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Carboxylic acids used in common buffer systems inhibit the activity of fungal laccases

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ABSTRACT

The inhibition of fungal laccases by carboxylic acids has been studied. Steady-state kinetics performed with recombinant laccase from *Trametes villosa* and ABTS as a substrate revealed an s-linear, i-parabolic mixed inhibition type for acetate, while formate exhibited a linear, non-competitive inhibition type. Although Ki values were several orders of magnitude higher than those for azide, inhibition levels for acetate were substantial (10–60% of initial activity) at concentrations commonly used in routine laccase assays (10–100 mM). The first order inactivation rate constant for acetate was low (0.39 min^{-1}) and similar to that of propionate and butyrate. However, inhibition by di- and tricarboxylic acids was considerably less pronounced (up to 20% at 100 mM) and instantaneous. Therefore, citrate and particularly succinate appear much more suitable for laccase assays and applications than acetate which should be avoided. Wild-type laccases from several *Trametes* species were found to be inhibited to a similar extent, while laccase from *Pleurotus eryngii* and some other species were not affected or even stimulated by carboxylic acids. These results collectively suggest that fungal laccases do not share a common structural feature responsible for their inhibition by carboxylic acids.

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1. Introduction

Laccase (EC 1.10.3.2; p-diphenol:dioxygen oxidoreductase) is one of the oldest enzymes ever mentioned being described as the active principle in the sap of the lacquer tree (*Rhus vernicifera*) by Yoshida in 1883 [1], and being characterized as a metal containing oxidase in 1895 [2,3]. Since then laccase-like enzymes have been detected in plants [4,5], fungi [6], insects [7,8] and even in some bacteria [9,11,12]. However, laccases from fungi are the most thoroughly investigated ones.

Unlike many other enzymes that are specific for a single substrate, laccase catalyses the oxidation of a large variety of organic and even inorganic substrates. Monophenols, o-diphenols, p-diphenols, polyphenols, methoxy-substituted phenols, and diamines are primary laccase substrates, but also non-aromatic compounds such as ascorbate, ABTS (2,2′-azino-bis(3-ethybenzthiazoline-6-sulphonate)) and metal complexes such as ferrocene are oxidized. With the utilization of low molecular weight compounds as so-called “redox mediators” the substrate range can even be extended to compounds that are not laccase substrates *per se* [13].

The oxidation of substrates by laccase takes place with the concomitant four-electron reduction of oxygen to water [14]. The use of oxygen as the terminal electron-acceptor, its reduction to water, the tolerance of organic solvents [15], as well as the wide substrate range collectively make laccase an ideal catalyst for biotechnological applications. Therefore, the use of laccase has been tested in many fields of application such as pulp bleaching [16], wood modification [10], bioremediation and waste water treatment [17,18], as a detoxification agent during the production of bioethanol from renewable raw materials [19], for the organic synthesis of dyes and drugs [20,21], and for the clarification of wine [22].

Laccase belongs to a group of enzymes termed “large blue copper oxidases”. Compared to the other blue copper oxidases, laccases represent the simplest form of a catalytic center comprising four copper atoms in their oxidized state. These copper atoms are historically classified as T1, T2, and T3 according to their spectroscopic and electronic properties [23,24]. There is one T1 copper that is highly conserved and responsible for the oxidation of the substrate. One T2 and two T3 copper atoms built up a trinuclear cluster where the reduction of atmospheric oxygen to water takes place. In its fully reduced state the enzyme undergoes a conformation change that makes the T2 copper accessible for complexation with halide-like substances [25] blocking the internal electron transfer from T1

