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Veratryl alcohol oxidases from the lignin-degrading basidiomycete *Pleurotus sajor-caju*

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The basidiomycete *Pleurotus sajor-caju* mineralizes *ring*-^{14}C-labelled lignin (dehydrogenative polymer) when grown in mycological broth. Under these conditions, two veratryl alcohol oxidase (VAO) enzymes were found in the culture medium. They oxidized a number of aromatic alcohols to aldehydes and reduced O_2 to H_2O_2. The enzymes were purified by ion-exchange and gel-permeation chromatography. The final step of purification on Mono Q resolved the activity into two peaks (VAO I and VAO II). Both enzymes had the same M_r, approx. 71000, but their isoelectric points differed slightly, 3.8 for VAO I and 4.0 for VAO II. Their amino acid compositions were similar except for aspartic acid/asparagine and glycine. Both enzymes are glycoproteins and contain flavin prosthetic groups. Their pH optima were around 5, and kinetic constants and specificities were similar. 4-Methoxybenzyl alcohol was oxidized the most rapidly, followed by veratryl alcohol. Not all aromatic alcohols were oxidized, neither were non-aromatic alcohols. Cinnamyl alcohol was oxidized at the γ position. The VAO enzymes thus represent a significantly different route for veratryl alcohol oxidation from that catalysed by the previously found lignin peroxidases from *Phanerochaete chrysosporium*. The role of the oxidases in biodegradation might be to produce H_2O_2 during oxidation of lignin fragments.

INTRODUCTION

Lignin is a three-dimensional polymer found abundantly in wood and plant tissue (Sarkanen & Ludwig, 1971). It is composed of phenylpropanoid units interconnected by stable C–C and C–O bonds. The heterogeneity and complexity of its structure confers resistance to microbial attack. However, lignin can be degraded slowly in Nature, mainly by white-rot fungi, and this has considerable impact in forestry and agriculture.

Lignin biodegradation by white-rot fungi is an oxidative process in which H_2O_2 plays an important role (Crawford & Crawford, 1984). The lignin-degrading system and enzymes involved have been studied quite extensively in the basidiomycete species *Phanerochaete chrysosporium*. When actively degrading lignin, this strain produces extracellular H_2O_2 (Faison & Kirk, 1985) and a number of peroxidases that were able to oxidize several lignin model compounds (Tien & Kirk, 1983; Glenn *et al.*, 1983; Huynh & Crawford, 1985). One of the peroxidases, named lignin peroxidase (Tien & Kirk, 1983), was found to be induced by veratryl alcohol, which is synthesized *de novo* by the fungus during its ligninolytic phase (Faison *et al.*, 1986). The enzyme is routinely assayed by the oxidation of veratryl alcohol to veratraldehyde in the presence of H_2O_2. In this same fungus many enzymes have been proposed for H_2O_2 generation, including the intracellular enzymes glucose 1-oxidase (Kelly & Reddy, 1986) and glucose 2-oxidase (Eriksson *et al.*, 1986) and, more recently, an extracellular glyoxal oxidase (Kersten & Kirk, 1987). H_2O_2-producing oxidases have also been reported from other lignin-degrading fungi, including an extracellular aromatic alcohol oxidase from *Polystictus versicolor* (Farmer *et al.*, 1960) and an $\alpha\beta$-unsaturated aromatic alcohol oxidase from *Fusarium* sp. (Iwahara *et al.*, 1980).

Enzymes involved in lignin degradation from *Pleurotus* spp. are still uncharacterized, even though this white-rot fungus has been recognized as a lignin degrader (Trojanowski & Leonowicz, 1969; Hiroi & Eriksson, 1976; Leatham & Kirk, 1983). Here we report on the purification and characterization of extracellular aromatic alcohol oxidases from *Pleurotus sajor-caju* and their possible implication in lignin degradation.

