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Oxidation of aromatic alcohols by laccase from *Trametes versicolor* mediated by the 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) cation radical and dication

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Abstract Oxidation of aromatic alcohols, such as non-phenolic lignin model compounds, by oxidised species of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) has been investigated. The cation radical and dication formed from ABTS were both capable of oxidising aromatic alcohols to aldehydes. The reactions terminated at the level of the aldehyde and no acids were formed. The cation radical and dication worked in a cycle as an electron-transfer compound between an oxidant and alcohol. In addition to the oxidation of the primary benzyl-hydroxyl group, an oxidation of the secondary α-hydroxyl group to the ketone by the dication was possible. All distinguishing features of these reactions corresponded to the results of the oxidation performed by the laccase of *Trametes versicolor* in the presence of ABTS. The decomposition products from the dication alone and ABTS with laccase confirmed the supposition that the dication was involved in the laccase mediator system. A reaction mechanism based on deprotonation of the alcohol cation radical was predicted to play a key role in the irreversible followup reaction and to be the driving force of the process.

Introduction

For over 100 years the biological oxidation of phenolic compounds to quinones or their polymerisation has been associated with the action of so-called phenol oxidases, enzymes produced by numerous plants and secreted by many wood-rotting fungi. Conversely, the commonest enzymes of this group, laccases (EC 1.10.3.2.), were considered to be enzymes capable of only acting on phenolic substrates. The ability of fungal laccases to cause a C_α–C_β cleavage of the side-chain of lignin models has suggested that they might also participate in lignin degradation (Kawai et al. 1988). A new biotechnological role for this enzyme in the bleaching of hardwood kraft pulp by depolymerising and solubilising lignin was presented in the presence of so-called mediator compounds (Bourbonnais and Paice 1990, 1992; Bourbonnais et al. 1995; Call 1996; Call and Mücke 1997). These novel catalytic activities of laccases in laccase mediator systems (LMS) enhanced their status in comparison to the generally more powerful peroxidases.

The supposition of a mediator effect based on a transfer of electrons via radical species from copper-containing oxidases to reduced pyridine nucleotides was reported by Kubowitz as early as 1937 (Kubowitz 1937a, b) and later extended to ceruloplasmin and aromatic amines (Walaas and Walaas 1961). Oxidation of natural compounds, such as vindoline or rotenone, by ceruloplasmin and by plant or fungal laccases has been achieved by addition of chlorpromazine or syringaldazine (Eckenrode et al. 1982; Sariaslani et al. 1984). The physiological role of some compounds as mediators in oxidative processes was also considered (Barrass and Coult 1972).

A notable action of this oxidation system was reported with regard to the oxidation of the aromatic methyl groups (Potthast et al. 1995) and various aromatic alcohols to the corresponding aldehydes (Kawai et al. 1989; Potthast et al. 1996; Rosenau et al. 1996). Other non-phenolic substrates, such as polycyclic aromatic hydrocarbons (PAH), were also demonstrated to be extensively oxidised by this system (Collins et al. 1996; Johannes et al. 1996; Majcherczyk et al. 1998; Johannes et al. 1998).

Incubation of the mediator, for example 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), with laccase and removal of the enzyme by ultrafiltration left no active species able to perform the expected oxidation and bleaching (Bourbonnais and Paice 1990).

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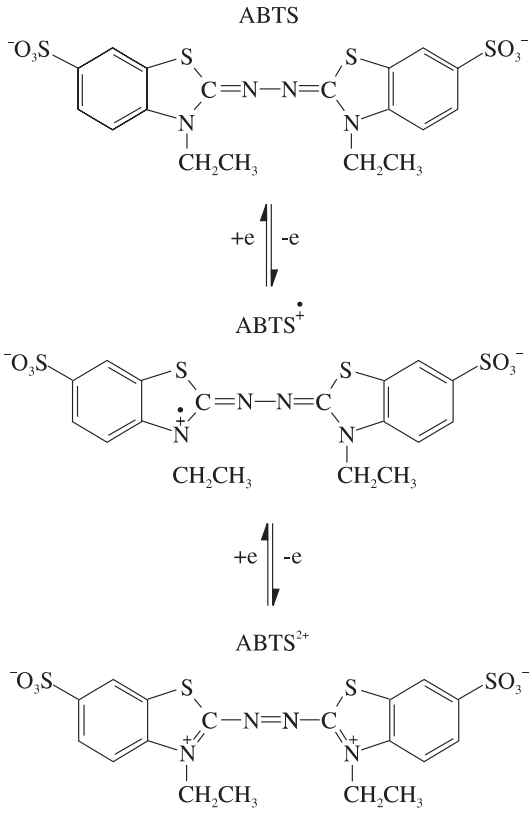
This implied the necessity for both components, enzyme and mediator, to be present in the reaction mixture. The presence of an enzyme-mediator complex capable of performing the redox reaction and exceeding the expected redox barrier of laccase alone was commonly assumed. *Ortho* substituents in aromatic alcohols prevented the oxidation, and the presence of *ortho*-hydrogen atoms was assumed to be essential, implying a direct contact of the substrate with the enzyme (Potthast et al. 1996). Furthermore, the role of a mediator as a co-oxidant "switching" laccase to the novel state capable of performing oxidation reactions by transfer of two electrons to the oxygen molecule in one step was also postulated (Potthast et al. 1995).

The so-called mediator compound plays an important part in the LMS and the choice of the proper substance may play a key role in determining the effect obtained. Over 100 possible mediator compounds have been described (Call 1996; Bourbonnais et al. 1997) but the most commonly used are the azine ABTS and the triazole 1-hydroxybenzotriazole (HBT). ABTS is readily oxidised by free radicals, various peroxidases and laccases to the cation radical $ABTS^{+\bullet}$, and the concentration of the intensely coloured, green-blue cation radical can be correlated to the enzyme activity.