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copper to the trinuclear cluster. Therefore, typical inhibitors for laccase are halogenides or pseudo halides such as azides or cyanides that are able to form stable copper complexes. Only iodides have a redox potential low enough to be oxidized to iodine by laccases [26]. The degree of inhibition depends on the accessibility of the trinuclear cluster which correlates with the size of the chelating ion (inhibitor) species [26]. Therefore, a decrease in the potency of inhibition can be observed from F^- to Br^-. Also hydroxyl ions are able to form a pH dependent complex with the T2 copper. This effect in combination with the higher oxidation rate of phenolic substrates at higher pH values often leads to a bell shaped activity profile of laccase with increasing pH [27], while non-phenolic substrates such as ABTS show increased oxidation rates with lower pH. The inhibitory effect of substances containing a sulfhydryl group such as thioglycolic acid, cysteine or diethyldithiocarbaminic acid is controversial. They have often been used as inhibitors for the characterisation of laccases from different sources [6,28–30] but it has later been shown that the inhibitory effect may be due to the interaction of these substances with the substrate used for activity testing or with products formed thereof [31]. Other laccase inhibitors described in the literature are metal ions (e.g. Hg^{2+}), EDTA or quaternary cationic ammonium compounds. At very low or very high pH values laccases are also inhibited which may be ascribed to ionizable amino acids in the catalytic center [32]. On the whole it can be said that none of these inhibitors are specific for laccase—their application for laccase originates from results obtained with other metalloenzymes [31].

Very little information exists on a possible inhibition of laccase by carboxylic acids [33] and, therefore, they are still commonly used in buffer systems for assaying laccase activity. Here we describe the interaction of laccases with such carboxylic acids and show that they can have a detrimental inhibitory effect on the activity of the enzyme.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated all chemicals were at least analytical grade and obtained from Sigma–Aldrich (St. Louis, MO, USA). Acetic acid, citric acid, formic acid, butyric acid, lactic acid, glycolic acid, Na_2SO_4, agar-agar and $MgSO_4 \cdot x7H_2O$ were from Merck (Darmstadt, Germany). Peptone from meat (peptic digest), succinic acid, propionic acid and malt extract agar were from Fluka (Buchs, CH). In some experiments 250 mM acetate buffer was prepared from high purity acetic acid (≥99.99%) and NaOH (99.998%) obtained from Sigma–Aldrich.

2.2. Standard laccase assays

Laccase activity was defined as the amount of enzyme required to oxidise 1 μmol of ABTS per minute at 30 °C. Unless mentioned otherwise, the assay contained sodium succinate (800 μl, 10 mM, pH 5.0), ABTS (100 μl, 4 mM) and prediluted laccase (100 μl). ABTS oxidation was monitored at 420 nm (ε_{420} = 43.2 cm^2 μmol^{-1}). The oxidation of 2,6-dimethoxyphenol, when used as a substrate, was monitored at 468 nm (ε_{468} = 14.8 cm^2 μmol^{-1} [34]). In some experiments laccase activity was monitored through dissolved oxygen consumption using the Oxygraph System with a DW1/AD electrode chamber (Hansatech Instruments, UK).

2.3. Laccase sources and production

Unless stated otherwise, experiments were conducted with the recombinant laccase Lcc1 from *Trametes villosa* expressed in *Aspergillus oryzae* [35] that was obtained from Novo Nordisk (Bagsværd, Denmark; Product No. 44008). Wild-type laccase from *Trametes pubescens* CBS 696.94 was produced by cultivating *T. pubescens* according to Galhaup and Haltrich [36] with slight modifications. Culture filtrates were collected and concentrated on a Minisette tangential flow device (Pall Life Sciences) using a 10 kDa cut-off Omega membrane. Precipitations were removed by centrifugation for 15 min at 10,000 × *g*. Wild-type laccases from other fungi were produced as just described for *T. pubescens* except that the cultivation temperature was 25 °C and no $CuSO_4$ was added to the basal medium.

2.4. Laccase purification

Recombinant laccase Lcc1 and wild-type laccase from *T. pubescens* were both purified on Q-Sepharose Fast Flow (Amersham-Pharmacia) equilibrated in 20 mM sodium acetate (pH 5.0). Laccase was eluted at 3 ml min^{-1} using a linear gradient of 0–0.3 M NaCl in the same buffer. Two laccase isoforms (LAP1, LAP2) were resolved by Q-Sepharose with *T. pubescens* culture fluid, and only fractions containing the predominant isoform, LAP2, were further purified and used in the experiments. As expected, only one isoform was found with the recombinant laccase. The pooled laccase fractions were again concentrated using a 10 kDa cut-off Omega membrane in a 150 ml stirred cell (Pall Life Sciences) and then subjected to size exclusion chromatography (SEC) on Bio-Gel 100 (BioRad) equilibrated in 20 mM acetate (pH 5.0) containing 100 mM NaCl. The column was eluted at 1 ml min^{-1} and fractions containing laccase activity were again pooled, concentrated on a Microsep 10K Omega membrane (Pall Life Sciences) and kept at 4 °C until further use. Both purified laccases had an A_{280}/A_{610} absorption ratio of ∼15 indicating that they were of high purity [35]. Laccases from other fungi were partially purified by SEC on Macroprep SE 100/40 (BioRad) equilibrated in 0.1 M sodium citrate buffer (pH 5.0). Active fractions were pooled and concentrated as above.