MATERIALS AND METHODS

Enzyme production and purification

Pleurotus sajor-caju strain 405 (Pulp and Paper Research Institute of Canada Collection) was maintained through periodic transfer on malt/agar plates. Shake flasks (16 × 500 ml), each containing 250 ml of mycological broth (Difco) and a 2 cm glass bead, were inoculated with a 1 cm mycelial disc from a 5-day-old malt/agar plate. Cultures were incubated at 25 °C and 200 rev./min for 12 days. Cultures were combined and centrifuged (8000 *g* for 30 min at 4 °C) and phenylmethanesulphonyl fluoride (0.2 mM) was added to the supernatant to minimize proteolysis. Proteins from the supernatant were precipitated by adding solid $(NH_4)_2SO_4$ up to 80% saturation at 0 °C and centrifuged at 8000 *g* for 2 h. The precipitate was resuspended in 300 ml of 0.05 M-sodium acetate buffer, pH 5.0, and dialysed overnight against 4 litres of the same buffer. Insoluble material was centrifuged and the supernatant was concentrated by ultrafiltration (Amicon PM-10 filter) to about 60 ml. The sample was applied to a DEAE-Bio-

Abbreviations used: VAO, veratryl alcohol oxidase; DHP, dehydrogenative polymer; ABTS, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate).
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Gel A column (2.5 cm × 35 cm) previously equilibrated with the same buffer. The column was washed with 20 ml of the buffer and then eluted with a linear salt gradient from 0 to 0.6 M-NaCl to a total volume of 550 ml with a flow rate of 13 ml/h. Fractions containing the VAO activity were pooled and concentrated on Millipore immersible ultrafiltration units (CX-10) to a final volume of 8 ml. The sample was chromatographed on a Sephacryl S-200 (Pharmacia) column (2.5 cm × 87 cm) equilibrated in 0.05 M-sodium acetate buffer, pH 5.0, with an upward flow of 10 ml/h. Pooled fractions were then dialysed against 0.01 M-sodium phosphate buffer, pH 7.0, concentrated on CX-10 to a final volume of 10 ml, and applied to a column (1.5 cm × 30 cm) of hydroxyapatite (Bio-Gel HT; Bio-Rad Laboratories) previously equilibrated in the same buffer. The column was washed with 50 ml of the starring buffer and eluted with a buffer gradient up to 0.2 M-phosphate buffer at a rate of 1 mM/ml with a flow rate of 20 ml/h. Enzyme fractions were dialysed against 0.02 M-Bistris/HCl buffer, pH 6.5, concentrated by ultrafiltration and finally chromatographed on a Mono Q HR 5/5 (Pharmacia) column previously equilibrated in the Bistris buffer. Enzymes were eluted with a linear salt gradient (0–0.15 M-NaCl in starting buffer) during 20 min.

Protein determination

The protein content of each fraction was determined spectrophotometrically at 595 nm with Coomassie Blue (Bradford, 1976). Bovine plasma γ-globulin was used as protein standard (Bio-Rad Protein Assay).

Biomass measurement

The dry biomass concentration was determined by filtering 10 ml of culture through a Millipore filter (8 μm porosity), washed with distilled water and oven-dried at 100 °C.

Assay for degradation of ^{14}C-labelled DHP to $^{14}CO_2$

The *ring*-^{14}C-labelled DHP was prepared by E. Odier (I.N.R.A., Paris, France) according to the procedure described by Faix *et al.* (1985). The specific radioactivity of the ^{14}C-DHP was 277 kBq/mg and was free of low-M_r fractions. Erlenmeyer flasks (500 ml) each containing 150 ml of mycological broth and 4×10^5 d.p.m. of ^{14}C-DHP were inoculated with 2 ml of a 5-day liquid culture of *Pleurotus*. The flasks were closed with rubber stoppers equipped with tube ports to permit periodic O_2 flushing and collection of CO_2 (Kirk *et al.*, 1975). Flasks were incubated at 25 °C with constant shaking (200 rev./min) and flushed with sterile O_2 at 3–4-day intervals. CO_2 was trapped in 10 ml of ^{14}C scintillation cocktail (cat. no. OX 161; R. J. Harvey Instrument Corp.). Radioactivity was measured with a Beckman LS 6800 liquid-scintillation counter.

