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Stabilization studies of *Fomes sclerodermeus* laccases

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Abstract

Stability of laccase isoenzymes from a crude extract obtained from *Fomes sclerodermeus* grown on wheat bran medium was studied. The variables assessed were temperature, pH and additives. As revealed by PAGE, three bands of laccase, each with different thermal inactivation pattern, were detected in the crude extract: after 6 h at 50 °C and pH 8, Lc2 was the most resistant, while the Lc1 and Lc3 bands were almost completely inactivated. This pattern of inactivation was observed at all temperatures and pH tested. Laccase activity was more stable in the 5–10 pH range when incubated at 40 and 50 °C; at 30 °C and 24 h the enzyme remained fully active in the 3–11 pH range. The effect of additives (veratryl alcohol, trehalose, glycerol, mannitol, glutaraldehyde, $CuSO_4$ and 1-HBT) on laccase stability was tested. The stability was enhanced with $CuSO_4$ (1.25 mM), glycerol (0.2%) and mannitol (1%). The presence of both $CuSO_4$ and glycerol caused a 3-fold increase in the half-life values.

Keywords: Laccase; Stability; *Fomes sclerodermeus*; Additives

1. Introduction

Lignin biodegradation is a key step for carbon recycling in terrestrial ecosystems, where white-rot basidiomycetes play an important role in the degradation of this recalcitrant wood polymer, enabling cellulose utilization by microbial populations (Kirk and Farrell, 1987). One of the enzymes involved in this process is laccase, an oxidase that catalyzes the one-electron oxidation of polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds (Thurston, 1994).

Production of extracellular fungal laccases can be enhanced by the presence of a wide variety of inducing substances, such as copper and aromatic or phenolic compounds related to lignin and different nitrogen sources (Galhaup et al., 2002; Muñoz et al., 1997; Palmieri et al., 2000). These strategies, however, usually require the use of high-cost defined media. On the other hand, there is an increasing interest in using solid state fermentation (SSF) techniques to produce a wide variety of enzymes. Among them, laccase is one of the most studied (Fenice et al., 2003; Gómez et al., 2005; Koroleva et al., 2002). Enzyme production by SSF offers numerous advantages over liquid fermentation systems, such as higher productivity (Viniegra-Gonzáles et al., 2003), simpler technique, and lower cost. Thus, a general aim is to use the enzymes produced by means of SSF without prior purification to avoid further increase of the cost of the process. With this in mind, in this investigation we used a low-cost solid medium, envisaging the potential industrial applications of this crude enzyme. In a previous report, *Fomes sclerodermeus* cultivated on wheat bran as a substrate was a high laccase producer (Papinutti et al., 2003).

The resistance of the catalytically active protein structure toward high temperature, pH and other potentially denaturing factors is one of the chief criteria for commercialization and for industrial applications of the enzymes (Renate and Ulrich, 1999). Increase of the stability of the enzymes under harsh pH and temperature conditions by the addition of salts, polyols, sugars and others has been previously reported for diverse enzyme systems (Andrew

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et al., 2000; Costa et al., 2002; Lozano et al., 1994). Also, it has been shown that the artificial primary laccase substrates ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate)] and 1-HBT (1-hydroxybenzotriazole) have the capacity to act as cooxidants allowing the oxidation of non-phenolic lignin model compounds that are not laccase substrates on their own (Bourbonnais et al., 1997; Collins et al., 1998); in turn, these compounds have been shown to elicit the destabilization of laccase (Bourbonnais et al., 1997).

In this context, the aim of this study was: (i) to assess the inactivation kinetics of laccase obtained from *F. sclerodermeus* BAFC 2752, a white-rot basidiomycete, grown on wheat bran and (ii) to study the stabilization of crude laccase against detrimental environmental conditions by means of various additives focusing on its use without prior purification.

2. Methods

2.1. Organism and culture conditions

F. sclerodermeus (Léveillé) Cooke BAFC 2752 (Science Faculty collection in Buenos Aires University) was maintained in malt extract-agar medium at 4 °C.

