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Catalytic behavior and detoxifying ability of a laccase from the fungal strain *Cerrena unicolor*

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Abstract

The kinetics and stability of a laccase isolated and purified from the fungal strain *Cerrena unicolor* were studied. The enzyme was produced in a great yield without inducers. Kinetic parameters were determined by using 2,2′-azinobis–(3-ethylbenzthiazoline-6-sulfonate) (ABTS) as substrate. At high ABTS concentrations (> 10 mM) a substrate inhibition phenomenon appeared and an inhibition constant K_i of 24 mM was determined. The pH- and temperature-profiles as well as the sensitivity of the enzyme to several deactivation agents were almost similar to those observed with laccase from different origins. Freezing–thawing treatment, high temperature, acidic pH (< 3.0) and acetonitrile strongly affected laccase activity. The laccase showed a good ability to oxidize different phenolic substances; a significant enhancing effect was showed by ABTS acting as co-substrate. These results seem to suggest that this new laccase preparation may be suitable for environmental purposes. © 1998 Elsevier Science B.V.

Keywords: Laccase; Kinetics; Stability; Phenol detoxification

1. Introduction

Laccases (benzenediol:oxygen oxidoreductase, E.C. 1.10.3.2) are copper-containing oxidoreductive enzymes, which reduce oxygen to water and, typically, oxidize a phenolic substrate. They have wide substrate specificity and a great potential in biotechnological and environmental applications. Laccases have been tested for the removal of natural phenolic compounds or xenobiotics from polluted soils [1,2]. Furthermore, the capability of these enzymes to transform lignin from the pulp and paper industry has been studied [3–5]. The ability of laccase in the oxidation of non-phenolic substrates has been also demonstrated [6].

Laccases are ubiquitous enzymes produced by higher plants and microorganisms, mainly fungi [3,7]. Little information has been reported on bacterial laccases [8]. In several fungi, laccase production is usually inducible, and elevated yields are obtained only after an induction period of time.

Recently, Leonowicz and Gianfreda demonstrated that various fungal strains are capable of producing high quantities of laccase without any induction (unpublished results).

In order to utilize a new enzyme preparation for environmental or biotechnological applica-

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tions, it is useful to have a specific and precise knowledge of the kinetic behavior of the enzyme. The kinetic parameters, the pH- or temperature-activity profiles, the effect of inhibitors and, mainly, the storage and, stability in general, must be known.

The purpose of this study is to provide a detailed characterization of this new laccase preparation in terms of kinetic parameters and dependence of enzymatic activity on experimental conditions such as pH, temperature, presence of additives, storage time, exposure to high temperature and/or chemical agents. The capability of the enzyme to degrade xenobiotics of aromatic nature was also investigated.

2. Experimental

2.1. Chemicals

2,4-dichlorophenol (2,4-DCP) (99% purity) catechol and methylcatechol were purchased from Aldrich Chemie. 2,2′-azinobis–(3-ethylbenzthiazoline-6-sulfonate) (ABTS) and proteinase K (E.C. 3.4.21.14) were obtained from Boehringer and Sigma Chemical, respectively. 2,4-D (99.6% purity) was obtained from Dr. Ehernstofer GmbH. Highly purified hydroxytyrosol and tyrosol were a kind gift of professor Renato Capasso of Dipartimento di Scienze Chimico Agrarie, University of Naples. All other chemicals, reagent grade, were from LabScan. The chemical structures of all compounds are reported in Fig. 1.

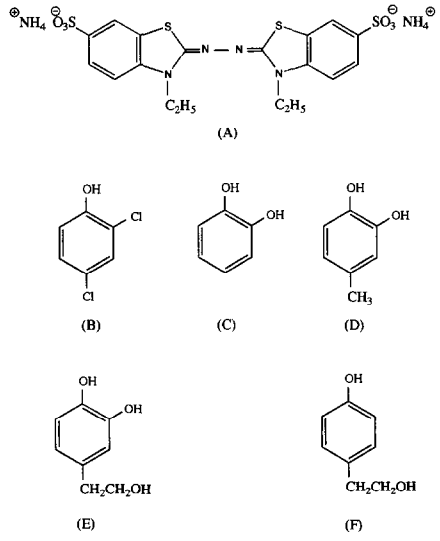

Fig. 1. Chemical structures of compounds used as substrates of laccase: (A) ABTS, (B) 2,4-dichlorophenol, (C) catechol, (D) methylcatechol, (E) hydroxytyrosol and (F) tyrosol.