Enzyme assays

For determination of VAO activity the reaction mixture contained 1 mM-veratryl alcohol, 0.25 M-sodium tartrate buffer, pH 5.0, and a suitable amount of enzyme in a total volume of 3 ml. Oxidation of the substrate at room temperature was monitored by an absorbance increase at 310 nm due to the formation of veratraldehyde ($\epsilon_{310} = 9300\ M^{-1} \cdot cm^{-1}$). One unit of the enzyme is defined as the amount producing 1 μmol of veratraldehyde/min under the assay conditions.

Lignin peroxidase was measured by veratryl alcohol oxidation at pH 3.0 in the presence of 0.4 mM-H_2O_2 (Tien & Kirk, 1983).

Laccase activity was determined by oxidation of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) (Wolfenden & Willson, 1982). The reaction mixture contained 0.03% ABTS, 0.1 M-sodium acetate buffer, pH 5.0, and a suitable amount of enzyme. Oxidation of ABTS was followed by absorbance increase at 420 nm ($\epsilon_{420} = 3.6 \times 10^4\ M^{-1} \cdot cm^{-1}$).

O_2 uptake during substrate oxidation was measured with a Clark oxygen electrode (Rank Brothers, Cambridge, U.K.) at room temperature in a reaction volume of 3 ml. The composition of the reaction mixture was the same as in the spectrophotometric assay for VAO described above. The H_2O_2 produced was determined by adding 3000 units of catalase to the reaction mixture.

Electrophoresis and isoelectric focusing

SDS/polyacrylamide-gel electrophoresis was performed on the Phast System (Pharmacia) with a Phast Gel gradient of 10–15% acrylamide. Protein M_r standards (Pharmacia) were α-lactalbumin, soya-bean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin and phosphorylase *b*. Isoelectric focusing was performed with Phast Gel IEF apparatus with a pH range of 3–9 (Pharmacia) with the use of the isoelectric-focusing calibration kit from Pharmacia. Protein bands were stained with Coomassie Blue. Schiff staining of the gels for glycoprotein determination was performed in accordance with Glossmann & Neville (1971).

Spectroscopic procedures

Absorption spectra were recorded on a Perkin–Elmer Lambda 3 spectrophotometer at room temperature in a 1 cm-light-path cuvette. The enzymes were dissolved in 0.02 M-Bistris/HCl buffer, pH 6.5. The reduced enzymes were obtained by adding a few crystals of $Na_2S_2O_4$, and the reoxidized enzymes were then obtained by passing O_2 through the solution.

Chemicals

All substrates tested for enzyme specificity were obtained from Aldrich Chemical Co.

Amino acid analysis

Protein samples were hydrolysed with 6 M-HCl at 150 °C in sealed evacuated tubes for 1 and 3 h. Hydrolysates were analysed on a Beckman 6300 amino acid analyser. Concentrations of each amino acid were calculated by extrapolation to zero time. Cysteine was determined as cysteic acid after performic acid oxidation.

RESULTS

VAO production

Lignin biodegradation, as determined by $^{14}CO_2$ release from *ring*-^{14}C-labelled DHP, occurred in nitrogen-rich agitated cultures of *Pleurotus sajor-caju* (Fig. 1). Culture supernatant oxidized veratryl alcohol to veratraldehyde in the absence of H_2O_2. The VAO was characterized further as described below. Polyphenol oxidase (laccase) was also present in the culture supernatant, but lignin peroxidase, determined by H_2O_2-dependent oxidation of veratryl alcohol at pH 3.0, was not detected.