The organism was cultivated in a basal medium containing 4 g wheat bran and 16 ml distilled water (Papinutti et al., 2003). The inoculation was carried out by using three agar cubes (0.25 cm^2) from the advancing margin of the colony. Enzymatic crude extract was obtained by adding distilled water to the solid culture medium 15 d after inoculation, to reach a ratio of 1:5 (w/v); this suspension was then stirred for 20 min, filtered and centrifuged (Papinutti et al., 2003). The supernatant was stored at −20 °C until used as the enzymatic source in all the experiments.

2.1.1. Enzyme and stability assays

Laccase activity was determined spectrophotometrically at 420 nm ($\varepsilon_{420} = 36\ mM^{-1}\ cm^{-1}$) and 30 °C by using 0.5 mM ABTS in sodium acetate buffer pH 3.6 as a substrate (Bourbonnais et al., 1995). Stability assays were carried out in 50 mM citrate–phosphate–borate buffer (pH 2–11). Sealed tubes containing 2 U of laccase in a total volume of 2 ml were incubated at 30, 40 and 50 °C and the activity was measured periodically. The effect of various additives after 24 h at 40 °C and pH 4.5 were tested at the following concentrations: veratryl alcohol (VA) (0.2%, 1%, 5% and 10%), glycerol (0.2%, 1%, 5% and 10%), trehalose (0.05%, 0.1%, 0.5% and 1%), 1-hydroxybenzotriazole (1-HBT) (0.1, 0.5, 1.25 and 2.5 mM), $CuSO_4$ (0.05, 0.1, 0.5 and 1.25 mM), mannitol (0.05%, 0.1%, 0.5% and 1%) and glutaraldehyde (0.05%, 0.25% and 1%). All chemicals used are commercially available and were used without further purification.

Native 12% pore-size PAGE was performed to determine the number of laccase bands and their thermal inactivation pattern. α_2-macroglobulin (180 kDa), β-galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa) and triosephosphate isomerase (26.6 kDa) were used as molecular mass standards (Sigma). After different periods of thermal treatment (30, 40 and 50 °C) and pH (4, 6, 8 and 10), enzyme extracts were concentrated 20-fold. The lanes of the gels were loaded with an activity of 10 mU. The bands were visualized by incubating the gels for 10 min at 37 °C in the presence of 0.5 mM ABTS in sodium acetate buffer pH 3.6 as substrate.

2.2. Decolorization activity

Decolorization activity toward malachite green (MG) after 2 h incubation period was spectrophotometrically estimated as described by Papinutti and Forchiassin (2004). Samples containing crude laccase and collected at different incubation intervals were dialyzed and concentrated around 20-fold prior to use. All reaction tubes contained 10 U of laccase activity in a total volume of 3 ml.

2.3. Data analysis, half-lives and fit to the biexponential equation

Effect of additives and pH data are from triplicate independent experiments and the results represent the mean with less than 10% of standard error.

For half-live (t_m) calculations, the mean values of activity as function of time were fitted to the biexponential equation (Eq. (1)) with three parameters as proposed by Aymard and Belarbi (2000). The processing of data was performed by direct curvilinear regression, without any prior transformation by using the software STATISTICA® (version 5.1) (StatSoft, Tulsa, Okla, USA)

$$Y = b_1 \exp(-b_2 X) + (1 - b_1) \exp(-b_3 X) \quad (1)$$

where Y is the variable response (remaining activity) expressed as the ratio of the activity measured at time X to the initial activity, b_i are the regression coefficients (parameters) given by the model, X (time) is the independent variable. Laccase activity was assayed at time intervals until it was 10% or less of the initial activity. Remaining activity was expressed as A_t/A_0, where A_t is the activity at time t and A_0 is the initial activity.