It is well known that cation radicals represent an intermediate oxidation step in the redox cycle of azines and, upon extended oxidation and abstraction of the second electron, the corresponding dications can be obtained (Hünig et al. 1964a); see Scheme 1, for example. The electrochemical properties and reversibility of the above ABTS redox cycle have been the subject of detailed studies and the decomposition products have also been investigated (Janata and Williams 1972; Scott et al. 1993). The two steps in the oxidation of ABTS are not pH-dependent within the range 2–11 (Hünig et al. 1964b). The redox potentials of $ABTS^{+\bullet}$ and $ABTS^{2+}$ were estimated as 0.680 V and 1.09 V (against NHE, e.g. Scott et al. 1993) respectively. ABTS can be readily oxidised to $ABTS^{+\bullet}$ by various chemical oxidants; however, most of these decompose the primary cation radical or oxidise it to the dication and even decompose the final one (Venkatasubramanian and Maruthamuthu 1989).

No experiments have so far been performed that demonstrate a possible role of the dication in LMS. An ability of the electrochemically generated dication to oxidise veratryl alcohol was recently clearly demonstrated (Bourbonnais et al. 1998); however, no evidence was shown for the participation of the dication in the oxidation of alcohols or lignin utilising ABTS and laccase. The formation of free radical from HBT upon chemical oxidation and its reactivity in inert solvents such as chloroform or carbontetrachloride was previously described (Aurich et al. 1977), but an involvement of this radical in LMS was not demonstrated. Although the LMS are of technical interest for various applications, ranging from synthesis to bleaching of wood-pulp or textile pigments, no direct experimental results have been presented concerning the reaction mechanisms.



Scheme 1 Formation of the cation radical and the dication by removal of one and two electrons from ABTS

Side-chain oxidation of non-phenolic aromatic alcohols was selected in our study as a simple model for α-oxidation of the lignin structure. In addition, the oxidation of benzyl alcohol and its analogues, such as veratryl alcohol, to the corresponding aldehydes was expected to be performed by enzymes with high oxidation potentials (e.g. peroxidases).

Materials and methods

Chemicals

3,4-Dimethoxybenzyl alcohol (veratryl alcohol), 1-phenylethanol, 4-methoxybenzyl alcohol, 2,6-difluorobenzyl alcohol, 2,4-difluorobenzyl alcohol, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid), 5,10-dihydro-5,10-dimethylphenazine (DMPZ), and promethazine [PMTZ, 10-(2-dimethylaminopropyl)phenothiazine] were purchased from Aldrich (Steinheim, Germany). Acetonitrile was obtained from Biomol (Hamburg, Germany). All other chemicals were purchased from Fluka (Neu-Ulm, Germany). Laccase of *Trametes versicolor* was prepared as previously described (Majcherczyk et al. 1998; Johannes et al. 1998).

Enzyme assay

Laccase activity was determined by oxidation of ABTS. The reaction mixture contained 5 mM ABTS in 0.1 M McIlvaine buffer, pH 4.5, and a suitable amount of enzyme. Oxidation of ABTS was

followed by an absorbance increase at 420 nm. The enzyme activity was expressed in units defined as 1 U = 1 μmol ABTS oxidised/min (ε_{420} = 36,000 $M^{-1}cm^{-1}$) (Childs and Bardsley 1975).

HPLC analysis

Reaction products were analysed by HPLC (1090 with a DAD detector, Hewlett Packard, Waldbronn, Germany) using a LiChrospher 100 RP-18 5 μm column (4 × 125 mm). Separation was obtained in isocratic mode at a flow rate of 1 ml/min, using 30% acetonitrile acidified with 0.1% acetic acid. The quantitative analysis was performed by monitoring the UV absorption at 200 nm or 216 nm; absorption spectra within the range 200–600 nm were recorded. Calibration with alcohols, aldehydes and acids was performed, using synthetic compounds within the appropriate concentration range (five – point calibration).

Analysis of ABTS-derived compounds was performed in gradient mode from 15% acetonitrile (solvent A) to 95% acetonitrile (solvent B) containing 10 mM tetrabutylammonium sulphate with a flow rate of 1 ml/min. After an isocratic run for 1 min in solvent A, the mobile phase was changed by a linear gradient over 18 min to solvent B and elution was continued for an additional 2 min. Absorption spectra within the range 200–600 nm were recorded.

Preparation of ABTS cation radical

$ABTS^{+\bullet}$ used in experiments was prepared from 5 mM ABTS solution in 50 mM acetate buffer pH 4.5, using laccase of *T. versicolor*. After ABTS had completely reacted, the cation radical was separated from the enzyme by double filtration through a YM3 Amicon filter. The filtrate was tested for a negative reaction on laccase and used after appropriate dilution. The same results were obtained in all experiments in which cation radicals were generated by a stoichiometric amount of potassium peroxomonosulphate, potassium peroxodisulphate or cerium (IV) sulphate.

Preparation of ABTS dication

$ABTS^{2+}$ was prepared from ABTS in a solution of potassium peroxodisulphate (Maruthamuthu et al. 1987). A 200-μl sample of 50 mM ABTS stock solution was added in four portions to 5 ml 200 mM peroxodisulphate in 20% acetonitrile. After the addition of each aliquot and mixing, the solution rapidly turned blue and changed colour to an intense red after 1 min; a red-brown brittle precipitate then formed. The suspension was left for an additional 15 min and centrifuged at room temperature for 10 min at 1400 *g*. The fine precipitate was separated carefully by decanting and washed three times with 5 ml 20% acetonitrile. The precipitate of $ABTS^{2+}$ was resuspended in 500 μl 20% acetonitrile and used in this fresh form for further experiments. The amount of the dication was determined by adding an aliquot of the suspension to 5 ml 5 mM ABTS in water. The amount of ABTS cation radical ($ABTS^{+\bullet}$), which formed immediately according to the comproportionation reaction: ABTS + $ABTS^{2+}$ → 2 $ABTS^{+\bullet}$, was measured by absorbance at 420 nm (ε_{420} = 36,000 $M^{-1}cm^{-1}$) in an appropriate dilution.