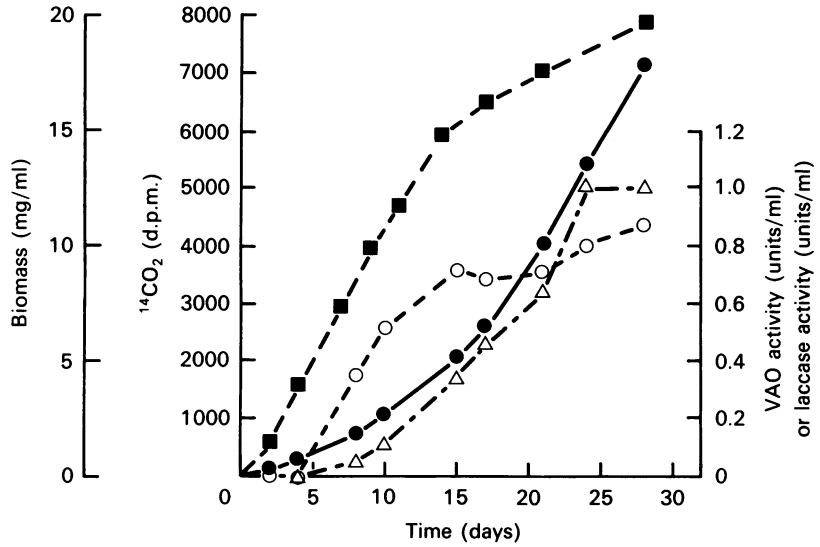

Fig. 1. Time course of ligninolytic activity (*ring*-^{14}C-labelled DHP → $^{14}CO_2$) (●), biomass concentration (■), VAO activity (△) and laccase activity (○) during growth of *Pleurotus sajor-caju* in agitated mycological broth cultures

For experimental details see the Materials and methods section.

Table 1. Purification procedure for the VAO enzymes

Purification step	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Purification factor	Yield (%)
Supernatant	440	0.88	387	1.0	100
$(NH_4)_2SO_4$ precipitation	237	1.40	332	1.6	85.8
DEAE-Bio-Gel	23	9.9	228	11.2	58.9
Sephacryl S-200	7.5	19.9	149	22.6	38.5
Hydroxyapatite	4	30.6	122	34.8	31.5
Mono Q					
VAO I	0.6	35.0	21	39.8	5.4
VAO II	1.0	30.7	32	36.4	8.3

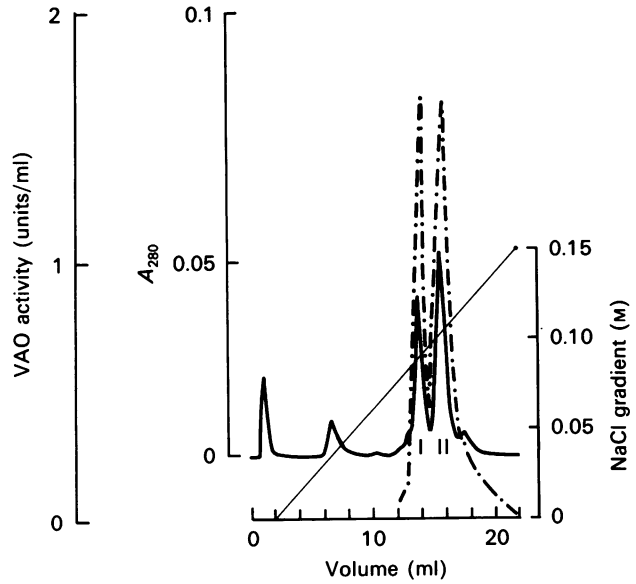

Fig. 2. Elution profile of VAO activities during the final step of purification on Mono Q anion-exchanger

Chromatography conditions are described in the Materials and methods section, ——, A_{280}; ·—·—·, VAO activity.

Purification of enzymes

Table 1 presents a summary of the VAO purification procedure. The last step of the purification, using the Mono Q anion-exchange chromatography, resolved the enzyme into two distinct peaks of activity (VAO I and VAO II), as shown in Fig. 2. The two enzymes have similar specific activities, between 31 and 35 μmol of veratryl alcohol oxidized/min per mg of protein. There was an overall recovery of 14% of the total activity present in the supernatant.

Characterization

SDS/polyacrylamide-gel electrophoresis (Fig. 3*a*) and isoelectric focusing (Fig. 3*b*) confirmed the purity of the two enzymes. They have similar M_r values of approx. 71000 as measured by SDS/polyacrylamide-gel electrophoresis, but their isoelectric points differed slightly, 3.8 for VAO I and 4.0 for VAO II. Schiff staining of the gels showed that the VAO enzymes were glycosylated.