3. Results

3.1. Effect of pH and temperature on enzyme stability

F. sclerodermeus crude laccase obtained under the conditions outlined above and stored at −20 °C was completely stable for over 2 years. When the enzyme was subjected to a pre-incubation period of 30 min at 40 °C, the activity increased by approximately 50% compared to non-incubated laccase. Consequently, all subsequent experiments were performed on the basis of this treatment, which was

taken as time zero. The fractions were incubated in buffers ranging from pH 2 to pH 11 at 30 °C, 40 °C and 50 °C and then assayed for activity (Fig. 1). At 30 °C (Fig. 1A), laccase remained fully active in the 3–11 pH range following 24 h incubation. Assays carried out at 40 °C (Fig. 1B) rendered completely different stability profiles: for a 1 h incubation, laccase retained >75% of its activity between pH 5 and 10, with maximal stability at pH 8, after 6 and 24 h, the enzyme showed a different stability pattern: in all cases, the residual activity was below 75%, and the optimum shifted to pH 7. At 50 °C (Fig. 1C), the enzyme residual activity was at all times below 75%, even after a relatively short (0.5 h) incubation period. pH optima of stability were again correlated to the incubation period: after 0.5 h, maximal activity was recorded at pH 7, while after 5 and 24 h maximal activities were detected at pH 5.

3.2. Pattern of isoenzyme inactivation

In an attempt to gain further insight into the inactivation kinetics of laccase activity, 12% polyacrylamide gel electrophoreses (PAGE) of crude enzyme extracts subjected to different thermal treatments and pH were performed. All lanes in the gel showed the same pattern of inactivation therefore only one of the treatments is shown. Fig. 2A shows that the untreated fraction (time zero) contains at least three isoenzymes. The molecular masses of the three bands were roughly estimated being of 148, 47 and 36.5 kDa for Lc1, Lc2 and Lc3 respectively (data not shown). After 1 h at 50 °C (pH 8), both Lc1 and Lc3 bands became less visible, and after 6 h of incubation at 50 °C

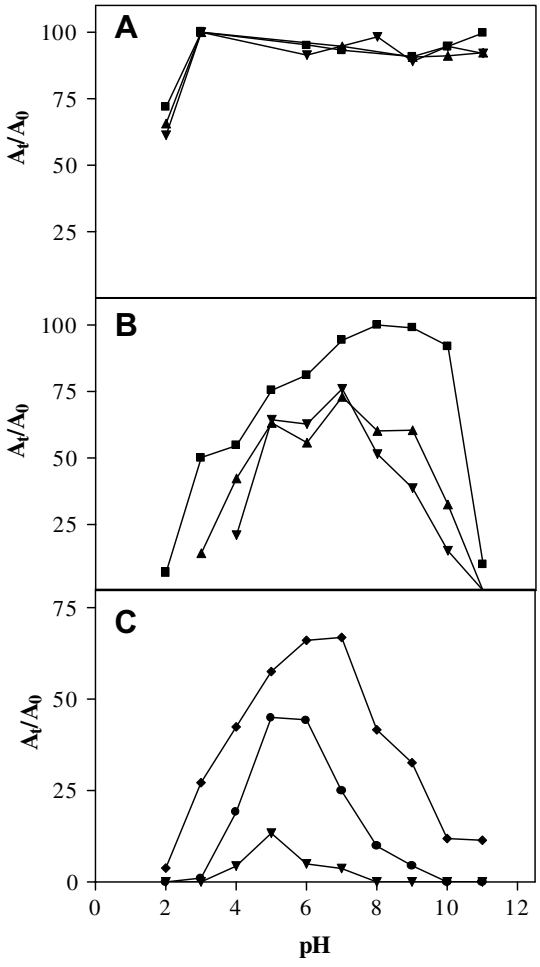

Fig. 1. pH profile of laccase inactivation at 30 (A), 40 (B) and 50 °C (C). ◆0.5, ■1, ▲6, ●5, ▼24 h. Data are mean values from triplicate experiments, SD was less than 10%.