2.2. Laccase preparation

Laccase was isolated and purified from the fungal strain *Cerrena unicolor* (Bull. ex Fr.) Murr., Einfarbige Tramete Strain T * 143, Collection Molitoris, Botanic Institute, University of Regensburg (Germany).

The fermentor cultures of *Cerrena unicolor* based on Lindeberg and Holm [9] medium were run at 28°C according to Rogalski et al. [10], without inducers. The enzyme was purified using the filtrate from the time of maximum extracellular laccase activity. The culture filtrates were saturated with ammonium sulphate at the saturation range 0–0.8 and centrifuged (15 min, 15 000 × g). The precipitates were dissolved in 0.1 M phosphate buffer, pH 6.0. The concentrated (both by ultrafiltration and ammonium sulphate) culture filtrates were dialyzed 7 times against de-ionized water and (before the end of dialysis) one time against 0.1 M phosphate buffer, pH 6.0. Subsequently, 25 ml of such enzyme preparations were applied onto a ConA–Sepharose column, which was then washed with 0.1 M phosphate buffer, pH 6.0, until all unbound proteins were removed. The specifically bound proteins, including laccase, were eluted from the column with 20% sucrose in 0.1 M phosphate buffer, pH 6.0. The fractions showing the highest laccase activity were concentrated by ultrafiltration; the concentrated samples were then applied onto a DEAE–Sephadex A-50 column, previously washed and equilibrated with 0.01 M phosphate buffer, pH 6.0. Laccase-rich fractions were dialyzed against

0.1 M phosphate buffer, pH 6.0, and applied separately onto a column of AH–Sepharose 4B coupled to syringaldehyde. The enzyme was eluted by 0.5 M ammonium sulphate dissolved in the same buffer, dialyzed against 0.01 M phosphate buffer, pH 6.0, and stored as freeze-dried.

The constitutive form of laccase showed a specific activity of 6.0 μmol min^{-1} mg^{-1}, using ABTS as substrate, at 25°C and pH 3.5.

2.3. Activity assay

The activity of laccase was tested with ABTS as substrate, with the activity assay being rapid, very easy and reproducible. ABTS oxidized product is a chromogenic compound which adsorbs at 420 nm and has an extinction coefficient of 36 mM^{-1} cm^{-1} [11]. All activity tests were carried out directly in a Perkin-Elmer, Lambda 3B, UV/Vis spectrophotometer, equipped with a Perkin-Elmer R100 A recorder, by continuously monitoring the production of the chromogenic compound. One unit of enzymatic activity was defined as the amount of enzyme required to transform 1 μmol of ABTS min^{-1} at 25°C and pH 3.5. The enzymatic specific activity was obtained by dividing the units per mg of protein. Protein concentration was determined [12] by the BIO-RAD, protein reagent method, using albumin as the standard protein.

The standard conditions adopted for the measurement of the enzyme activity were: ABTS = 3 mM, in 100 mM glycine/HCl buffer pH = 3.5 and $T = 25$°C, unless otherwise specified.

As described below, enzyme in solution showed low stability to freezing–thawing treatment. Consequently, laccase solutions were prepared from freeze-dried samples, when needed, and immediately utilized.

2.4. Kinetic studies

To determine the kinetic parameters, activity assays were performed at 25°C and pH 3.5 in 100 mM glycine/HCl buffer, by ranging the ABTS concentration from 0.025 to 20 mM. Because at ABTS concentrations higher than 5 mM, a substrate inhibition phenomenon was evident, the experimental data were fitted to the modified Michaelis–Menten equation which takes into account substrate inhibition [13]:

$$v = \frac{V_{max} \times S}{K_m + S + (S^2/K_i)} \quad (1)$$

where v is the specific enzymatic activity, V_{max} and K_m are the maximum velocity and the Michaelis–Menten constant in the absence of inhibition and K_i is the substrate inhibition constant. The values of V_{max}, K_m and K_i were obtained by non-linear regression analysis. Although the previous equation applies primarily to single-substrate reaction, this kinetic treatment may be used because the concentration of oxygen, the other substrate participating to the reaction, may be considered in excess and constant over the time span of the reaction.

2.5. Dependence on pH and temperature

The pH-activity profile was obtained by performing activity tests at 3 mM substrate concentration and ranging the pH of the solution from 2.13 to 7.00. The following 100 mM buffers were utilized: glycine/HCl, pH 2.13–3.65; acetate, pH 3.65–5.60; and phosphate, pH 5.60–7.00. Control tests were performed at the extreme pH values of each buffer pH range.