2.5. Steady-state kinetics

Unless stated otherwise, *T. villosa* recombinant laccase was used to determine the activity of laccase in various buffer systems at 30 °C using ABTS as the substrate. The buffer systems were carboxylic acids in the range of 10 mM and 250 mM with sodium as the counter ion, and one synthetic buffer (McIlvaine buffer; 0.1 M di-sodium phosphate adjusted to the desired pH with 0.05 M citric acid) at pH values of 3.0, 4.0 and 5.0. The buffers were only used in a pH region of ±1 around the pK_a of the corresponding carboxylic acid. Each buffer concentration was individually prepared and accurately adjusted to the desired pH value.

The time course of laccase inactivation by carboxylic acids was determined by preincubating laccase from 1 min to 37 min at 30 °C. Residual activity was then measured by adding ABTS. Rate constants for laccase inactivation were calculated by fitting the data points by non-linear regression to the equation $A = A_r + (A_0 - A_r)\,e^{-kt}$ for exponential decay, where A, A_r, and A_0 are the actual, residual, and initial laccase activities, respectively, t is the reaction time, and k is the first order inactivation rate constant.

Kinetic constants of the uninhibited enzyme were determined in 5 mM succinate buffer rather than in pure water to ensure reproducibility of measurements. A preliminary experiment showed that the difference of laccase activity in 5 mM succinate at pH 4.0 and pure water adjusted to pH 4.0 with H_2SO_4 was less than 3%. The mechanism of inhibition by formate and acetate was determined by recording the initial velocity of ABTS oxidation at varying ABTS concentrations and at four different acetate or formate concentrations at pH 4.0. The enzyme was equilibrated for at least 30 min (which was found to be sufficient to reach inhibition equilibrium

in the respective buffer) before the reaction was started by adding the substrate. The inhibition kinetics for sodium azide were also determined for comparison.

The inhibition constants were determined graphically according to Dixon and Webb [37] and Cornish-Bowden [38]. The results and the inhibition mechanisms derived from this classical approach were cross-checked against calculated values that were obtained by fitting the data by non-linear least-squares regression to the built in equations of the Sigmaplot Enzyme Kinetics module (Sigmaplot 9.0) where the regression coefficient is used to determine the best fit. The molar enzyme concentration required for calculating k_{cat} was determined spectrophotometrically from the extinction of the purified laccase at 615 nm ($\varepsilon = 5.3\,cm^2\,\mu mol^{-1}$).

2.6. Substrate and product dependency

To determine whether the amount of substrate available for oxidation is lowered by unwanted side reactions, 0.03 mM ABTS was incubated once with 250 mM acetate buffer and once with 250 mM citrate buffer and the extinction was monitored until a stable end-point was reached.

Enzyme-free ABTS cation radicals ($ABTS^{+\bullet}$) were prepared as described by Majcherczyk et al. [39] via oxidation with laccase in 10 mM succinate buffer at pH 5.0. The enzyme was removed by ultrafiltration. To ensure the complete removal of residual catalytic activity, the obtained solution was heated for 30 min slightly below its boiling point. The dark green solution was diluted 1:9 either with water or with 250 mM sodium acetate buffer and the change of absorption at 420 nm was followed. It has been shown elsewhere [39] that the coloured ABTS radical cation is stable for at least 22 h under acidic pH values.

2.7. Ionic strength dependency

A possible effect of the varying ionic strengths in buffer systems was elucidated by comparing the activity of laccase in assays containing either 250 mM acetate buffer (ionic strength 43 mM), 250 mM formate buffer (ionic strength 177 mM), or 250 mM acetate buffer brought to the same ionic strength as the formate buffer by adding 6.062 g/l Na_2SO_4.

