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Anthracenediethanol inhibits lignin degradation by *Phanerochaete chrysosporium* by competing for oxidation by lignin peroxidase, and not by trapping singlet oxygen

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Abstract

The biodegradation of anthracene-9,10-diethanol by the ligninolytic fungus *Phanerochaete chrysosporium*, previously thought to involve singlet oxygen, is shown to be catalyzed by lignin peroxidases. Veratryl alcohol stimulated the enzymatic degradation of anthracenediethanol, and anthracenediethanol inhibited enzymatic oxidation of veratryl alcohol. Competition for oxidation by lignin peroxidase is suggested as the mechanism of the inhibition of lignin biodegradation by anthracenediethanol and related anthracene derivatives.

Abbreviations: ADE – anthracene-9,10-diethanol, AES – anthracene-9,10-bisethanesulfonic acid, DHP – dehydrogenative polymerizate, DMF – N,N-dimethylformamide, EPX – 9,10-endoperoxide of ADE, PMR – proton magnetic resonance

Introduction

The ability of ligninolytic fungi to oxidatively degrade a wide variety of structures in lignin and in other compounds led to the suggestion that these fungi might employ one or more 'active oxygen species', rather than enzymes with high substrate specificity, to fragment the lignin polymer (Hall 1980). Subsequent attempts to identify and determine the role of species such as superoxide anion radical ($O_2^{\cdot}$), hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) have been summarized by Kirk & Shimada (1985). Since the discovery of powerful extracellular peroxidases in cultures of *Phanerochaete chrysosporium* (Tien & Kirk 1983; Glenn et al. 1983), the model has evolved to initial enzymatic oxidation of the aromatic rings in lignin and other substrates to cation radicals, followed by nonenzymatic reactions of those radicals (Kirk & Farrell 1987). Formation and participation of both oxygen-centered radicals ($OH^{\cdot}$, $OOH^{\cdot}$) and carbon-centered radicals in these reactions remain active topics of discussion (Momohara et al. 1990; Schmidt et al. 1989; Harvey et al. 1989).

One of the difficulties in elucidating the role of active oxygen species in ligninolytic cultures has been the lack of specific and reliable assays for superoxide, hydroxyl radical, and singlet oxygen. Botsivali & Evans (1979) described anthracene-9,10-bisethanesulphonic acid (AES) as a specific trapping agent for singlet oxygen. Nakatsubo, Reid & Kirk (1981) found that the ligninolytic fungus *P. chrysosporium* could bleach AES, as $^1O_2$ does, and that AES inhibited lignin degradation by the fun-

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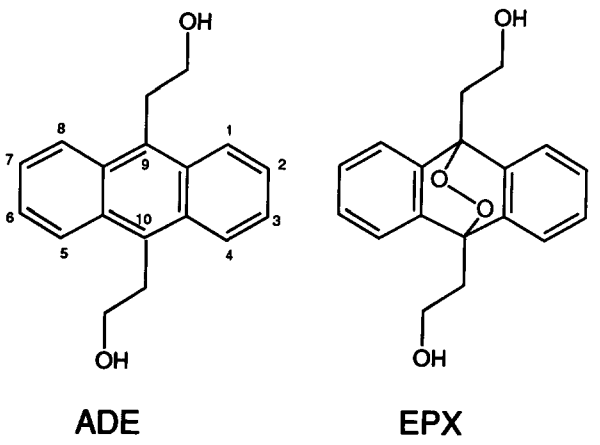

Fig. 1. Structures of anthracene-9,10-diethanol (ADE) and its endoperoxide (EPX).

gus. On this evidence, plus similarities in the fungal and photochemical oxidation of a lignin model dimer, they suggested that *P. chrysosporium* generates $^1O_2$, and that $^1O_2$ is involved in lignin degradation. Further study showed that the photochemical oxidation of the lignin model compound did not involve $^1O_2$ (Kutsuki, Enoki, & Gold 1983, Kirk, Nakatsubo & Reid 1983) and the suggestion that $^1O_2$ is involved in lignin degradation has been retracted (Kirk et al. 1983). However, the possible involvement of $^1O_2$ in the degradation of AES by the fungus and the mechanism by which AES inhibits lignin biodegradation have not been resolved.