The dependence of the enzymatic activity on the temperature was assessed by activity tests at 3 mM ABTS and pH 3.5, and temperatures from 20 to 80°C. The activation energy E_a of the reaction was calculated by a regression of natural logarithms of the activity on the reciprocal of the absolute temperature.

2.6. Inhibition tests

The inhibitory effect of sodium azide (NaN_3), EDTA (ethylenediaminetetraacetic acid), or-

ganic solvents (methanol and acetonitrile) was studied under standard conditions, by activity tests at 3 mM ABTS, pH 3.5, $T = 25$°C and in the presence of various amounts of each additive.

2.7. Stability studies

The enzyme stability to freezing and thawing, high temperatures, pH, organic solvents (methanol, acetonitrile and dioxane) and presence of protease was examined. Laccase specimens were stored in a freezer at −20°C, and their residual activity was determined after one or more freezing–thawing cycles. As enzyme samples were very small (at the most 50 μl), freezing and thawing of enzyme can be considered instantaneous. The thermal stability of the enzyme was assessed by exposing laccase samples at 50°C. The sensitivity of the enzyme to chemical and proteolytic deactivation was measured at 25°C and at variable pH values (2.16, 3.0, 3.5, 6.0 and 7.0), in organic solvent–water (1:1, v:v) solutions, and in the presence of proteinase K (20:1, proteinase K/laccase, w:w), respectively. At predetermined time intervals, enzyme samples were withdrawn and their residual activities were determined, under standard conditions. From a semilog plot of specific activity versus time a deactivation constant and the half-life ($t_{1/2}$) were calculated.

2.8. Transformation of phenolic substances

The oxidative transformation of 2,4-D, 2,4-DCP, catechol, methylcatechol, tyrosol and hydroxytyrosol was usually determined by incubating at 25°C for 24 h 2.5 ml of 0.27 mM substrate in 100 mM citrate–phosphate buffer, pH 6.0 [2], containing suitable amounts of laccase.

Activity tests at 2,4-DCP concentrations ranging from 0.03 to 0.34 mM were carried out as reported above, and the kinetic parameters V_{max} and K_m were evaluated by a non-linear regression analysis, according to the Michaelis–Menten equation. The oxidation of 2,4-DCP was linear throughout 24 h incubation time.

The effect of ABTS on the transformation of 2,4-DCP was assessed by measuring the disappearance of 0.27 mM 2,4-DCP in the presence of 1 mM ABTS after 24 h incubation at 25°C and pH 6.0.

After centrifugation at $12\,000 \times g$ for 15 min at 10°C, using a Sorvall SS34 Rotor in a Sorvall RC-5B refrigerated superspeed centrifuge, the amount of residual phenols in the supernatants was analyzed by HPLC. The HPLC analyses, on samples filtered through cellulose acetate filter 0.2 μm (Sartorius, Germany), were carried out with a Varian apparatus, equipped with a Varian Mat pump, and the Vari-Chrom variable-wavelength absorbance detector set at 280 nm. A Spheri-5-RP18 22 cm by 4.6 mm C_{18}–80 column BrownLee of 5 μm particle size was used; isocratic elution was performed at a flow rate of 1.8 ml min^{-1} with a mobile phase composed of acetonitrile and phosphate buffer (40 mM, pH 2.95) (35:65, v:v) [14]. The removal of phenols was measured by the difference between levels in the experimental assay and in a control (a substrate solution lacking the enzyme).

All the experiments were carried out at least in triplicate and all data were normalized on the basis of a specific activity of 6 μmol min^{-1} mg^{-1} (with ABTS as substrate).

3. Results and discussion

3.1. Laccase purification

As reported in Table 1, a good yield both in terms of recovered units of enzyme activity and purification factor, was achieved after four purification steps. In particular, the ConA–Sepharose chromatography and the affinity chromatography step (Syringyl–AH–Sepharose 4B) allowed a 10-fold and 2-fold increase of purification factor, respectively.

Table 1
Isolation and purification of laccase preparation

Purification step	Activity		Yield	Purification
	total (nkat./vol.)	specific (total/mg prot.)	(%)	
Filtrate	40.26	0.05	100.0	1.0
Ultrafiltration	36.04	0.18	89.6	3.6
ConA–Sepharose	30.87	1.88	75.8	37.2
DEAE–Sephadex A-50	7.76	4.58	19.0	90.4
Syringyl–AH–Sepharose 4B	3.70	6.00	9.1	118.3

Taking in account that the purified enzyme was a no-induced form, these results can be considering satisfactory and satisfying.