Oxidation using ABTS dication

The stepwise oxidation of benzyl alcohol (30 μmol) was performed in 1.5 ml 200 mM potassium peroxodisulphate (300 μmol) in 20% acetonitrile. At 6-min intervals aliquots of 2 μl reaction mixture were analysed by HPLC. Approximately every ten injections (approximately each hour) a portion of 1.2 μmol ABTS was added and mixed.

Oxidation of aromatic alcohols was performed using an excess of 100 μmol alcohol and 1 μmol $ABTS^{2+}$ in 0.5 ml 20% acetonitrile. After addition of the dication, the reaction mixture was incubated at 30 °C and the aliquot was analysed after the desired time had elapsed. Control samples were prepared and analysed in the same manner but with the dication omitted. Small amounts of aldehydes present in the initial samples of aromatic alcohol were subtracted.

Oxidation using laccase and mediators

Oxidation of aromatic alcohols was performed by addition of 1 U/ml laccase from *T. versicolor* to the solution of 100 mM alcohol and 1 mM mediator compound in 50 mM McIlvaine buffer pH 4.5 containing 20% acetonitrile and through incubation at 30 °C for the desired time. Control samples were incubated without the enzyme or a mediator. Other concentrations of substrates or enzyme are indicated when applied.

Results

All reactions performed in our study, if not otherwise specified, were performed in buffer at pH 4.5. Addition of 20% of acetonitrile was necessary to solubilise some alcohols or the resulting aldehydes and was added equally in all experiments. Under these conditions, laccase of *T. versicolor* retained 65% of its initial activity measured in pure buffer. Acetonitrile did not participate in the reactions and the same effects were obtained for soluble alcohols in buffer.

Oxidation reactions with ABTS and laccase

Oxidation of veratryl alcohol to veratraldehyde by laccase from *T. versicolor* in the presence of ABTS was studied for different concentrations of alcohol (Fig. 1). The reaction rate decreased in the same manner independently of the initial concentration of the alcohol. Within a range of 1–20 mM substrate, the initial reaction rates were linearly proportional to the substrate concentration. The slower initial reaction rate found at a higher concentration of alcohol (100 mM) resulted from a lowering of the activity of laccase by the substrate. This was also observable through the slower formation of $ABTS^{+\bullet}$. The amount of veratraldehyde produced was not a linear function of the amount of laccase in the enzyme concentration within the range 0.1–5 U/ml (Fig. 2). In this case the reaction rate also decreased during the incubation time of over 22 h; this was not the result of a decreased amount of substrate, which was still in excess.

Upon incubation for a few hours, a small amount of red product with a broad absorption maximum at 560–570 nm was detected in addition to the typical green-blue $ABTS^{+\bullet}$ (418, 645, 728, 810 nm). This was considered to be an addition product of the protein moiety and the cation radical because a similar red product with a corresponding absorption maximum (562 nm) was also obtained from the enzyme-free $ABTS^{+\bullet}$ and the bovine serum albumin; a slow reaction

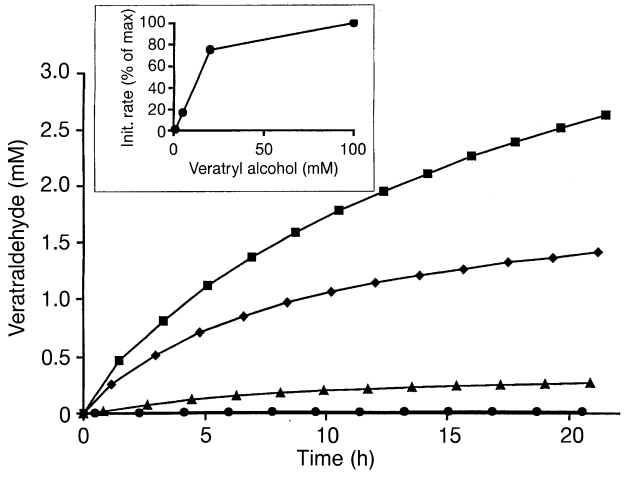


Fig. 1 Veratraldehyde obtained by oxidation of veratryl alcohol by laccase from *Trametes versicolor* (0.175 U/ml) in the presence of 1 mM 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Initial concentration of alcohol: ● 1 mM, ▲ 5 mM, ◆ 20 mM, ■ 100 mM. *Insert*: initial oxidation rates

of $ABTS^{+\bullet}$ with blood serum has also been previously reported (Romay et al. 1996). The detection of traces of a possible ABTS dication (518 nm) was thus not possible; however, this effect did not significantly influence the stability of the laccase: only about 7% (±2%) of the initial enzyme activity was lost after 22 h incubation of 1 U/ml laccase with 1 mM ABTS in buffer at pH 4.5 and room temperature. Approximately 80% (±6%) of the initial activity remained after 22 h in a control sample without ABTS. The decrease in the oxidation rate observed above was ascribed to the high amount of ABTS lost during the incubation: only 27% of the initial mediator was detected as the cation radical after 22 h (Fig. 3A). Otherwise, the cation radical separated from the enzyme by ultrafiltration was found to be very stable in the above buffer. Its absorption did not decrease significantly after 22 h. The expected decomposition of $ABTS^{+\bullet}$ by the nucleophilic addition of water (or hydroxyl anion) was detected only in neutral or alkaline solutions (Fig. 3B). Two colourless products with similar absorption spectra (220, 254, 284, 292 nm and 210, 254, shoulder at 290 nm) were separated by HPLC (data not presented). Only traces of these products were detected in the samples of ABTS incubated with laccase as described above.