Table 2 shows the amino acid content of each enzyme based on an M_r of 71000. Within the limit of error (approx. 5%) the content of most of the amino acids for the two enzymes is the same except for aspartic acid and asparagine (Δ 9%) and glycine (Δ 11%). The difference

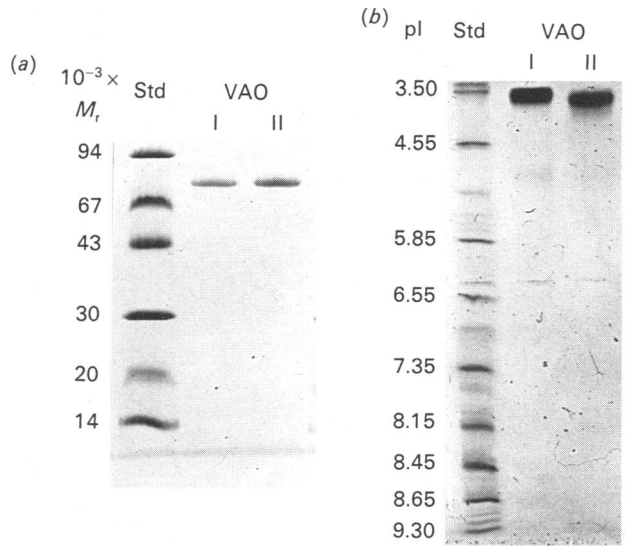

Fig. 3. (*a*) SDS/polyacrylamide-gel electrophoresis and (*b*) isoelectric focusing of VAO I and VAO II relative to standards (Std)

For experimental details see the Materials and methods section.

Table 2. Amino acid compositions of the VAO I and VAO II

The analyses were performed as described in the Materials and methods section, and were calculated on the basis of an M_r value of 71000 for VAO I and VAO II. Abbreviation: N.D., not determined.

	Residues	
Amino acid	VAO I	VAO II
Asp + Asn	83	92
Thr	42	44
Ser	57	59
Glu + Gln	48	47
Gly	77	66
Ala	62	63
Cys	N.D.	4
Val	48	46
Met	6	9
Ile	33	31
Leu	55	55
Tyr	14	11
Phe	32	31
His	9	11
Lys	12	11
Trp	N.D.	N.D.
Arg	30	30
Pro	42	41

between the amount of the charged amino acids aspartic acid and asparagine could explain the different isoelectric points of the two enzymes.

Spectral properties

The visible-region-absorption spectra of VAO II are shown in Fig. 4. The spectra for VAO I are similar. The native enzymes have two maxima at 385 and 460 nm,

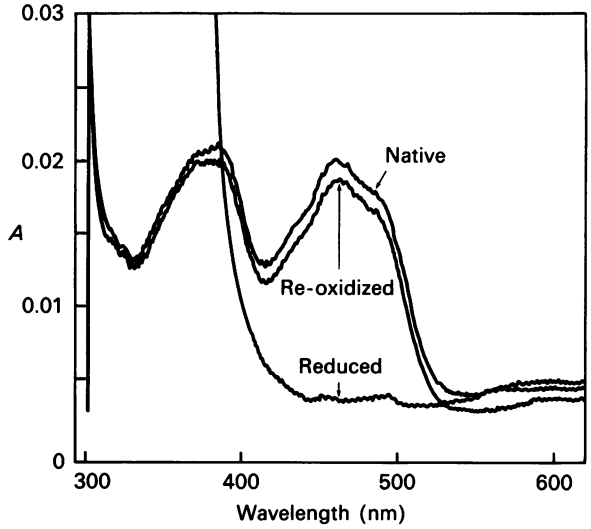

Fig. 4. U.v.–visible-absorption spectra of native, reduced and re-oxidized VAO II

For experimental details see the Materials and methods section.