3.2. Catalytic behavior

3.2.1. Kinetic studies

Fig. 2 shows the oxidation of ABTS by laccase at increasing ABTS concentrations. At ABTS concentrations higher than 5–10 mM a substrate-inhibition phenomenon appeared. The kinetic parameters calculated by the modified Michaelis–Menten equation [13], are reported in Fig. 2.

As compared to laccase from other fungal origins, a lower affinity for ABTS was observed (higher K_m value). *Trametes versicolor* [15] and *Pleurotus ostreatus* [16] laccases showed K_m values of 0.42 mM and 0.28 mM, respectively, compared to 0.8 mM in this study. Inhibition by ABTS has been observed also with other laccases. In studies of the role of ABTS in the demethylation and delignification of kraft pulp by *Trametes versicolor* laccase, Bourbonnais and Paice [3] reported that an inhibition at high ABTS concentrations seemed to be evident, but no numerical evaluation was made. Maremonti et al. [15] demonstrated that laccase from *Trametes versicolor* was actually inhibited by ABTS and a K_i value of 20 mM was estimated according to Eq. (1). On the contrary, no ABTS inhibition effect was demonstrated for *Pleurotus ostreatus* laccase, at least at the ABTS concentrations utilized [16]. The value of K_i evaluated for *C. unicolor* laccase is sufficiently high (24 mM) to assume that substrate inhibition is negligible at the standard conditions (ABTS 3 mM) adopted in all enzymatic activity tests.

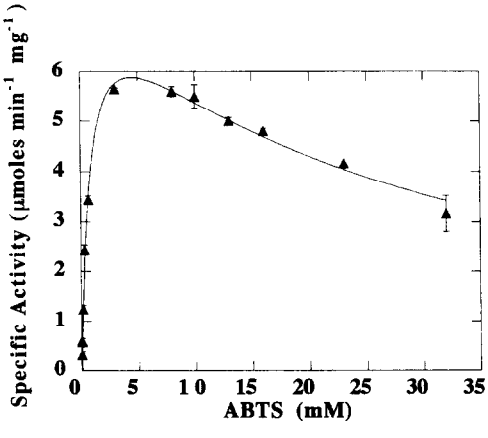

Fig. 2. Specific activity versus ABTS concentration. The values of kinetic parameters were: $V_{max} = 8$ μmol min^{-1} mg^{-1}, $K_m = 0.8$ mM and $K_i = 24$ mM. The standard deviation ranged from 0.02 to 0.70.

Substrate inhibition shown in Fig. 2 is usually explained by the reaction between the substrate and the enzyme–substrate complex (ES) to form a non-productive ternary complex ES_2. This complex is un-reactive to split into enzyme and product. The high value of K_i suggests that the phenomenon predominates only when substrate concentration is elevated.

Laccase showed a maximum of activity in the pH range 3.5–4.0 (Fig. 3a). At acidic pH values, the activity sharply fell, whereas a smooth decrease was observed in the alkaline region (pH 5–7). At pH 2 (1.5 pH units below the maximum) the activity was reduced more than 97%, while at 1.5 pH units above (pH 5.0) only 69% decrease of activity was detected.

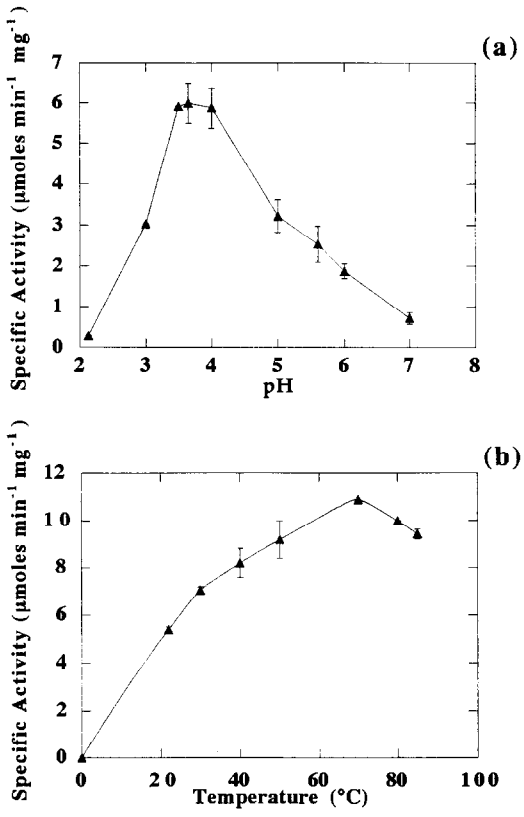

Fig. 3. Effect of pH (a) and temperature (b) on laccase activity. The standard deviation ranged from 0.00 to 0.36.