This report describes the degradation of the related compound anthracene-9,10-diethanol (ADE, Fig. 1) by cultures of *P. chrysosporium* and by an extracellular enzyme, characterizes the endoperoxide product of $^1O_2$ oxidation of ADE, and shows that the endoperoxide is not formed during degradation of ADE by *P. chrysosporium*. ADE was used instead of AES in this study because it can be easily extracted with organic solvents.

Materials and methods

Fungus

Phanerochaete chrysosporium Burds., strain ME-446, (= PRL 2750, ATCC 34541) was used in most experiments. Strain BKM F-1767 (= PRL 3040, ATCC 20696) was used occasionally as noted.

Culture conditions

Cultures were grown at 39°C without shaking in a nitrogen-limited medium buffered with polyacrylic acid (Reid 1983), 10 ml per 125 ml Erlenmeyer flask. The cultures were inoculated with conidia, and incubated for 3 days with an air atmosphere. Then they were flushed with O_2, sealed, and incubated for another 3 days.

In studies on the production of the ADE-bleaching enzyme, some cultures were supplemented with extra trace element solution (Kirk et al. 1986, 9 ml/l) and/or with 0.2 mM veratryl alcohol.

Chemicals

Synthesis of anthracene-9,10-diethanol was according to the scheme of Botsivali & Evans (1979), and the product was recrystallized several times from acetonitrile. Photoreaction of 10 mg ADE in 20 ml of methanol under air was carried out at room temperature (water bath cooling) in a 150 ml stoppered Pyrex Erlenmeyer flask using a 275 watt General Electric Suntanner Bulb at a distance of 20 cm. An exposure of 4 h gave complete conversion to the endoperoxide, and a shorter exposure (10 min) was used to prepare the mixture of ADE and its endoperoxide used in the experiment shown in Fig. 2.

To confirm synthesis of the endoperoxide by photoreaction with oxygen, it was synthesized also by the hydrogen peroxide-hypochlorite system (Murray 1979). To 17.5 mg of ADE in 5 ml of methanol, cooled in an ice bath, was added 0.15 ml of 30% H_2O_2, and 0.05 ml of 1 M NaOH. Over a period of 90 min, 2 ml of 6% NaOCl was added in small portions with stirring. Most of the methanol was evaporated at 30°C or cooler, water was added, and the aqueous mixture was extracted with CH_2Cl_2, and either stored in this solvent at −12°C until used, or evaporated and the residue dissolved

in methanol and stored at −12°C. Proton magnetic resonance (PMR) spectra had the same resonances for this product as for the material obtained by photoreaction.

PMR spectroscopy

PMR spectra were run at 23°C and 360 MHz with a Bruker NMR spectrometer, model AM-360-WB, using a pulse angle of 45° and an acquisition time of 3.3 s. These conditions were sufficient to establish thermal equilibrium between pulses for the signals of interest. The spectrum was surveyed for the resonances of ADE and its endoperoxide, and where these resonance peaks were judged to be sufficiently separated from other signals, the pattern was integrated, and its proton content calculated from the integral of the reference compound.

Metabolism by whole cultures

A fine suspension of ADE was produced by dissolving a weighed amount (10-20 mg) in 0.4 ml of DMF, and adding the solution with vigorous stirring to 25 ml of sterile H_2O. Portions (1.0 ml) of this suspension were added to 6 day old cultures of *P. chrysosporium* ME-446. The cultures were flushed with O_2, and then incubated at 39°C in the dark. At intervals, some of the cultures were killed by adding 10 ml of methanol. The cultures were extracted with 10 ml of CH_2Cl_2, followed by 8 ml of CH_2Cl_2 plus 2 ml of methanol. The combined extracts were washed with brine, passed through a column of sodium sulfate to dry them, and then evaporated with a rotary evaporator. The residue was evacuated to 0.5 torr to remove traces of solvent, and then was dissolved in a small portion of $CDCl_3$, and filtered, followed by 2-3 washings of the flask and filter, to give a combined volume of ca. 1.5 ml in NMR tubes of 0.5 cm diameter. Hexamethyltrisiloxane (8.30 μmoles in $CDCl_3$) was added to each tube as an internal reference for integration. The tubes were stored in a freezer until PMR spectra could be obtained. All manipulations were carried out in dim light.