3. Results and discussion

Initially we have observed unsatisfactory linearity and reproducibility in routine assays for fungal laccase activity. These problems were then traced back to a slight but significant inhibition of laccase by carboxylic acids that are commonly used as buffering substances in laccase assays and, therefore, we examined this effect in more detail.

Fig. 1 shows the time-dependent inhibition of laccase activity by monocarboxylic acids. Formate instantly and completely inhibited laccase activity, while inhibition by acetate was slow and significantly less extensive under the same conditions suggesting that the inhibition mechanisms of formate and acetate differ from each other. When formate is disregarded, the extent of inhibition is increasing with chain length from acetate to butyrate. However, the inactivation kinetics were nevertheless inconsistent within this group: inactivation followed first order kinetics with similar rate constants for acetate (0.390 min^{-1}, with $R^2 = 0.9980$) and propionate (0.505 min^{-1}, with $R^2 = 0.9978$), while the rate constant for butyrate was significantly lower (0.160 min^{-1}, with $R^2 = 0.9989$), possibly indicating the onset of steric hindrance. Therefore, a clear-cut correlation between the degree of inhibition and the size of the inhibitor molecule, as it has been reported previously for laccase inhibition by lower fatty acids [33], and as it is well known for the

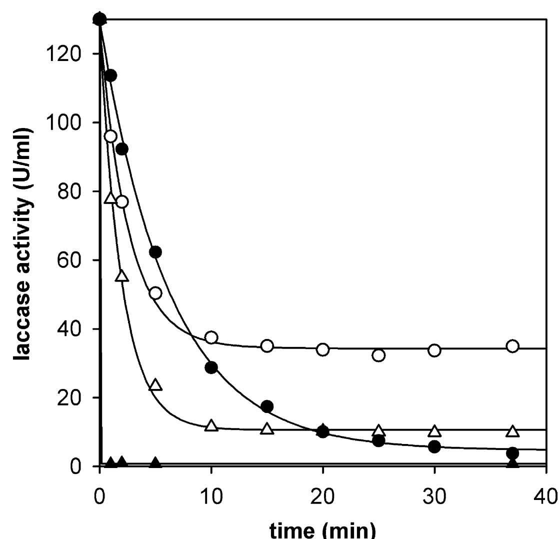

Fig. 1. Laccase activity in 250 mM acetate (○), 250 mM propionate (△), 250 mM butyrate (●), and 250 mM formate (▲) at pH 4.0 with an initial activity of 130 U/ml as measured in 5 mM succinate at pH 4.0. Curve-fitting was obtained by non-linear regression assuming first order kinetics.

inhibition of laccase by halides [26], apparently does not exist for the inhibition of laccase by carboxylic acids.

When laccase was first inhibited by preincubation in 250 mM acetate or 50 mM formate at pH 4 for 30 min and then diluted 1:1000 with water followed by incubation for another 30 min, the enzyme regained at least 95% of its initial activity as measured under standard conditions. This means that the inhibition process for acetate and formate is reversible. This has also been shown for the inhibition of laccase with halides [26].

Fig. 2 shows that the time profile of laccase inhibition in di- and tricarboxylic acids was different from that observed with monocarboxylic acids. All of the tested acids showed moderate inhibition from 20% to 40% but inhibition was almost instantaneous. The rate constants were estimated to be well above 50 min^{-1} and could therefore not be calculated from standard spectrophotometric assay data.

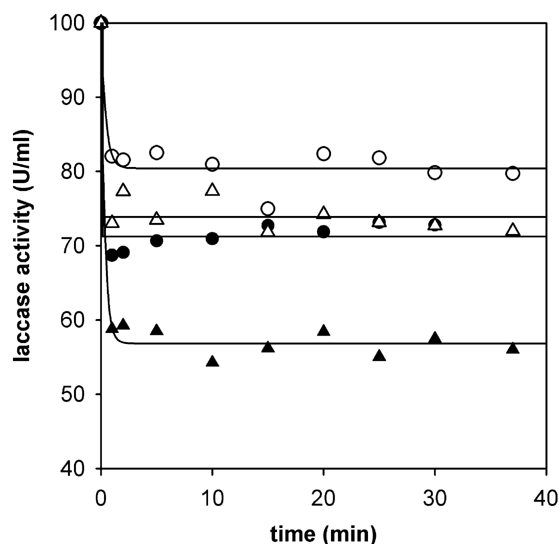

Fig. 2. Laccase activity in 250 mM succinate (○), 250 mM malate (△), 250 mM citrate (●), and 250 mM tartrate (▲) at pH 5.0 with an initial activity of 100 U/ml as measured in 5 mM succinate at pH 5.0. Curve-fitting was obtained by non-linear regression assuming first order kinetics.