Oxidation reactions with ABTS dication

As repeatedly reported from previous studies, the cation radical separated from laccase by ultrafiltration was not able to perform the oxidation/bleaching reactions of LMS. These findings give rise to the supposition that no other low-molecular-mass compounds derived from LMS could be responsible for these processes. To prove whether the oxidised ABTS, e.g. $ABTS^{2+}$, can act as an oxidant for aromatic alcohols and produce aromatic aldehydes without further oxidation to the aromatic acids, we used a reaction mixture consisting of a chemical oxidant and ABTS. $ABTS^{2+}$, obtained as a purple-red solution in 200 mM potassium peroxodisulphate (Maruthamuthu et al. 1987), reacted readily upon addition of aromatic alcohols: the colour of the solution turned back to green-blue, indicating a rapid reduction of $ABTS^{2+}$ to $ABTS^{+\bullet}$. To prove that $ABTS^{2+}$ is capable of catalysing oxidations by working in a cycle, we applied the above system to the oxidation of benzyl alcohol. Although potassium peroxodisulphate possesses a high oxidation potential (E_{ox} = 2.01 V), it oxidised

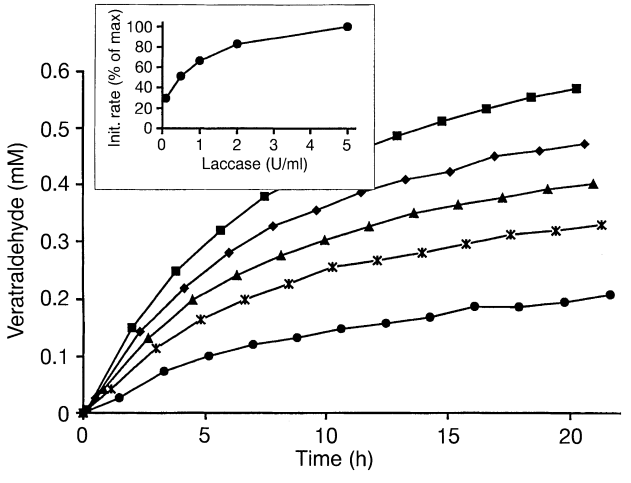


Fig. 2 Oxidation of veratryl alcohol (5 mM) to veratraldehyde by 0.1 (●), 0.5 (×), 1 (▲), 2 (◆), and 5 (■) U/ml laccase from *T. versicolor* in the presence of 1 mM ABTS. *Insert*: initial oxidation rates

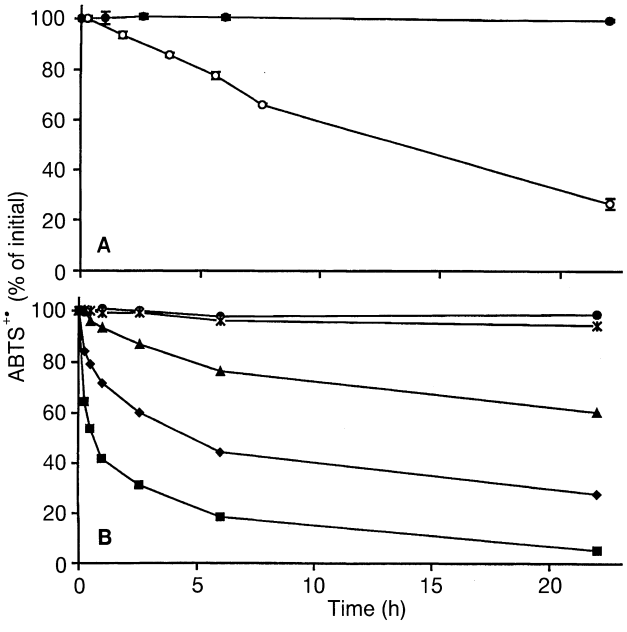


Fig. 3A, B Stability of $ABTS^{+\bullet}$ (1 mM). **A** With (○) and without (●) laccase (1 U/ml) in buffer pH 4.5. **B** Without laccase in universal buffer at pH 3 (●), 5 (×), 7 (▲), 9 (◆), and 11 (■)

benzyl alcohol only very slowly; the system consisting of an excess of the peroxodisulphate (300 μmol) and substrate (30 μmol benzyl alcohol) showed no significant production of the aldehyde. This changed rapidly when 1.2 μmol ABTS was added (Fig. 4). Approximately 6 μmol aldehyde was produced, indicating that about 10 cycles of $ABTS^{+\bullet}/ABTS^{2+}$ occurred before ABTS decomposed and the reaction slowed. As in the reaction utilising LMS, the oxidation stopped at the aldehyde step and no significant amount of benzoic acid was produced. New additions of ABTS repeated the cycle but the reaction became slower after the third addition because of the lower concentration of benzyl alcohol present. Oxidation was also obtained when a suspension of oxidant-free $ABTS^{2+}$ was used.

Reaction mechanism

It is obvious that the dication oxidises alcohol by transfer of two electrons in two one-electron steps. The oxidation potential of $ABTS^{2+}$ is less positive than that of most aromatic alcohols (e.g. veratryl alcohol, VA) and the reaction can be described as an equilibrium:

$$ABTS^{2+} + VA \longleftrightarrow ABTS^{+\bullet} + VA^{+\bullet}$$

The equilibrium constant, K, calculated from the electrochemical data for veratryl alcohol ($E_{a,p} = 1.4$ V, Bourbonnais et al. 1998) is 6.98×10^{-6}. A corresponding constant calculated for the reaction with $ABTS^{+\bullet}$ would be 1.06×10^{-12}. The above reaction and a similar reaction using $ABTS^{+\bullet}$ are thermodynamically unfavourable but they can be achieved if coupled with a further, irreversible follow-up reaction removing the cation radical of the alcohol and shifting the entire equilibrium to the right. In addition, increasing the concentration of substrates, e.g. alcohol, would also shift the equilibrium and would result in an increased amount of products. This hypothesis was confirmed by oxidation of veratryl alcohol by $ABTS^{+\bullet}$. Low concentrations of alcohol (1 mM) added to 1 mM $ABTS^{+\bullet}$ resulted in a very small, non-significant amount of veratraldehyde, which conformed with the previously reported data; however, the aldehyde was detected by increasing the concentration of alcohol (Fig. 5). According to the two-step one-electron oxidation, a reaction yield as high as 95% (calculated for $ABTS^{+\bullet}$) would be obtained if 500 mM veratryl alcohol and 1 mM $ABTS^{+\bullet}$ were used.