Inhibition of lignin degradation

Solutions of radioactive synthetic lignin (Dehydrogenative polymerizate, DHP, *ring*-^{14}C, 9.2×10^5 dpm/mg, 0.33 mg) in 175 μl of DMF supplemented with 3 mg of ADE or EPX, or nothing (controls), were added with vigorous stirring to 3.0 ml of sterile H_2O, to form fine suspensions. Samples (0.5 ml) were added to 6-day old *P. chrysosporium* PRL 2750 cultures, 5 replicate cultures for each of the three treatments. The cultures were immediately flushed with O_2 and returned to a dark 39°C incubator. At intervals, the cultures were flushed with O_2, and the radioactive CO_2 produced was trapped and counted (Reid 1979).

Enzyme assays

Assays were carried out on undiluted culture supernatants, or on purified fractions after appropriate dilution with water.

ADE bleaching enzyme

Degradation of ADE was followed by the decrease in absorbance at 375.5 nm. Cuvets received 2.0 ml of enzyme solution, 1.0 ml of 0.25 M sodium tartrate, pH 3.0, 6 μg of ADE dissolved in 10 μl of DMF, and 10 μl of 50 mM H_2O_2. The assays were incubated at room temperature (23 °C) and the absorbances were recorded at 5 min. intervals in a Cary 210 spectrophotometer set to 0.2 absorbance units full scale. The rates were multiplied by 96.2 to convert from absorbance units/min to μmoles/min.

Lignin peroxidase

The oxidation of veratryl alcohol to veratraldehyde was monitored at 310 nm. An assay mix, containing 0.2 mg of veratryl alcohol per ml of 0.25 M tartrate buffer, pH 3.0, and 0.5 mM H_2O_2, was prepared fresh daily. For each assay, 2.0 ml of enzyme solution was mixed with 1.0 ml of assay mix in a cuvet, and the absorbance at 310 nm was recorded in a Cary 210 spectrophotometer.

Results

ADE metabolism by whole cultures

Most of the 0.5 mg of ADE added to a culture as a fine suspension disappeared (loss of fluorescence and typical ultra-violet absorbance of ADE) in 5-10 h; this disappearance did not occur in the absence of the organism, or in a culture in which the organism had been killed. The ultraviolet spectrum of the endoperoxide is such (absorption below 300 nm) that end absorption in the culture medium would mask its presence in such spectra. The disappearance of ADE in the culture did not result in the formation of any new peaks in the longer wavelength (above 300 nm) region, which would be compatible with the formation of an endoperoxide, but not with the formation of a quinone as an end product. Therefore, an NMR method was devised to examine culture extracts for the presence of endoperoxide.

The aromatic protons of ADE dissolved in $CDCl_3$ had PMR resonances (360 MHz) at 8.37 and 7.52 ppm which were multiplets, each with four distinguishable peaks of approximately equal height, separated by 2-4 Hz, a pattern similar to that of the aromatic protons of 9,10-dimethylanthracene (Pouchert 1983). The CH_2-CH_2-O protons of ADE had resonances at 4.09 and 3.94 ppm, with a coupling of approximately 6.5-7.0 Hz. The lower frequency resonance was a triplet, but the triplet structure at 4.09 ppm was sometimes obscured by coupling to the hydroxyl proton; this coupling could be removed by appropriate homonuclear decoupling of OH protons.

The patterns of the resonances from the ADE endoperoxide at 360 MHz were very similar to those of ADE, but with different chemical shifts, 7.35 and 7.25 ppm for the aromatic protons, 4.15 for the CH_2-O protons and 2.99 for the other protons of the 2-ethanol substituent.