One possible explanation for the observed differences between mono-, di- and tricarboxylic acids results from literature data demonstrating at least two potential binding sites for anions in laccase. Firstly, anions may bind to the highly charged, coordinatively unsaturated trinuclear cupric cluster [40] as it is known for halides or pseudo halides [26]. Secondly, anions may also interact with protonated side chains of amino acids such as those of lysine, histidine or arginine. *T. versicolor* laccase, for example, has eight lysine residues and at least four of them are located on or near the surface of the enzyme [41]. Due to their size and electronic properties, di- and tricarboxylic acids may not be able to enter the active site of laccase and preferably interact with the surface of the enzyme molecule, while monocarboxylic acids may be able to interact with the trinuclear cluster resulting in a diffusion-controlled, slow inhibition type. However, at the current state of knowledge, these implications remain rather speculative.

Table 1 summarizes laccase activity data obtained in various buffer systems at different pH values and may be useful to determine the most suitable carboxylic acid based buffer for *Trametes* laccases at a desired pH value and buffer strength. For example, citrate appears to be the ideal choice for assaying laccase at pH 4 and below, irrespective of the chosen buffer concentration. However, succinate buffer at concentrations up to 100 mM may be preferable at pH 5. Overall it can be said that carboxylic acids with more than one carboxy group are preferable buffer compounds for laccase assays not only because of their relatively minor inhibitory effect but also because they inhibit instantly contrary to monocarboxylic acids. Apparently time-independent inactivation was also observed with the synthetic McIlvaine buffer, but the extent of inactivation was high (around 40%) independent of the chosen pH. Table 1 also shows that α-hydroxy carboxylic acids – glycolate and lactate – were relatively strong inhibitors of laccase activity. Glycolate and lactate exhibited slow first order inactivation rates similar to those of their monocarboxylic acid homologues (data not shown). Table 1 further shows that the extent of inactivation was relatively independent of pH and therefore also independent of ionic strength. This was confirmed for acetate in a control experiment in which the ionic strength of 250 mM acetate at pH 4.0 was brought to the same value as that of 250 mM formate at pH 4.0 by addition of 0.1 M Na_2SO_4. The extent of inhibition was almost the same with (74.7%) and without (75.3%) Na_2SO_4 and can therefore not be attributed to differences in ionic strength in the various buffer systems.

Table 1
Residual activity [%][a] of recombinant *T. villosa* laccase after preincubation for 37 min in various buffer systems.

	10 mM	50 mM	100 mM	250 mM
pH 3.0				
Citrate	86.8	98.0	82.6	86.4
Succinate	94.7	74.4	82.8	64.2
Tartrate	70.1	84.3	79.2	72.4
Malate	89.2	85.9	80.6	55.4
McIlvaine		63.7		
Glycolate	86.0	57.4	53.2	40.9
Lactate	88.3	71.0	50.8	23.7
Formate	32.1	5.0	1.6	0.5
pH 4.0				
Citrate	96.4	99.2	96.8	98.2
Succinate	91.4	89.1	87.2	74.7
Tartrate	94.0	98.8	88.5	86.6
Malate	96.2	82.5	98.5	87.4
McIlvaine		56.8		
Glycolate	94.0	71.2	49.4	35.0
Lactate	95.2	86.2	69.7	66.7
Formate	41.4	6.3	2.3	0.6
Acetate	84.6	56.3	37.2	26.9
Propionate	75.5	28.9	15.9	7.5
Butyrate	71.3	34.1	16.6	2.9
pH 5.0				
Citrate	86.3	87.0	77.5	72.8
Succinate	94.4	91.1	90.5	79.7
Tartrate	89.8	75.2	73.8	56.0
Malate	90.0	77.6	77.7	72.0
McIlvaine		58.6		
Acetate	90.3	66.4	49.2	26.4
Propionate	86.4	42.3	18.1	5.4
Butyrate	84.8	57.7	36.4	7.1

[a] 100% = activity in 5 mM succinate at respective pH value.