The reaction responsible for the continuous removal of the alcohol cation radical could be a deprotonation and the formation of the alcohol radical:

$$ABTS^{2+} + VA \longleftrightarrow ABTS^{+\bullet} + VA^{+\bullet} \quad \text{or}$$

$$ABTS^{+\bullet} + VA \longleftrightarrow ABTS + VA^{+\bullet}$$

$$VA^{+\bullet} + H_2O \rightarrow VA^{\bullet} + H_3O^{+}$$

This supposition was confirmed by experiments performed in universal buffer at various pH values. The oxidation of veratryl alcohol to the aldehyde was increased approximately tenfold by increasing the pH of the solution to 11 (Fig. 6). A low but repeatedly measured decrease in the oxidation of alcohol at pH 7 could be the result of the decomposition of the cation radical detected in neutral solution (compare Fig. 3). Owing to the overlapping of these two processes, the increase of the oxidation in alkaline solutions can be assumed to be much higher than the measured values indicate.

Reactions with various alcohols and mediators

The oxidation capabilities of $ABTS^{2+}$ and $ABTS^{+\bullet}$ were tested with various alcohols (Table 1), using the laccase-generated free cation radical, but the same results were obtained with $ABTS^{+\bullet}$ obtained *via* chemical oxidation. The dication obtained in larger amounts as a red-brown precipitate with a metallic shine was

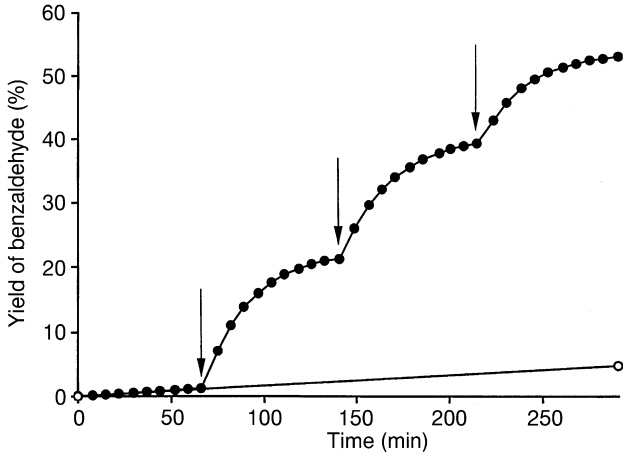


Fig. 4 Benzaldehyde obtained by the oxidation of benzyl alcohol with $ABTS^{2+}$ (●) in a solution of peroxodisulphate (percentage of the theoretical oxidation of alcohol). *Arrows* the addition of ABTS. ○ Control sample without ABTS

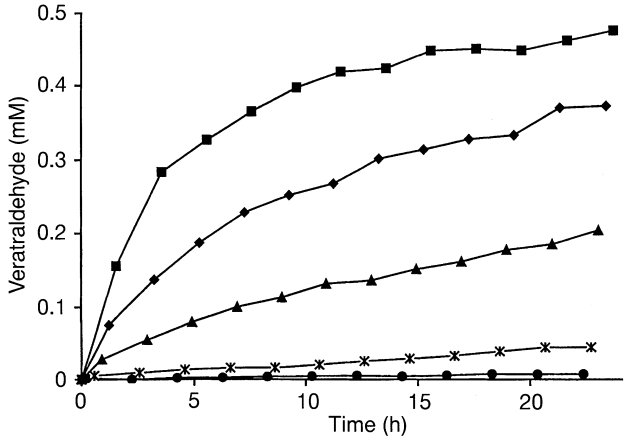


Fig. 5 Oxidation of veratryl alcohol to veratraldehyde by 1 mM $ABTS^{+\bullet}$ (enzyme-free). Initial alcohol concentrations: ● 1 mM, × 5 mM, ▲ 20 mM, ◆ 100 mM, ■ 500 mM

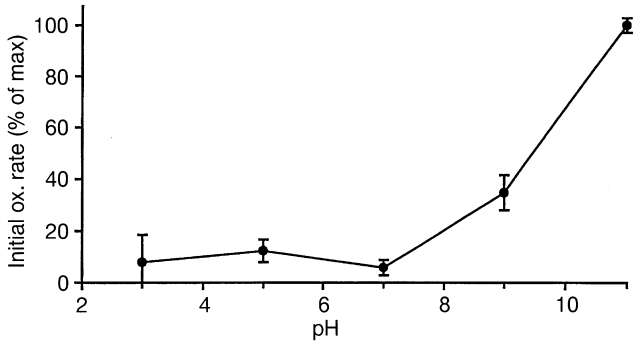


Fig. 6 Initial oxidation rates of veratryl alcohol (100 mM) to veratraldehyde by $ABTS^{+\bullet}$ (1 mM) at different pH values

washed from the oxidant and used in a suspension. The low solubility resulted in a much slower oxidation than in the reaction observed in the solution of $ABTS^{2+}$, and some of the dication was definitely lost by decomposition; therefore, the results obtained for the dication are qualitative and can only describe a relative reactivity with different alcohols. The dication was able to oxidise not only the primary hydroxyl groups but also a secondary alcohol at the α position: 1-phenylethanol was oxidised to acetophenone. Both oxidants reacted with benzyl, 4-methoxybenzyl, and veratryl alcohols. No significant amounts of acids were detected in any of the cases and no changes were observed when the reactions were performed under argon. The reactivity of the alcohols [benzyl < 4-methoxybenzyl < 3,4-dimethoxybenzyl (veratryl)] was proportional to the number of methoxyl groups and, accordingly, to the decrease of the redox potential (Zweig et al. 1964).