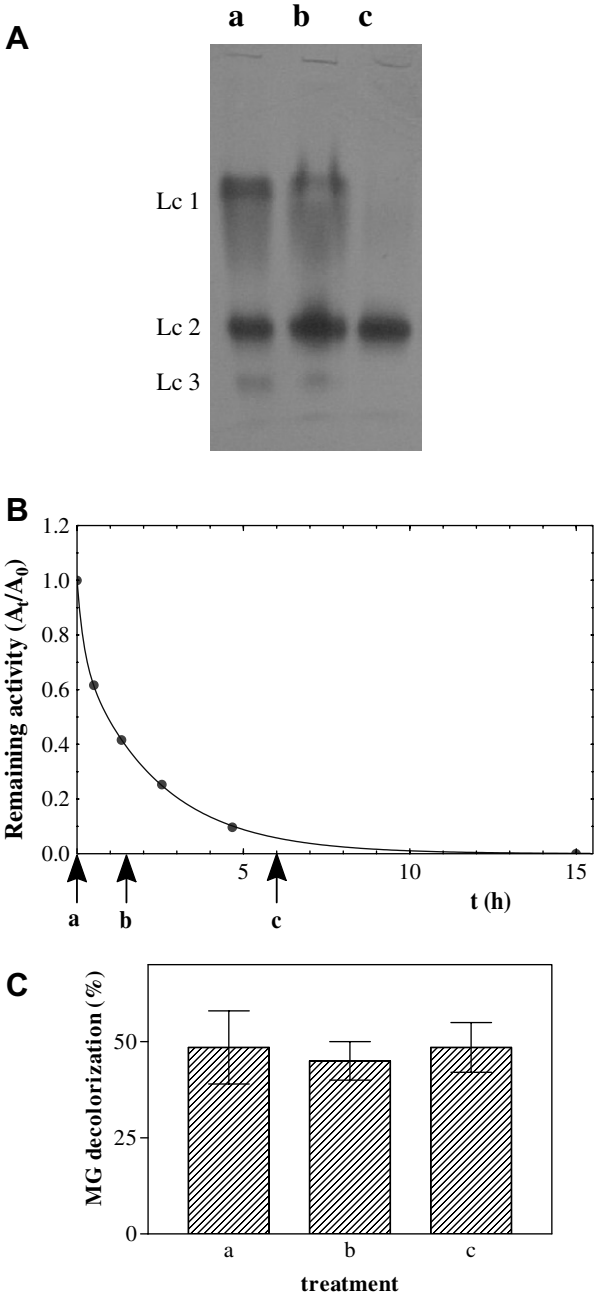

Fig. 2. Thermal responsiveness and inactivation kinetics of laccase. (A) Zymogram of laccase. Enzymatic crude extract was incubated at 50 °C and pH 8 for 0 (a), 1.5 (b) and 6 h (c), the fractions concentrated 20-fold, and applied to PAGE. (B) Inactivation kinetics of laccase at 50 °C. R^2 value of the fitted curve was 0.99. Incubation times used for the zymogram are indicated. (C) Decolorization of MG after 2 h incubation period with 10 U of crude laccase. Data are expressed as mean values ±SEM.

they were practically undetectable when revealed through ABTS oxidation, on the other hand, the central (Lc2) band remained as the unique band visualized for the duration of the incubation period. This fact could explain the drastic decrease of activity (75%) during the first 2 h, after which it decreased more slowly. The inactivation between 0 and 6 h is accompanied by the complete disappearance of the Lc1 and Lc3 bands, while the Lc2 showed the lowest rate of inactivation, thus the remaining activity (10%) observed in the 6 h incubation period, was due to Lc2 band (Fig. 2B).

Irrespectively of the crude laccase used (a, b or c), decolorization percentages of MG did not show differences (Fig. 2C).