Steady-state kinetic measurements were performed to determine the inhibitory mechanism of acetate as a representative of the monocarboxylic acids. Formate was also examined because of its anomalous behaviour compared to acetate and its longer-chained homologues. Reciprocal (Lineweaver–Burk) plots of the steady-state data at various acetate concentrations were almost linear (Fig. 3A) except for some non-linearity at high substrate concentrations (Fig. 3B) indicating a weak substrate-inhibition which was neglected for the determination of the inhibition type. The regression lines did not intersect in one point indicating a non-linear

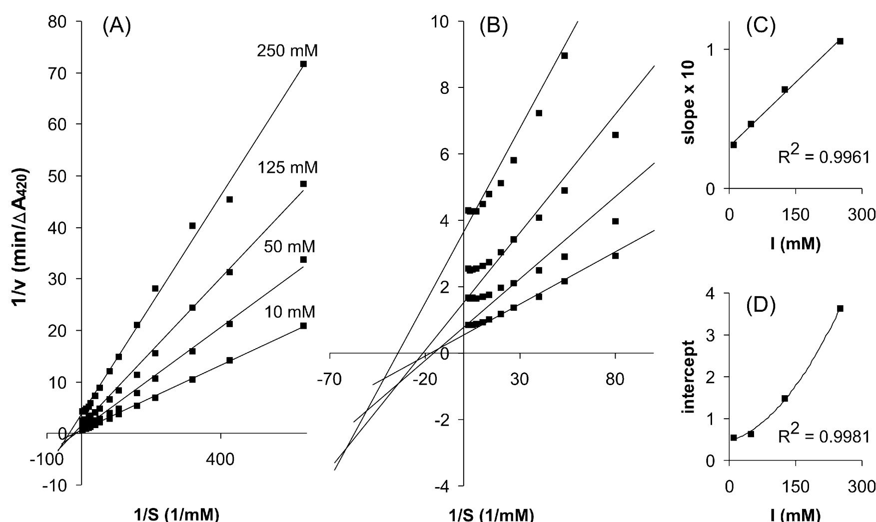

Fig. 3. Steady-state kinetics of laccase inhibition by acetate at 30 °C and pH 4. Reciprocal plots at different scales (A and B) as well as plots of slopes (C) and intercepts (D) against acetate concentration are shown.

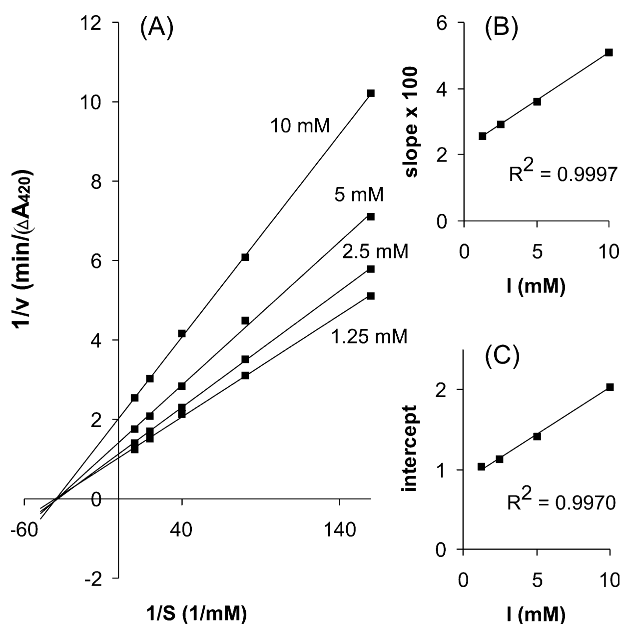

Fig. 4. Steady-state kinetics of laccase inhibition by formate at 30 °C and pH 4. Reciprocal plots (A) as well as plots of slopes (B) and intercepts (C) are shown.