Provided that photooxidation was avoided by handling ADE solutions in subdued light, and using amberized tubes for NMR samples, no endoperoxide was found in any cultures to which ADE alone had been added, regardless of the fact that ADE was disappearing during the course of incubation. This suggested that either no endoperoxide was formed, or it was formed but was so unstable in cultures that it could not accumulate in detectable amounts. In control experiments in which endoperoxide was incubated in non-inoculated culture medium at 39°C for 16 h, there was no detectable decomposition of the endoperoxide. However, when the endoperoxide was added to cultures and incubated at 39°C, it did disappear. To assess whether this disappearance was rapid compared to the metabolism of ADE, a mixture of ADE and its endoperoxide were added to cultures, and analysed for these compounds after incubating for various times. In the experiment of Fig. 2, the endoperoxide was metabolized at a slower rate than ADE itself, and is therefore relatively stable. The rate of EPX disappearance in this experiment was similar to the rate observed when EPX alone was added to cultures. The disappearance of both ADE and EPX from the cultures followed first-order kinetics, with a half-life of 4.6 h for ADE and 7.3 h for EPX. If EPX was an intermediate in the degradation of ADE, it should have accumulated to easily detected levels (Fig. 2). Since the endoperoxide was not found in cultures grown in the presence of ADE, it follows that ADE was not acting as a trapping agent for singlet oxygen, but was metabolized by the cultures by a method that did not involve free endoperoxide as an intermediate.

Inhibition of lignin degradation by ADE and EPX

Both ADE and its endoperoxide transiently inhibited degradation of lignin ([ring-^{14}C]DHP) to CO_2 (Fig. 3). The endoperoxide was somewhat more inhibitory than ADE. The inhibition had disappeared by ca. 40 h after addition of the compounds.

Extracellular ADE-degrading enzyme

Cell-free supernatants from *P. chrysosporium* cultures could degrade ADE, if they were supplemented with H_2O_2 at an appropriate concentration. This enzymatic degradation could be conveniently

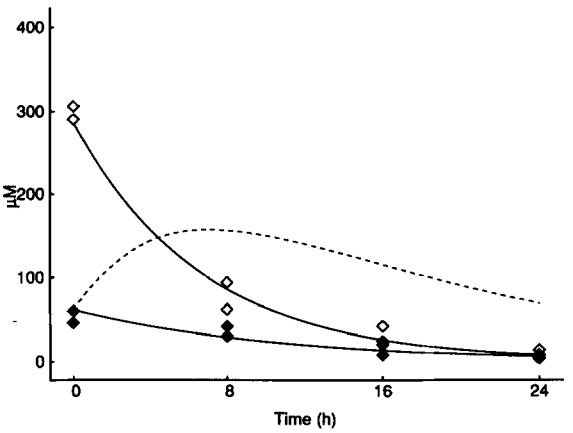

Fig. 2. Rate of disappearance of ADE (◇) and EPX (◆) in cultures, and expected kinetics of EPX concentration if it was formed from ADE (dashed line).

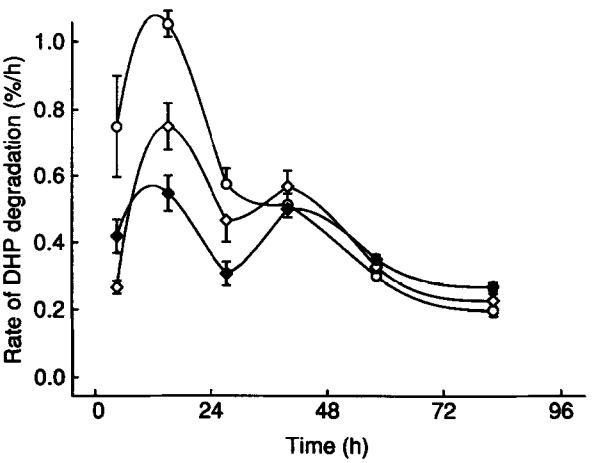

Fig. 3. Effect of 170 μM ADE (◇) and EPX (◆) on rate of degradation of [ring-^{14}C]DHP to CO_2, compared to a control culture (○).

monitored by following the disappearance of the characteristic trio of ADE absorbance peaks in the near UV (Fig. 4). Routinely, only the intensity of the central peak, at 375.5 nm, was followed. The extinction coefficient (ε) of ADE is 10,400 $M^{-1}cm^{-1}$ at this wavelength.