These results suggest that acidic pH values may influence not only the activity but also the stability of the enzyme (see below). Analogous behavior was observed with *T. versicolor* and *P. ostreatus* laccases. An optimum pH of 3.55 and a similar shape of the activity/pH profiles were found [15–17].

The activity of laccase gradually increased when temperature increased from 20 to 70°C, then it decreased (Fig. 3b). At 50 and 85°C, the activity was about 85% of the value measured at the optimum temperature (70°C). Being the activity evaluated by product formation at zero time incubation, it can exclude any deactivation phenomenon. Thus, the behavior illustrated in Fig. 3b indicates a flat dependence of the activity on temperature. It was confirmed by the small value (7.5 kJ mol^{-1}) of the activation energy (E_a). This low value of E_a suggests a high efficiency of laccase as a catalyst in the ABTS oxidation reaction.

The enzyme was extremely sensitive to NaN_3 (Fig. 4a). The enzymatic activity was inhibited by about 30% by 0.02 mM NaN_3, more than 80% by 0.2 mM, and totally by 2 mM of NaN_3. No inhibition by EDTA concentrations up to 3.76 mM was detected.

Azide also inhibits laccases from diverse fungi [2,16,18,19] and bacteria [20], thus suggesting similar features among fungal and bacterial laccases.

The activity of laccases in water–organic solvent mixtures has been demonstrated [4,5,16,19]. Organic solvents, however, affected differently the activity of the enzymes. A detectable effect on the activity of *Cerrena*-laccase resulted from the addition of methanol and

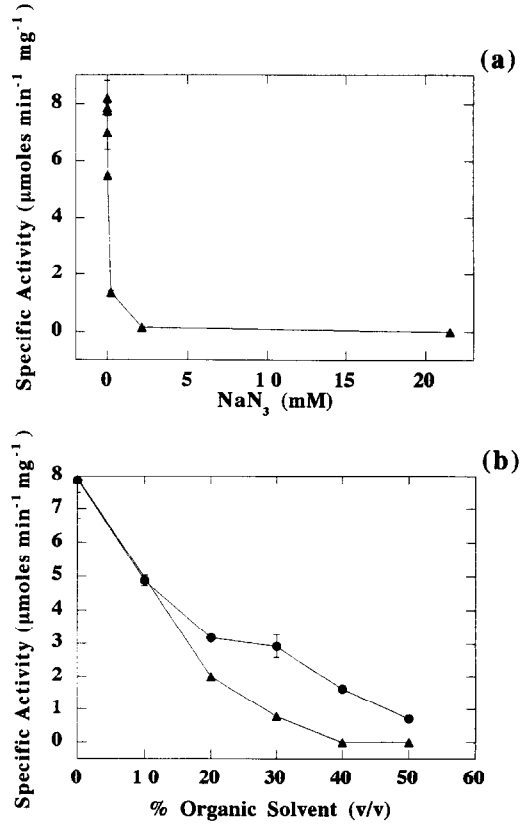

Fig. 4. Effect of NaN_3 (a) and organic solvents ((●) methanol and (▲) acetonitrile) on laccase activity. The standard deviation ranged from 0.00 to 0.60.

acetonitrile in the activity assay (Fig. 4b). Methanol at 10% decreased laccase activity by 40%, and 30% methanol inhibited it by 60%. Inhibition by acetonitrile increased abruptly from 10% at 10% solvent to 85% at 20% solvent, but did not increase further at 30% solvent. The high reduction of activity observed in the presence of acetonitrile indicate that this latter behaves as stronger inhibitor than methanol. Due to the experimental procedure adopted (direct and continuous monitoring of product formation), any deactivation by solvents might be ruled out. *Trametes*- and *Phlebia*-type laccases also showed a lower sensitivity to methanol than to other organic solvents [19].

3.2.2. Stability studies

The freezing–thawing treatment strongly affected laccase activity. One freezing–thawing cycle decreased the activity of the enzyme by 25%, whereas a reduction of more than 60% occurred after two repeated freezing–thawing cycles. The significant sensitivity to freezing–thawing processes is in agreement with the results previously reported for the inducible form of laccase from *T. versicolor* [17,21].