According to the proposed reaction scheme, oxidation of veratryl alcohol (reaction time 22 h) was also obtained with other mediators at 1 mM, 1 U/ml *T. versicolor* laccase and an excess of substrate (100 mM) (Table 2). PMTZ and DMPZ were both able to produce veratraldehyde while being oxidised by laccase to their coloured cation radicals. As previously reported (Bourbonnais et al. 1997), a comparatively high amount of aldehyde was produced by LMS utilising HBT but this was still lower than the results obtained by LMS with ABTS (compare Fig. 1) with less laccase.

Steric effects and reaction products

As the participation of $ABTS^{2+}$ in LMS could be proved only indirectly, we were forced to demonstrate that steric effects observed by oxidation of aromatic alcohols by ABTS/laccase (Potthast et al. 1996) also apply to the above reaction. The presence of at least one hydrogen atom in the position *ortho* to the α-hydroxyl group was found to be necessary for the oxidation by LMS; even small substituents, such as fluorine, in both *ortho* positions prevented the oxidation. Incubation of 2,4-difluorobenzyl alcohol and 2,6-difluorobenzyl alcohol with $ABTS^{2+}$, as described above, resulted in the same selectivity as previously reported for LMS. The formation of some coloured addition products was observed in both samples after a few hours; nevertheless, the corresponding aldehyde was detected only in the sample of 2,4-difluorobenzyl alcohol. It was not our intention to study this effect in detail, but we did observe the same selectivity in a simple attempt to oxidise both alcohols with 1% chromoxide (CrO_3) in water. More extensive oxidation conditions using 10% CrO_3 in acetic acid overnight were necessary to oxidise both alcohols at a higher yield, but even under these conditions a significant difference in the reactivity of these alcohols was observed. Therefore, the observed effect can not confirm a direct enzyme interaction.

As mentioned above, $ABTS^{2+}$ decomposes readily in water. The resulting products were well separated by means of HPLC under ion-pair conditions (Fig. 7A). Two products detected revealed similar absorption spectra with maxima at 224, 264, 294 nm and 224, 260, 292 nm respectively. The final hint of the possible reaction pathway of ABTS and laccase was obtained from the analysis of the already colourless sample of 1 mM ABTS incubated with 10 U/ml laccase in buffer for 48 h. Neither the unchanged ABTS nor its cation radical was found in this sample; however, the same two decomposition products as in the case of $ABTS^{2+}$ –

Table 1 Oxidation of aromatic alcohols by $ABTS^{+\bullet}$ and $ABTS^{2+}$ (precipitate). *ABTS* 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)

Substrate	Oxidant	Aldehyde/ketone produced	(μmol)
Veratryl alcohol	– (control)	Veratraldehyde	0.021 ± 0.002
	$ABTS^{+\bullet}$	Veratraldehyde	0.272 ± 0.001
	$ABTS^{+\bullet}$ (Ar)[a]	Veratraldehyde	0.259 ± 0.011
	$ABTS^{2+}$	Veratraldehyde	0.577 ± 0.011
	$ABTS^{2+}$ (Ar)[a]	Veratraldehyde	0.579 ± 0.011
Benzyl alcohol	– (control)	Benzaldehyde	0.013 ± 0.007
	$ABTS^{+\bullet}$	Benzaldehyde	0.066 ± 0.002
	$ABTS^{2+}$	Benzaldehyde	0.253 ± 0.005
Anise alcohol	– (control)	Anisaldehyde	0.009 ± 0.002
	$ABTS^{+\bullet}$	Anisaldehyde	0.152 ± 0.004
	$ABTS^{2+}$	Anisaldehyde	0.391 ± 0.007
1-Phenylethanol	$ABTS^{2+}$	Acetophenone	0.104 ± 0.009

[a] Reaction under argon

Table 2 Oxidation of veratryl alcohol (100 mM) to aldehyde by laccase mediator system. *HBT* 1-hydroxybenzotriazole, *PMTZ* promethazine [10-(2-dimethylaminopropyl)phenothiazine], *DMPZ* 5,10-dihydro-5,10-dimethylphenazine

Mediator	Veratraldehyde (mM)
–	0.002 ± 0.002
HBT	2.038 ± 0.026
PMTZ	1.195 ± 0.011
DMPZ	0.277 ± 0.012

detected by comparison of their retention times and absorption spectra – were found (Fig. 7B). On the other hand, no more than traces of these degradation products were found in samples of enzyme-free $ABTS^{+\bullet}$ after the same period. The degradation products that resulted from the decomposition of cation radicals with water (or hydroxyl anion), as reported above, were detected only in traces in these samples and were not indicated on the chromatograms.

Discussion

Oxidation of an aromatic alcohol to the corresponding aldehyde requires the transfer of two electrons and can be performed via an intermediate radical species or by formation of a covalent bond between the substrate and oxidant. The kinetics of the reaction is influenced by the oxidation potential of the oxidant and steric parameters. Thermodynamically unfavourable oxidation processes, as in the case of a negative difference in the oxidation potentials of substrate and oxidant, are possible if a follow-up process irreversibly removes one of the products from the equilibrium of the first reaction. Very interesting preparative applications of this reaction type were presented for electrochemical oxidations. Oxidative decarboxylation has been reported to be possible at potentials 500 mV lower than the redox potentials of carboxylates if a mediator compound, tris-(*p*-bromophenyl)amine, is added to the reaction mixture (Schmidt and Steckhan 1978a). Despite the very low value of the equilibrium constant, K, of only 4×10^{-9} the electrocatalytic cycle was driven forwards easily by the extremely fast irreversible decarboxylation of the acyloxy radicals formed.