3.3. Effect of additives on enzyme stability

The crude extract may contain proteases and a wide range of compounds that could interfere with the stability of the enzymes along with intrinsic mechanisms like unfolding or depletion of a prosthetic group. In this work, we used various compounds to increase the stability of laccase focusing on the avoidance of hydration as well as the depletion of copper, which were described as important events on enzyme deactivation. The effect of the cooxidant 1-HBT was evaluated also. Table 1 shows how different compounds affect laccase stability after 24 h at pH 4.5 and 40 °C. An increase of additives concentration was in general associated with a decrease in laccase activity under the above conditions, with the exception of $CuSO_4$ and mannitol. When this salt was added at a relatively high concentration (1.25 mM), the enzyme maintained 81% of its activity, whilst the control retained 57% of its activity.

Taking into account the results described above, a more comprehensive study was carried out in order to better understand how copper affects laccase stability (Table 2). Half-life time (t_m) value for each treatment was estimated from the fitted curve, all r^2 values were $\geqslant 0.9$ this means that at least the 90% of the variability in laccase activity observed can be accounted for by the Eq. (1). At pH 4.5 and 40 °C, the highest t_m values were obtained in the presence of $CuSO_4$ 1.25 mM/glycerol 0.2% ($t_m = 114$ h) and $CuSO_4$ 1.25 mM ($t_m = 71$ h), while at 50 °C $CuSO_4$ 2.5 mM/glycerol 0.2% and $CuSO_4$ 2.5 mM stimulated the best stabilization effect. Half-lives values of the controls were 40 and 9.81 h at 40 and 50 °C, respectively.

Table 1
Effect of additives on laccase stability, after incubation for 24 h at pH 4.5 and 40 °C. SD was less than 10%

Additive	Concentration	Remaining activity (A_t/A_0)
Veratryl alcohol		
	0.2%	0.63
	1%	0.51
	5%	0.26
	10%	0.11
Glycerol		
	0.2%	0.60
	1%	0.50
	5%	0.49
	10%	0.48
Trehalose		
	0.05%	0.61
	0.1%	0.35
	0.5%	0.04
	1%	0.07
1-HBT		
	0.1 mM	0.59
	0.5 mM	0.39
	1.25 mM	0.11
	2.5 mM	0.07
$CuSO_4$		
	0.05 mM	0.58
	0.1 mM	0.53
	0.5 mM	0.73
	1.25 mM	0.81
Mannitol		
	0.05%	0.55
	0.1%	0.53
	0.5%	0.54
	1%	0.78
Glutaraldehyde		
	0.05%	0.46
	0.25%	0.35
	1%	0.37
Control		0.57

Table 2
Half-life time of crude laccase treated with different combinations of $CuSO_4$ and glycerol at pH 4.5

$CuSO_4$ (mM)	Glycerol (%)	t_m (h)	
		40 °C	50 °C
1.25	0.2	114	14.51
	0	71	12.53
2.5	0.2	53	15.46
	0	44	14.01
5	0.2	30	9.35
	0	43	7.98
0	0.2	36	8.91
	0	40	9.81

Data are from the fitted curves to Eq. (1), all r^2 values were $\geqslant 0.9$.

4. Discussion

This investigation used crude supernatants from *F. sclerodermeus* to assess the stability of laccase activity. Ligninases production by SSF has been extensively studied, most of them focused on the optimization of the process and the screening of high-producer strains with the objective to be used in bioremediation processes. An alternative to the use of enzymatic crude, could be the cultivation of the fungus on the contaminated substrate, but the time necessary for growth is another factor to be considered. In addition, the toxicity of compounds further delays the fungal growth. SSF has a considerable potential for the production of laccase, particularly where the crude fermented

product may be used directly as the enzyme source. Thus, to utilize crude laccases more efficiently for these biotechnological applications it is necessary to better understand the properties of this enzyme at a physicochemical and kinetic level, rather than the requirement of large amounts of enzyme production.

The results of this study indicate that different factors influencing the enzyme stability such as the presence of proteases and other contaminant compounds, as well as intrinsic factors of the protein, are expressed under the conditions assayed. The stability of purified proteins seems to be dependent upon various extrinsic factors, namely, pH, the buffer, and temperature; these, in turn, may influence the deactivation of enzymes by affecting intrinsic mechanisms.

