguchi, M. (1995) Appl. Environ. Microbiol. 61, 1876–1880.
[4] Eggert, C., Temp, U., Dean, J.F.D. and Eriksson, K.-E.L. (1996) FEBS Lett. 391, 144–148.

[5] Collins, P.J., Kottermann, M.J.J., Field, J.A. and Dobson, A.D. (1996) Appl. Environ. Microbiol. 62, 4563–4567.
[6] Chefetz, B., Chen, Y. and Hadar, Y. (1998) Appl. Environ. Microbiol. 64, 3175–3179.
[7] Yaropolov, A.I., Skorobogat'ko, O.V., Vartanov, S.S. and Varfolomeyev, S.D. (1994) Appl. Biochem. Biotechnol. 49, 257–280.
[8] Ducros, V., Brzozowski, A.M., Wilson, K.S., Brown, S.H., Østergard, P., Schneider, P., Yaver, D.S., Pedersen, A.H. and Davies, G.J. (1998) Nat. Struct. Biol. 5, 310–316.
[9] Wariishi, H., Valli, K. and Gold, M.H. (1991) Biochem. Biophys. Res. Commun. 176, 269–275.
[10] Jensen, K.A., Bao, W., Kawai, S., Srebotnik, E. and Hammel, K.E. (1996) Appl. Environ. Microbiol. 62, 3679–3686.
[11] Bogan, B.W., Lamar, R.T. and Hammel, K.E. (1996) Appl. Environ. Microbiol. 62, 1788–1792.
[12] Hofrichter, M., Scheibner, K., Schneegaß, I. and Fritsche, W. (1998) Appl. Environ. Microbiol. 64, 399–404.
[13] Archibald, F. and Roy, B. (1992) Appl. Environ. Microbiol. 58, 1496–1499.
[14] Munoz, C., Guillén, F., Martínez, A.T. and Martínez, M.J. (1997) Appl. Environ. Microbiol. 63, 2166–2174.
[15] Collins, P.J., Dobson, A.D. and Field, J.A. (1998) Appl. Environ. Microbiol. 64, 2026–2031.
[16] Schlosser, D., Grey, R. and Fritsche, W. (1997) Appl. Microbiol. Biotechnol. 47, 412–418.
[17] Grey, R., Höfer, C. and Schlosser, D. (1998) J. Basic Microbiol. 38, 371–382.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[20] Kenten, R.H. and Mann, P.J.G. (1955) Biochem. J. 61, 279–286.
[21] Heinzkill, M., Bech, L., Halkier, T., Schneider, P. and Anke, T. (1998) Appl. Environ. Microbiol. 64, 1601–1606.
[22] Fukushima, Y. and Tien, T.K. (1995) Appl. Environ. Microbiol. 61, 872–876.
[23] Leonowicz, A., Edgehill, R.U. and Bollag, J.-M. (1984) Arch. Microbiol. 137, 89–96.
[24] Xu, F. (1996) Biochemistry 35, 7608–7614.
[25] Xu, F. (1996) Appl. Biochem. Biotechnol. 59, 221–230.
[26] Tollin, G., Meyer, T.E., Cusanovich, M.A., Curir, P. and Marchesini, A. (1993) Biochim. Biophys. Acta 1183, 309–314.
[27] Koudelka, G.B. and Ettinger, M.J. (1988) J. Biol. Chem. 263, 3698–3705.
[28] Urzúa, U., Kersten, P.J. and Vicuna, R. (1998) Appl. Environ. Microbiol. 64, 68–73.