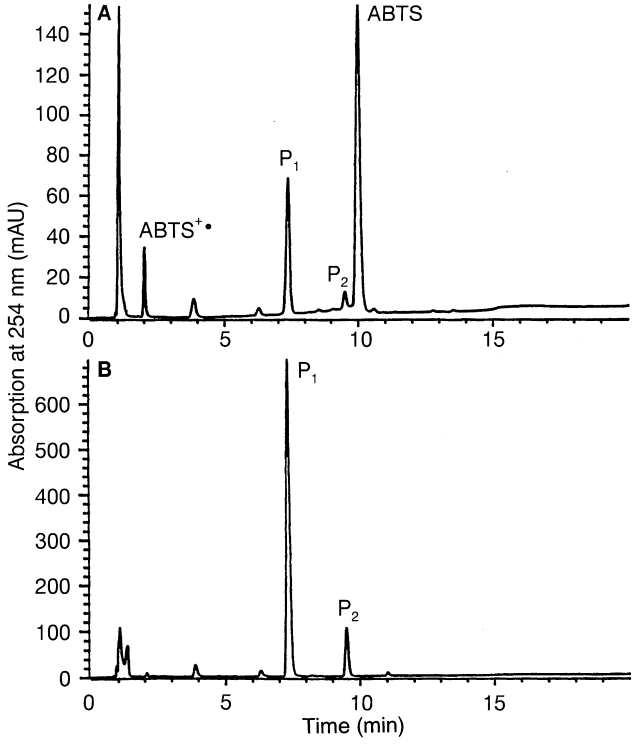


Fig. 7A, B HPLC of degradation products resulted from (**A**) $ABTS^{2+}$ and (**B**) ABTS/laccase

Preparative application of the electrocatalytic cycle to the oxidation of alcohols to aldehydes and ketones at potentials lower than the E_{ox} of alcohols has been demonstrated, with thioanisole as the mediator compound (Shono et al. 1979); the removal of a 1,3-dithiane protecting group was successful at potentials lower by 300 mV (Platen and Steckhan 1980). The deprotonation of the alcohol cation radical as the driving force of the irreversible follow-up reaction was reported following the oxidative hydrolysis of benzyl ether protecting groups (Schmidt and Steckhan 1978b). Oxidation of benzyl ethers to benzaldehydes was thus possible at potentials 550 mV lower than the irreversible oxidation potential of ethers, when the electrocatalytic cycle with triphenylamines was used (Schmidt and Steckhan 1979a, b). The same reaction was possible through a direct titration with the cation radical of the mediator.

The findings from electrochemical experiments have not been included in the discussions of the possible mediator cycle of LMS. This is possibly because the reactivity of cation radicals separated from the enzyme and the complexity of the system has been underestimated. The expected switching of laccases to a novel powerful state with higher redox potential in the presence of a mediator could not be experimentally demonstrated. We have not found an experimental confirmation for this supposition and we also excluded the possibility that the *ortho*-hydrogen atoms hint at a direct enzymatic interaction by the oxidation of aromatic alcohols.

Contrary to the previous reports on LMS, we were able to demonstrate clearly that the oxidation reactions with aromatic alcohols can be performed with an enzyme-free cation radical, $ABTS^{+\bullet}$. The ability of the dication, $ABTS^{2+}$, to oxidise aromatic alcohols is therefore not unexpected, and also demonstrated in the homogeneous system. It is rather evident that the driving force for the reaction is an irreversible deprotonation in the follow-up reaction corresponding to the electrocatalytic oxidation of the benzyl ether discussed above. This is also inline with the pH dependence of the reaction. The deprotonation step may be even faster and, there-

fore, more helpful in the oxidation of benzyl alcohol than its methoxylated derivatives (Brown et al. 1972a, b). On the basis of molecular orbital calculations, Elder (1997) suggested that the oxidation of veratryl alcohol is controlled by the relative endothermicity associated with a proton transfer and the formation of a neutral radical. Although several products can be formed from the initial alcohol cation radical, the step determining the rate of the oxidation, the proton-transfer reaction, also has the lowest reaction heat for the pathway leading to the aldehyde. The step suspected is the initial reaction, as in the case of oxidation with lignin peroxidase, for example; however, the participation of molecular oxygen in the second step was not found. We found the same amount of veratraldehyde in air and argon atmospheres and this could be the result of a rapid abstraction of a second electron by the oxidant at potentials equal to or lower than the initial step (Lund 1957).

Owing to the higher redox potential, the oxidation capability of the dication is much higher than that of the ABTS cation radical; however, it is diminished by its instability in solution and low solubility. Depending on the reaction conditions, it is probable that both of the oxidised states of ABTS participate in the oxidation of the aromatic alcohols studied (Fig. 8). The application of other heterocyclic aromatic compounds, PMTZ and DMPZ, reacting with laccase from *T. versicolor* to cation radicals confirmed our supposition of the general nature of the laccase mediator system. A comparison of the reactivity of the mediators with the yield of oxidation products does not correlate easily to their redox potential because of the different kinetics of their oxidation with the enzyme and substrate, their decomposition and their ability to deactivate the enzyme.