In Fig. 5, the log of residual activities of laccase after incubation at 50°C are reported versus deactivation time. A bi-linear behavior

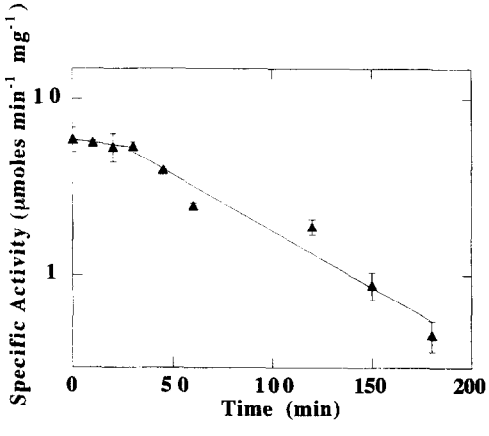

Fig. 5. Thermal deactivation of laccase at 50°C. Semi-log plot of specific activity versus time. The standard deviation ranged from 0.00 to 0.50.

was obtained. In the first period of time (about 50 min) the deactivation constant was low (0.19 h^{-1}), then it increased to 0.91 h^{-1}. After about 3 h exposure at 50°C, the activity decrease was greater than one order of magnitude.

As reported by Sadana [22] and reviewed by Gianfreda and Greco [23], the deactivation of laccase at 50°C can be described by a complex no-first-order kinetics. In particular, the initial stable phase of little activity loss, followed by an accelerating deactivation period, indicates a typically 'grace period' behavior. This 'grace period' behavior has been explained by the occurrence of enzymatically active intermediate forms in the thermal denaturation of the protein. Several phenomenological approaches have been utilized to explain a complex behavior of enzyme inactivation; they include a number of reversible (dissociation and denaturation) as well as irreversible (decomposition, aggregation and coagulation) reactions [24,25].

In contrast, a typical one-step transition was observed when laccase was exposed to proteolysis. The deactivation constant of 0.086 h^{-1} reflects the lower sensitivity of laccase to proteolytic than thermal deactivation. In fact, half-lives ($t_{1/2}$) of 8 and 0.8 h were obtained for the proteolytic and thermal laccase deactivation (the latter value was calculated on the second thermal deactivation step). Similar results were reported for other fungal laccases [21,26].

Fig. 6 shows the residual activities of laccase detected at different times of exposure to various pH values (Fig. 6a) and water or water–organic solvent (1:1, v:v) solutions (Fig. 6b). The enzyme appeared particularly stable in the pH range 3.0–6.0 and showed the greatest stability at pH 6.0, where the activity remained practically unaltered, even after > 24 h exposure. In contrast, the enzyme displayed a lower stability at extreme pH. At pH 2.2 and 7.0, about 30 min were enough to reduce the enzyme activity to 10 and 32% of the initial one, respectively. The residual activities at extreme pH values, mainly in the acidic zone (pH 2.2), confirm the data shown in Fig. 3a and are in

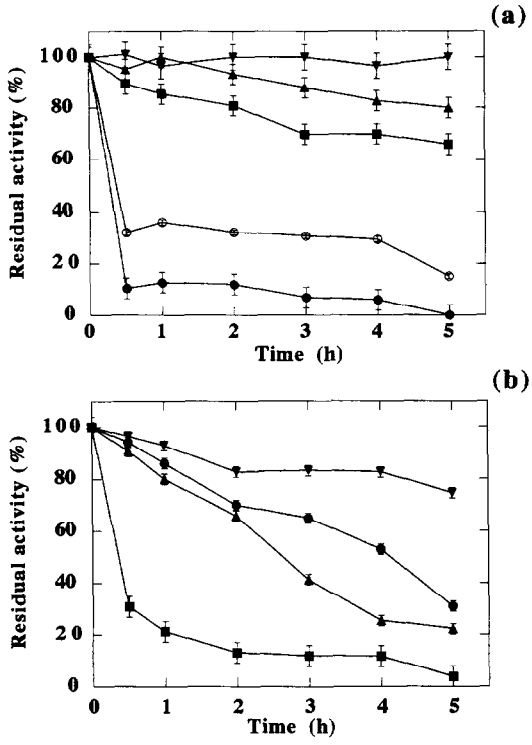

Fig. 6. Enzyme stability to various pHs (2.2, ●; 3.0, ■; 3.5, ▲; 6.0, ▼; 7.0, ○) (a) and solvents (water, ▼; acetonitrile, ▲; methanol, ●; dioxane, ■) (b). The standard deviation ranged from 0.00 to 4.00.

agreement with the findings reported for several laccases [16,21].