Peroxide could be supplied either by adding glucose oxidase, or by adding low concentrations of H_2O_2. An initial H_2O_2 concentration of 0.17 mM was optimal, and H_2O_2 concentrations above 0.25 mM progressively inhibited the ADE-bleaching enzyme (Fig. 5). The shapes of the reaction progress curves at initial H_2O_2 concentration of 0.5 mM and higher, with gradual decreases in the reaction rate which cannot be attributed to exhaustion of ADE or H_2O_2, indicate inactivation of the enzyme.

The bleaching of ADE was fastest when the culture supernatants were acidified to pH 3, and was negligible at pH values higher than 5. The effect of pH on the rate of ADE bleaching by a purified enzyme preparation is shown in Fig. 6.

Factors affecting enzyme production

A series of experiments to determine the effect of culture age, isolate of *P. chrysosporium* (ME-446 or BKM F-1767), supplementary veratryl alcohol, and supplementary trace elements on the activity of the ADE-bleaching extracellular enzyme were performed. Lignin peroxidase activity was also measured in the culture supernatants, and was closely correlated with the ADE-bleaching activity (Fig. 7). The effects of supplementary trace elements and veratryl alcohol on lignin peroxidase activity were as previously described (Kirk et al. 1986). Isolate BKM F-1767 generally gave higher and earlier activity with both substrates, and responded more strongly to the presence of veratryl alcohol and additional trace elements than did isolate ME-446. The highest enzyme activities were obtained in 6-day old cultures of BKM F-1767 which had received veratryl alcohol and extra trace elements.

Purification of ADE-bleaching enzyme

The medium from 6-day old cultures of *P. chrysosporium* BKM F-1767, filtered through glass wool, was supplemented with 0.05% of cetyltrimethylammonium bromide (CTAB), and adjusted to pH 5.5. The enzyme was adsorbed on DEAE-BioGel A by passing the culture filtrate through a column 5 cm diameter × 10 cm high of gel equilibrated with 20 mM 2,2-dimethylsuccinate, pH 5.5, 0.05% CTAB, at room temperature during 3

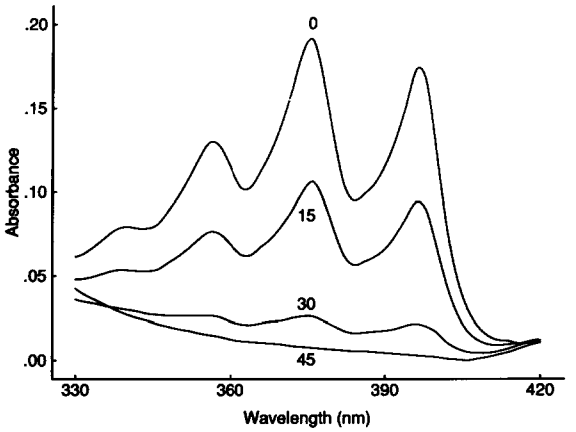

Fig. 4. Bleaching of ADE by cell-free supernatant from *P. chrysosporium* cultures. Supernatant (1.5 ml, pH 4.4) was mixed with 1.5 ml of a saturated aqueous solution (37 μM) of ADE in a cuvet, 30 μl of glucose oxidase solution (1 mg/ml) was added to generate H_2O_2, and the spectrum of the reaction mixture was scanned at intervals of 15 min. The glucose concentration in the reaction mixture was ca. 14 mM.