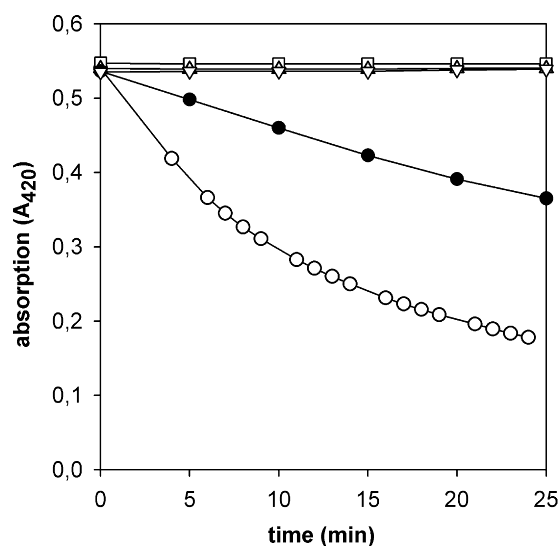

Fig. 5. Stability of the ABTS cation radical. (□): water; (△): 200 mM acetate; (▽): 50 mM formate; (●): 50 mM malonate; (○): 200 mM malonate.

mixed inhibition type where the inhibitor affects both oxidation-dependent and -independent reaction steps. Slope plots (Fig. 3C) against acetate concentration were linear demonstrating a linear inhibition of the oxidation-dependent step with increasing inhibitor concentrations, while plots of the intercepts with the $1/v$ axis (Fig. 3D) were hyperbolic indicating that the fully liganded enzyme at high inhibitor concentration would be completely inactive. In summary, the recombinant *T. villosa* laccase showed an s-linear, i-parabolic mixed inhibition type [38] for acetate at pH 4 with graphically determined K_i and K_i' values of 42.2 mM and 85.9 mM. Using the equations for non-linear mixed inhibitions provided with the enzyme kinetics module of Sigmaplot, K_i and K_i' values of 38.8 mM and 117.5 mM (R^2 = 0.9973), respectively, were calculated which is in good agreement with the graphically obtained values.

In contrast to acetate, the fitted lines of the Lineweaver–Burk plot at different formate concentrations (Fig. 4A) were intersecting in one point on the *x*-axis ($1/S$) to the left of the *y*-axis ($1/v$) indicating a linear non-competitive inhibition. This was confirmed by the linearity of both the slope (Fig. 4B) and the intercept (Fig. 4C) plots against formate concentration. Therefore, the recombinant *T. villosa* laccase exhibited a simple linear non-competitive inhibition type for formate at pH 4 with a K_i value of 7.7 mM (R^2 = 0.9988). The K_i value for formate was therefore one order of magnitude lower than the K_i values for acetate.

Since the inhibition mechanisms for both acetate and formate were found to be non-competitive, it can be ruled out that the inhibition was due to acetate and formate merely acting as laccase substrates which should have resulted in a competitive type of inhibition.

For comparison, steady-state kinetic measurements were also performed with azide as a classical laccase inhibitor. A linear mixed type of inhibition with K_i and K_i' values of 0.0176 mM and 0.0106 mM (R^2 = 0.9852), respectively, was obtained by partial least-squares regression for recombinant *T. villosa* laccase which is in good agreement with previous findings for other laccases. Thus the affinity of monocarboxylic acids for laccase is several orders of magnitude lower than that of azide. However, it is still significant because carboxylic acids are often used in relatively high concentrations in laccase applications.

Several control experiments were performed to verify that the observed inhibition of laccase by carboxylic acids was not due to false data resulting from some sort of substrate–buffer or product–buffer interaction. For example, the primary product of ABTS oxidation, the ABTS cation radical, is known to be reduced by malonate if used as a buffer in the assay [31]:

(1) No significant change in percent inhibition by 250 mM acetate was observed when analytical grade acetic acid and NaOH were replaced by high purity reagents to prepare the buffer as described in the methods.
(2) A 0.03 mM ABTS solution that was oxidized with laccase in either 250 mM acetate or in 250 mM citrate buffers until completion of the reaction resulted in identical final absorption values (within 1%) of the ABTS cation radical for both buffers that were stable for at least 30 min. Therefore, it can be assumed that acetate does not affect the availability of ABTS as a laccase substrate.
(3) EDTA had no significant effect on laccase inhibition by 250 mM acetate (<5%), and no significant difference in the extent of inhibition by 250 mM acetate was observed when using buffer components of differing purity levels. This largely excludes the possibility that metal ions or other impurities in the reagents were responsible for laccase inhibition.
(4) Similar results were obtained when ABTS was replaced by 2,6-dimethoxyphenol in some experiments. For example, incubation of laccase for 5 min in 250 mM acetate buffer at pH 5.0 with ABTS or 2,6-dimethoxyphenol resulted in 59% or 55% inhibition, respectively. This excludes the possibility that laccase inhibition was related to the type of substrate used.
(5) As shown in Fig. 5, the initial absorption values for the enzyme-free ABTS cation radical in distilled water, 200 mM acetate, 50 mM formate, and in 50 mM or 200 mM malonate were identical confirming that the extinction coefficient of the ABTS cation radical is not altered by carboxylic acids. Furthermore, the ABTS cation radical was stable for at least 25 min in acetate as well as in formate, although reduction by formate might be expected due to its potential reactivity as an aldehyde. The reduction by malonate is also shown as a "positive" control.
(6) No dissolved oxygen consumption was observed when 0.15 U/ml recombinant laccase was incubated with 200 mM

Table 2
Relative activities of various wild-type laccases in selected buffers.

Laccase source	pH 5.0/4.0	pH 5.0				pH 4.0		Purity[a]
	Succinate	Succinate		Acetate		Formate		
	10 mM	100 mM	250 mM	100 mM	250 mM	10 mM	50 mM	
Trametes pubescens CBS 696.94	100	101	88	46	33	33	5	+
Trametes pubescens CBS 696.94	100	108	92	44	31	56	7	+++
Trametes versicolor CoA-217	100	99	110	64	33	51	10	++
Coriolus versicolor CTB 863 A	100	100	–	59	45	47	11	++
Pycnoporus sanguineus CBS 357.63	100	104	–	117	104	52	16	++
Pycnoporus cinnabarinus CBS 393.61	100	119	134	119	104	43	13	++
Pleurotus eryngii CBS 613.91	100	102	100	100	106	90	50	++
Phlebia tremellosa FPRL 2845	100	82	75	75	53	29	3	++
Ganoderma pfeifferi CBS 747.84	100	117	131	131	120	93	78	++
Lentinus tigrinus CBS 753.83	100	96	105	70	55	44	5	++
Dichomitus squalens s.p.	100	104	113	97	79	42	6	++

[a] Concentrated culture filtrate (+), partially purified by GPC (++), or purified by IEC and GPC (+++).

acetate at pH 5.0 or 10 mM formate at pH 4.0 (data not shown) confirming that neither acetate nor formate are directly oxidized by laccase. Oxygen consumption was observed only upon addition of ABTS.

These results collectively suggest that the observed inhibitory effects are not an artefact but actually related to an interaction between the carboxylic acid anion and the enzyme.

In order to determine whether our findings with recombinant and wild-type *Trametes* laccases are generally applicable to fungal laccases, we have partially purified various laccases from other genera and tested their behaviour towards carboxylic acids buffers. The results are presented in Table 2 and confirm that *Trametes* (*Coriolus*) laccases are moderately inhibited by acetate and highly sensitive to formate. While laccases from *Phlebia*, *Lentinus*, and *Dichomitus* species showed a similar behaviour, it is quite surprising that laccase from *Ganoderma pfeifferi* was only slightly inhibited by formate and even activated by high concentrations of succinate and acetate. Similarly, laccase from *Pleurotus eryngii* was only slightly inhibited by formate and not affected at all by acetate and succinate. It is also interesting that laccases from both *Pycnoporus* species were strongly inhibited by formate but rather stimulated by acetate and succinate. This inconsistent behaviour of the various fungal laccases strongly suggests that that their inhibition by carboxylic acids is determined by structural features of the protein itself rather than the chemistry of primary or secondary reactions possibly involved in enzyme catalysis. It may eventually be possible to identify these structural features by comparative structure function studies.

4. Conclusion

This work has shown that carboxylic acids may significantly inhibit the activity of fungal laccases. However, steady-state kinetics did not establish a common inhibition type for all carboxylic acids. Furthermore, inconsistent behaviour of laccases from different fungal species renders it unlikely that laccases share a common structural feature accounting for their inhibition by carboxylic acids.

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