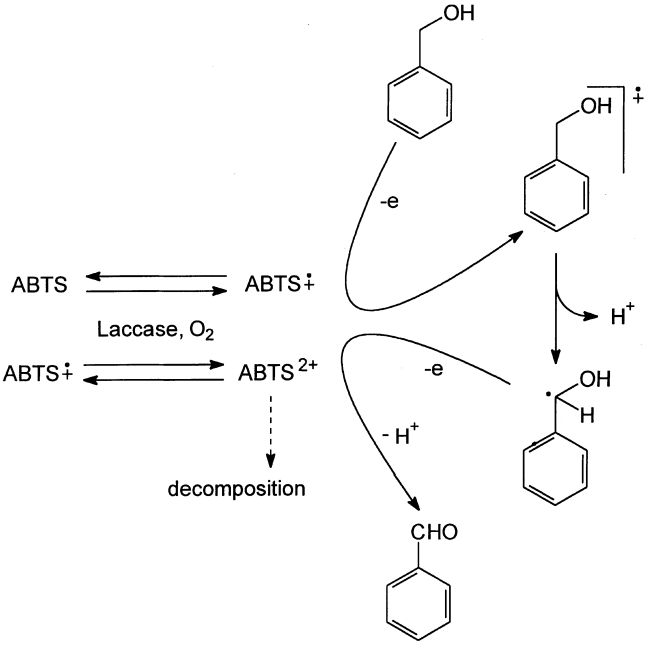


Fig. 8 Proposed reaction scheme for the oxidation of aromatic alcohols with $ABTS^{+\bullet}/ABTS^{2+}$ couple

Approximately half of the oxygen uptake measured for ABTS and laccases of *T. versicolor* was reported for the promazine, a structural isomer of PMTZ (Bourbonnais et al. 1997). A correspondingly large amount of aldehyde was produced by PMTZ, revealing a redox potential (E^0) for the cation radical of 0.86 V (Pelizzetti and Mentasti 1979). A participation of the dication, at least in this reaction, is not very probable. DMPZ (two reversible one-electron oxidation steps in acetonitrile at 0.35 V and 1.07 V) was less effective in the system studied. The cation radical produced from DMPZ was assumed to be an insufficient oxidant; the oxidation was performed instead by the water-sensitive DMPZ dication or by the dication of its decomposition product 5,10-dihydro-5-methylphenazine (cation radical at 0.31 V and dication at 0.87 V, Nelson et al. 1967).

Oxidation of substrates can be expected to be performed by a simple electron transfer to the cation radical or the dication, but the formation of sandwich complexes with the oxidant, thus lowering the oxidation potential of the substrate, or the association of two cation radicals to a complex, can not be excluded. Such mechanisms are known in reactions of aromatic compounds with cation radicals and are dependent on the substrate to oxidant ratio (Svanholm and Parker 1976). A disproportionation of cation radicals to dications can not be absolutely excluded, but at least in the case of the ABTS cation radical, it is not expected (Hünig et al. 1964a). The resulting decomposition of the dication in water would not be compatible with a very high stability of the cation radical, and the oxidation of alcohols has occurred even in the presence of some unreacted ABTS.

The oxidation potential of $ABTS^{2+}$ exceeds the commonly measured redox potential of fungal laccases within a range of approximately 0.5–0.8 V (against NHE). This means that, for thermodynamic reasons, $ABTS^{+\bullet}$ will not be readily oxidised by laccase to the dication at a high rate. However, this does not exclude the possibility of such a reaction; a slow reaction of laccases with compounds possessing a comparatively high redox potential such as phenol (0.93 V against NHE at pH 4) (Penketh 1957) is well known. This thermodynamically unfavourable reaction can proceed through the formation of stable radical products or removal of the products by quick follow-up reactions, e.g. polymerisation.

The reactivity of the system is also generally better than expected because the compounds can be oxidised at a high rate below their nominal redox peak. In the case of phenols, this was measured by potentials generally 0.136 V lower then their E_{ox} values (Fieser 1930). A partial stability of the dication could have been a result of its low solubility. The supposition that the thermodynamically unfavourable oxidation of $ABTS^{+\bullet}$ to $ABTS^{2+}$ by laccase proceeds via the reduction of the dication by veratryl alcohol, which oxidises to aldehyde (Bourbonnais et al. 1998), is not a sufficient explanation

because the oxidation of the alcohol to the aldehyde is also thermodynamically unfavourable in this reaction and does not proceed in one simple step. Moreover, we measured the decomposition of ABTS by laccase in water without alcohols, and the reaction products corresponded to the products obtained by decomposition of the pure dication. We did not attempt to identify these compounds but they could correspond to the 3-ethyl-2-benzthiazolone-6-sulphonic acid and 3-ethyl-2-benzothiazolinone-6-sulpho-imine (Janata and Williams 1972). The other possible decomposition product, 3-ethyl-2-benzothiazolinone-6-sulpho-hydrazone, seems to be less probable because of its lower stability and possible final decomposition to the imine and nitrogen.

These suppositions accord with the absorption spectra of the compounds found. None of the possible products seems to be involved in the oxidation reactions studied because their redox potentials are even higher than that of $ABTS^{2+}$ (Janata and Williams 1972). However, the formation of hydrazone can explain the production of the coloured by-products that we observed: its non-sulphonated analogue is well known as a sensitive reagent (3-methyl-2-benzothiazolinonehydrazone) for the determination of aldehydes and other carbonyl compounds (e.g. Soda 1967).

The reaction scheme proposed above may be universal for many types of mediator compounds producing cation radicals (or dications). The reaction products obtained from the system consisting of laccase of *T. versicolor* and HBT did not differ from that of ABTS; however, it cannot be denied that other reaction mechanisms, at least in their primary step, are also possible. The benzotriazolyl oxide radical was obtained from HBT ($E_{1/2} = 1.0$ V against NHE in buffer pH 7; Aurich et al. 1977); however, its participation in LMS was never shown. It is not certain whether it can act as an electron acceptor in reactions with alcohols or proceeds with the abstraction of a hydrogen atom. The HBT radical obtained in chloroform by oxidation with PbO_2 also oxidised veratryl alcohol to aldehyde (data not presented).

The laccase mediator system studied is certainly very interesting as far its application to the oxidation of lignin in wood pulp, chemical synthesis, or the oxidative degradation of persistent aromatic compounds is concerned. However, the system itself is not limited to laccases and was originally described for ceruloplasmin, as mentioned above. It would thus be better described as the oxidase mediator system. The description of the mediator can be confirmed as it acts as a catalytic agent but the mediation can not yet be properly described as an electron-transfer process, or at least not all mediator compounds can be understood as electron-transfer compounds, as in the case of ABTS.

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