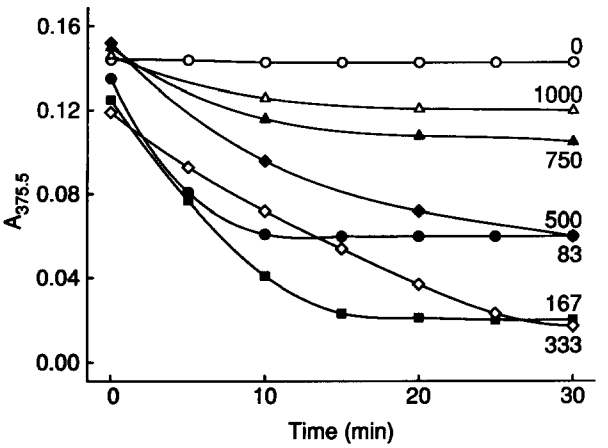

Fig. 5. Effect of initial H_2O_2 concentration on bleaching of ADE by crude extracellular enzyme. Assays included 1.5 ml of supernatant from a 6-day old culture, and 1.5 ml of a saturated solution (37 μM) of ADE in water. The reaction was started by adding an appropriate volume of 50 mM H_2O_2, and followed by recording near-UV absorbance spectra at 5 minute intervals. The labels on the curves indicate the initial H_2O_2 concentration in μM.

hours. After washing the column with the equilibration buffer, the enzyme was eluted with a gradient from 0 to 0.3 M NaCl in 400 ml of the same buffer. The A_{408} of the column effluent was monitored; a peak of adsorbing material was eluted by the salt gradient. The part of the effluent with an A_{408} higher than 0.02 was collected, and concentrated by ultrafiltration, first in an Amicon pressure cell with a Millipore PTGC membrane (NMWL 10,000 daltons), and then with a Millex CX-10 immersion filter. The recovery of ADE-bleaching activity in the eluate was 149%.

This concentrate was applied to a Mono Q HR5/5 column, and eluted with a gradient from 0.1 to 0.25 M NaCl (Fig. 8). Several peaks with absorbance at 410 nm were resolved. Two of these had substantial ADE-bleaching activity. The fractions were also assayed for veratryl alcohol peroxidase (lignin peroxidase) activity; the activity profiles for the two enzymes coincided very closely (Fig. 8). The absorbance spectrum of the most active fraction was typical of a hemoprotein lignin peroxidase (Tien & Kirk 1988) with a strong maximum at 408 nm and a weaker maximum at 503 nm; absorbance at 280 nm was much lower than at 408 nm.

The rate of ADE bleaching by the purified enzyme was proportional to enzyme concentration, at least up to rates of 0.016 absorbance units / min. The A_{408} of the enzyme solution producing this rate was 0.011 cm^{-1}; assuming that the extinction coefficient of 168 $mM^{-1}cm^{-1}$ found for the Soret maximum of lignin peroxidase isozyme H8 (Tien & Kirk 1988) is applicable, the enzyme concentration in this solution is 65 nM and the apparent turnover number of the enzyme for ADE oxidation under the assay conditions is 24 min^{-1}. The rate showed a Michaelis-Menten pattern of dependence on ADE concentration, with a K_m of 0.53 μM.

Products of ADE bleaching

No products from enzymatic degradation of ADE could be detected by UV spectroscopy or by thin-layer chromatography of extracts from either crude culture filtrates or purified enzyme preparations. Particular attention was paid to the possible presence of anthraquinone, but it was not detected.

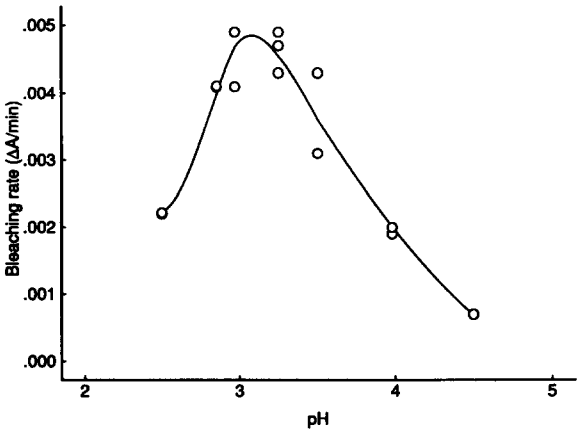

Fig. 6. Effect of pH on bleaching of ADE by extracellular enzyme. Reaction mixtures contained 3.00 ml of 0.25 M sodium tartrate buffer at an appropriate pH, 6 μg of ADE dissolved in 10 μl of DMF, 10 μl of 50 mM H_2O_2, and 10 μl of an enzyme solution purified by FPLC (A_{408} = 0.654). The pH of each reaction mixture was measured after determining the reaction rate.