When the enzyme was incubated in water and 1:1 (v:v) water–organic solvent solutions (0.5% solvent concentration in the enzyme assay), the activity decreased linearly with incubation time (Fig. 6b). The greatest stability was detected in water ($t_{1/2} = 10$ h, at pH 6.0), whereas a faster inactivation occurred in dioxane solution, in which the activity reduced by 70% after 30 min incubation. A moderate stability was observed with acetonitrile ($t_{1/2} = 2.8$ h) and methanol ($t_{1/2} = 4$ h).

3.3. Detoxifying properties

3.3.1. Phenol removal

The oxidizing ability of this new laccase preparation was demonstrated with some substances of aromatic character, which behave usually as pollutants of natural environments. Table 2 reports the removal of 2,4-D, 2,4-DCP, an intermediate of 2,4-D biodegradation and four phenols, catechol, methylcatechol, tyrosol and hydroxytyrosol, which are commonly present in waste waters such as olive oil mill waste waters.

Table 2
Removal of various phenols at 25°C

Substrate	Removal (%)
2,4-D	< 2
2,4-DCP	60
Catechol	100
Methylcatechol	76
Tyrosol	66
Hydroxytyrosol	73

The standard deviation values ranged from 0 to ±3. Substrate = 0.27 mM in 100 mM citrate–phosphate buffer pH 6.0. Incubation time = 24 h. Laccase = 4 μg ml^{-1}.

A negligible activity was measured with 2,4-D. Higher oxidation ranging from 60 to 100% was determined with the other substrates.

Fig. 7 shows the enzymatic activity expressed as nmol 2,4-DCP transformed in 1 min by 1 mg enzyme at different 2,4-DCP concentrations. The behavior of the data indicates typical Michaelis–Menten kinetics. Extrapolated values of 21 nmol min^{-1} mg^{-1} and 0.35 mM, respectively, for V_{max} and K_m, were determined by

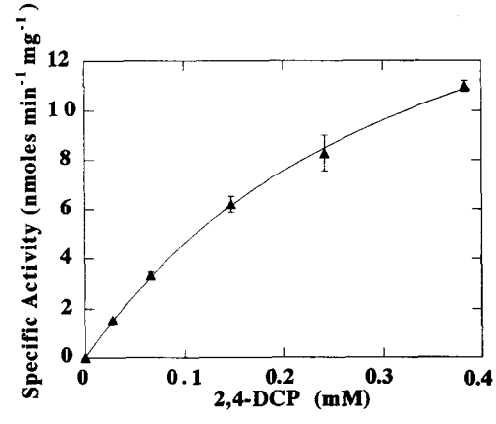

Fig. 7. Specific laccase activity versus 2,4-DCP concentration. The values of kinetic parameters were: $V_{max} = 21$ nmol min^{-1} mg^{-1}, $K_m = 0.35$ mM, The standard deviation ranged from 0.00 to 0.70.

non-linear regression analysis of the experimental data. The lower value of the Michaelis–Menten constant suggests a higher affinity of laccase for 2,4-DCP than the synthetic substrate ABTS.

By increasing 2,4-DCP concentration, the ability of laccase to transform the compound reduced from 96% at the lowest 2,4-DCP concentration utilized (0.03 mM) to about 50% at the highest one (0.34 mM).

The results described above are consistent with several studies demonstrating that phenoloxydases in general, and laccases in particular, can be used as detoxifying agents for the remediation of phenol polluted systems. Laccases from *R. praticola* and *T. versicolor* were shown to be able to polymerize different phenolic contaminants such as chlorophenols, bromophenols, methylphenols and methoxyphenols [27]. Moreover, Gianfreda and Bollag [2] demonstrated that a laccase from *T. versicolor* and a peroxidase from horseradish, immobilized on natural supports, retained high activity levels in the removal of 2,4-DCP, also in the presence of different soils.

Therefore, the use of laccases for the bioremediation of terrestrials, and aquatic systems could be envisaged. An effective and useful utilization of a catalyst, for practical application, however, needs the knowledge of the final catalyst localization and, mainly, of its possible re-usability. Consequently, when an enzyme is studied for detoxification purposes, it is a necessary prerequisite to have detailed information on the fate of the catalyst at the end of the catalytic process.