Laccase can be used for biotechnological purposes, such as the degradation of a wide range of toxic aromatic compounds (Pointing, 2001). Because of their recalcitrance in the environment, the remediation of these compounds using traditional techniques can often be extremely difficult. In addition, owing to their toxicity, PAHs are frequently not amenable to microbiological biodegradation, hampering the implementation of in situ bioremediation strategies (e.g. direct inoculation of fungus) as plausible reclamation options. Therefore, the use of enzymatic crude extracts may represent feasible, cost-effective solutions. However, the successful realization of such processes is not only dependent on high enzyme activities, but on their stability as well.

We focused our investigation on the enzyme stability without a prior purification step using the decay model outlined in a recent publication that dealt with the deactivation of enzymes in solution with other enzyme contaminants (Aymard and Belarbi, 2000).

The effect of increased activity after a pre-incubation of the crude is in agreement with previously published work, where a similar effect was observed for other fungal laccases e.g. from *Chaetomium thermophilium* (Chefetz et al., 1998), *Coriolus hirsutus* and *Coriolus zonatus* (Koroleva et al., 2001).

F. sclerodermeus crude extract laccase activity was much more stable than other purified fungal laccases, indicating an advantage from the standpoint of the purification cost. For example laccase of *Pycnoporus sanguineus* shows a rapid loss of activity at temperatures above 35 °C (Pointing et al., 2000); in *Pleurotus sajor-caju* losses of 40% and 75%, respectively were recorded after 5 min pre-incubation at 55 and 65 °C (Lo et al., 2001); laccase of *Agaricus blazei* at 40 °C retained 50% of activity after 25 min but only 10% after 120 min (Ullrich et al., 2005).

The PAGE of the different thermal treatments of the crude suggested that the alternative forms of laccase display distinct thermal sensitivity patterns, which could account for the gradual loss of activity of the enzyme toward the final stages of incubation (Fig. 2B). Probably purified Lc1, Lc2 and Lc3 also have different affinity toward the dye, MG, but these differences were not detected in the crude and could be due to the major presence of Lc2 (visible by band intensities) in the crude along with low but measurable activity in the Lc1 and Lc3.

The analysis of the data by means of the biexponential equation used in this work is a phenomenological description of the enzyme decay rather than a molecular identification of the mechanisms involved in the process. These mechanisms are difficult to elucidate due to the presence of many compounds in the crude extract.

Previous studies have shown that subjecting laccase to high temperatures can cause the release of copper ions. The depletion of copper ions not only inactivated the enzyme but also probably uncoupled the domains of the copper-depleted protein; this depletion, in turn, was shown to be reversible (Koroleva et al., 2001). These findings could be related to the observed stabilizing effect of this cation under the tested conditions. Probably, as a result of the reversibility of the depletion, the presence of copper in solution could delay its release from the protein, preventing its inactivation for a longer period.

Despite the possible presence of proteases, the half-lives obtained for *F. sclerodermeus* laccase were similar to those obtained for purified laccase from *Chaetomium thermophilium* which is a thermophilic fungus showing half-lives of 24 and 12 h at 40 and 50 °C, respectively (Chefetz et al., 1998). Protein stabilization by low molecular weight solutes is a widely used strategy. Compounds like sugars (Baptista et al., 2000), polyols (Costa et al., 2002) and salts (Baptista et al., 2000) may increase the thermal stability of enzymes. In particular, the role of polyols in enzyme stabilization is as a water-structure maker, which depresses the hydration of the enzyme and hence its denaturation (Costa et al., 2002; Lozano et al., 1994). Thus, because of the observed enhanced stability at 40 °C in response to the combination of both copper (1.25 mM) and the widely used glycerol, it is possible that laccase inactivation operates through at least two events: the lack of a hydration-depresser compound and the depletion of the copper ions.

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