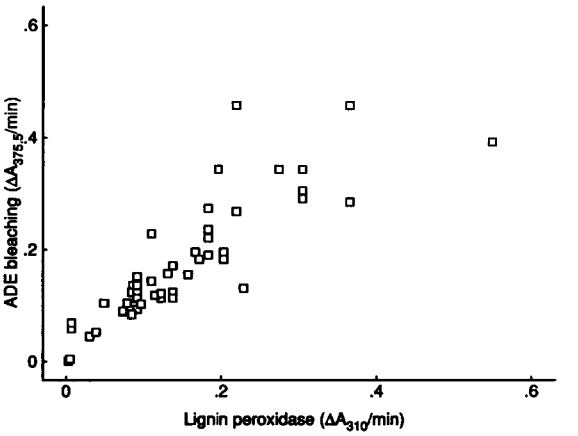

Fig. 7. Correlation between ADE-bleaching and lignin peroxidase activities in supernatants of *P. chrysosporium* cultures. Enzyme activities were measured with 2.0 ml of culture supernatant in a final reaction volume of 3.0 ml, as described in 'Materials and methods'. Cultures varied in age from 4 to 6 days, in supplementation with trace elements and/or veratryl alcohol, and in fungal strain (ME-446 or BKM F-1767).

Interaction with veratryl alcohol oxidation

When a mixture of ADE and veratryl alcohol was treated with the purified enzyme and H_2O_2, the bleaching of the ADE was accelerated compared to a reaction mixture without veratryl alcohol (Fig. 9). The oxidation of veratryl alcohol to veratraldehyde was retarded, compared to a reaction mixture without ADE, until the ADE had disappeared. The final extent of veratryl alcohol oxidation was lower in the reaction mixture which contained ADE (Fig. 9).

Discussion

Our results show that bleaching of ADE (and presumably AES) is not a reliable indication of singlet O_2, unless the formation of the endoperoxide is confirmed. Anthracene derivatives can be oxidized by a variety of oxidants in addition to singlet O_2. Cultures of *P. chrysosporium* bleach ADE, but do not form the endoperoxide as an intermediate; thus they cannot be using singlet O_2 to degrade ADE, AES, or lignin.

There is strong evidence that lignin peroxidase catalyzes at least the first step in ADE degradation. The bleaching of ADE by *P. chrysosporium* culture supernatants requires H_2O_2, but the activity is inactivated by H_2O_2 concentrations higher than 200 μM, as has been reported for lignin peroxidase (Tien et al. 1986). ADE bleaching has a low pH optimum, around 3, similar to lignin peroxidase (Tien et al. 1986). Production of ADE bleaching activity depended on strain of *P. chrysosporium* and supplementation with veratryl alcohol and trace elements as described for lignin peroxidase (Kirk et al. 1986). ADE bleaching was highly correlated with lignin peroxidase activity across a range of culture ages and medium compositions. Finally, the ADE bleaching enzyme co-purified with lignin peroxidase on high resolution ion exchange chromatography.

Veratryl alcohol stimulated degradation of ADE by the purified extracellular enzyme, and ADE inhibited veratryl alcohol oxidation. This type of interaction between veratryl alcohol and a variety of other lignin peroxidase substrates has been noted previously (Haemmerli et al. 1986, Harvey et al. 1986, Momohara et al. 1990) and has been attributed to participation of the radical cation of veratryl alcohol as a mediator (Harvey et al. 1986, 1989), or to protection of lignin peroxidase from