In this light, further experiments were performed for evaluating the fate of laccase after its contact with 2,4-DCP. In particular, for each 2,4-DCP concentration used in the kinetic tests (Fig. 7), the supernatants, obtained after centrifugation of the mixtures containing substrate and enzyme, were analyzed not only for the amount of 2,4-DCP transformed but also for the residual activity of laccase still present in the samples. The activity of the enzyme was tested

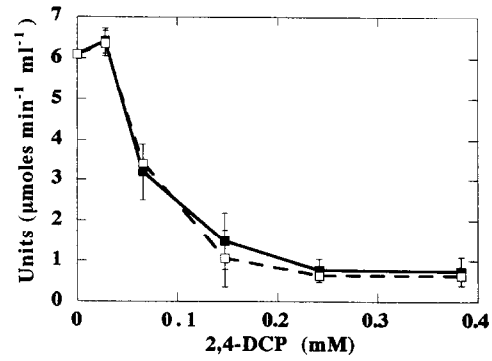

Fig. 8. Residual ABTS–laccase activities of filtered (—) and un-filtered (---) 2,4-DCP–enzyme mixtures. The standard deviation ranged from 0.00 to 0.70.

under standard conditions. Activity tests were also performed after filtering the supernatants with filters (Minisart cellulose acetate filters < 0.2 μm) that specifically adsorb 2,4-DCP, as evaluated by HPLC analyses on filtrate samples. Both the samples were analyzed for protein concentration.

Fig. 8 shows the laccase activity of filtered (continuous line) and un-filtered (dashed line) samples. The higher the 2,4-DCP concentration, the more pronounced was the loss of enzymatic activity. Furthermore, the preliminary filtration of the samples did not affect the enzyme activities.

In order to explain the results reported in Fig. 8 two hypotheses may be made: 2,4-DCP behaves as an inhibitor of ABTS–laccase activity or the enzyme is progressively inactivated or removed from 2,4-DCP–laccase mixtures, as the 2,4-DCP oxidative transformation proceeds. The first hypothesis can be ruled out because (a) ABTS-activity tests performed on the filtered samples (i.e. lacking 2,4-DCP) gave activity values similar to those obtained without sample filtration (Fig. 8, dashed and continuous lines); and (b) ABTS-activity tests performed under standard conditions, but in the presence of 2,4-DCP, showed no decrease of laccase activity at any 2,4-DCP concentration. Therefore, the results reported in Fig. 8 seem to infer that the oxidative transformation of 2,4-DCP gave rise

to the formation of polymers in which enzyme molecules were entrapped and progressively removed from the solution. However, the inactivation of the enzyme by the reaction products cannot be excluded.

This hypothesis was partially confirmed by activity tests performed on the supernatants of the phenolic–enzyme mixtures of Table 2, after centrifugation and filtration as described above. A residual laccase activity of about 55% was determined for catechol and metylcatechol mixtures. As expected, no detectable reduction was measured for 2,4-D, which was not oxidized by the enzyme. Contrasting and not easily understandable results were obtained with tyrosol and hydroxytyrosol. An increase of 18 and 60% of laccase activity was measured, respectively. This activation of laccase activity was also observed in experiments performed in the presence of humic substances from different origins (Filazzola et al., unpublished data).

The results of previous experiments seem to be of a certain interest, because they give information on the fate of laccase, when used as catalyst in a polymerization process.

When 2,4-DCP and 2,4-D were mixed together and treated with laccase, the removal of 2,4-DCP increased up to 65% whereas no effect was observed on 2,4-D transformation.

Further investigations were performed to verify the ability of ABTS to behave as co-substrate of 2,4-DCP transformation. Preliminary results seem to suggest that the removal of 2,4-DCP can be remarkably improved by the presence of ABTS as co-substrate. When 1 mM ABTS was present, 0.27 mM 2,4-DCP completely disappeared as compared to only 60% transformation measured without ABTS. ABTS promotion of chlorophenolic oxidation by laccases from *Trametes versicolor* have been reported by Roy-Arcand and Archibald [28].

In conclusion, a laccase preparation, separated and purified in high yield as a constitutive form from the fungal strain *Cerrena unicolor*, displayed catalytic properties similar to those of other fungal laccases. Furthermore, useful information on the fate of the enzyme was obtained after its application as catalyst in the polymerization of phenolic substrates. These results suggest that this new laccase preparation may have some potential for environmental applications such as water and soil detoxification processes.

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