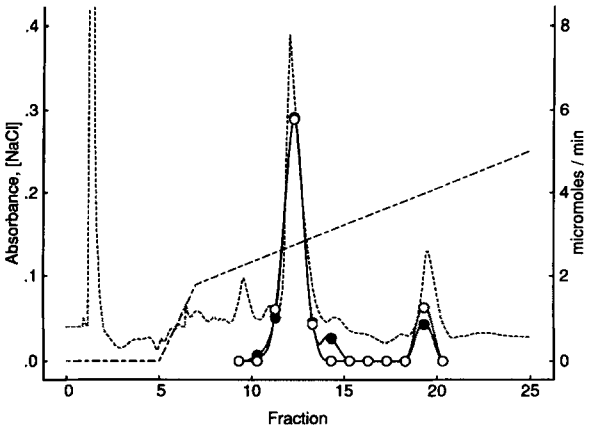

Fig. 8. Purification of extracellular ADE-bleaching enzymes by anion-exchange chromatography on a Mono Q column. , absorbance at 410 nm, ⁻ ⁻, salt concentration, (○), veratryl alcohol oxidizing activity, (●), ADE bleaching activity. Enzyme assays were performed with 50 μl samples from each fraction.

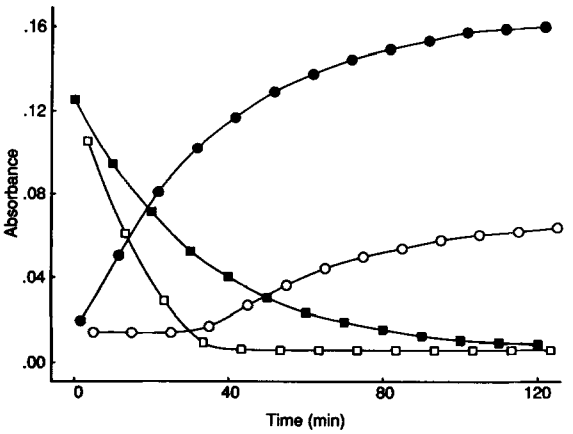

Fig. 9. Kinetics of oxidation of veratryl alcohol (A_{310}, circles) and bleaching of ADE ($A_{375.5}$, squares) by purified enzyme separately (filled symbols) and in a mixture (open symbols). Reaction mixtures contained 1.0 ml of 0.25 M sodium tartrate, pH 3.0, 20 μl of purified enzyme (major peak from Fig. 8, A_{408} = 0.654), 0 or 3 μg of ADE dissolved in 5 μl of DMF, 0 or 20 μl of 20 mM veratryl alcohol in DMF, and 5 μl of 50 mM H_2O_2. The spectrum of each reaction mixture from 400 to 250 nm was scanned repeatedly at 10 min. intervals.

inactivation by H_2O_2 (Haemmerli et al. 1986, Tonon & Odier 1988, Wariishi & Gold 1989, 1990, Valli et al. 1990).

Others have found quinones as the products of lignin peroxidase oxidation of polynuclear aromatic molecules (Hammel 1989, Hammel et al. 1986, Haemmerli et al. 1986), and anthracene-9,10-diethanol-1,5-dione is a plausible product from ADE. However, we could not detect this or any other identifiable product in reaction mixtures or fungal cultures. The initial attack on ADE must be the rate-limiting step in its degradation, and the initial products must be rapidly broken down further. Lignin peroxidase has been found to degrade 2-hydroxy-1,4-naphthoquinone in the presence of veratryl alcohol (Momohara et al. 1990); a similar reaction could participate in the degradation of ADE.

Both ADE and its endoperoxide transiently inhibit lignin degradation. The fact that the endoperoxide is no less inhibitory than ADE supports the argument that trapping of singlet O_2 is not the mechanism of inhibition by ADE or AES. The fact that ADE is a substrate for lignin peroxidase, and retards the oxidation of veratryl alcohol by that enzyme, suggests that ADE, AES, and EPX inhibit lignin degradation by competing with lignin for oxidation by lignin peroxidase, and possibly other oxidative enzymes. The K_m of ADE for lignin peroxidase is much lower than the reported K_m of veratryl alcohol or of a lignin model dimer (Farrell et al. 1989), indicating a high affinity of ADE for the enzyme. Thus the original idea that AES intercepts an oxidant in cultures of *P. chrysosporium* (Nakatsubo et al. 1981) is supported, but the identity of that oxidant has changed.

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