which were bleached upon addition of dithionite. The reduced enzymes, which have lost the maxima, could be reoxidized with O_2 to restore the spectral properties of the native enzymes. These results indicate that flavin is the prosthetic group of the VAO enzymes. The ϵ_{460} and ϵ_{385} were respectively 6.21×10^6 and 7.10×10^6 $M^{-1}\cdot cm^{-1}$ for VAO I and 5.20×10^6 and 5.54×10^6 $M^{-1}\cdot cm^{-1}$ for VAO II.

pH optimum, K_m and molecular activity

The activities of the VAO enzymes as a function of pH are shown in Fig. 5. Sodium tartrate buffer (0.25 M) was used for pH 3–5.5 and 0.25 M-sodium phosphate buffer was used for pH 5.5–8.0. The two enzymes showed their maximum activities around pH 5. These enzymes have a quite large range of pH for their activity, more than 90%

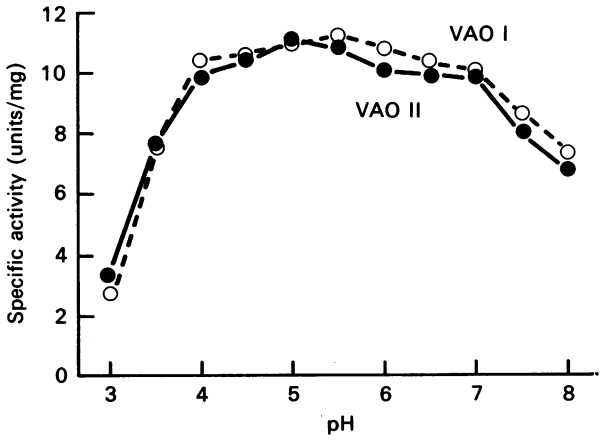

Fig. 5. Specific activities of VAO I (○) and VAO II (●) different pH values

For experimental details see the Materials and methods section.

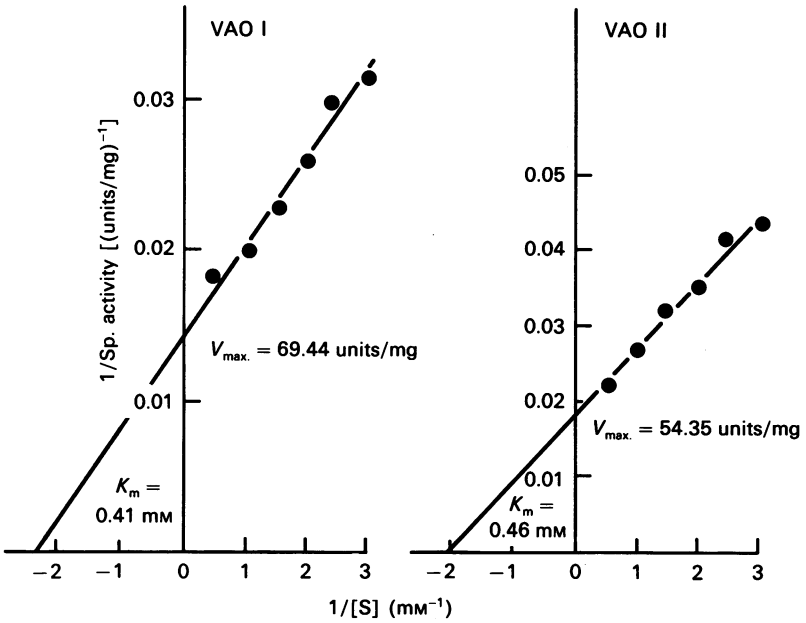

Fig. 6. Lineweaver–Burk plots of VAO I and VAO II with veratryl alcohol substrate

Table 3. Relative rate of oxidation of various alcohols by the VAO I and VAO II

Measurements of O_2 consumption were performed as described in the Materials and methods section and results are expressed as percentages of the activity observed with veratryl alcohol. All alcohols were tested at a concentration of 1 mM. Values less than 5% were considered negligible and expressed as –.

	Relative oxidation rate (%)	
Alcohol	VAO I	VAO II
Veratryl	100	100
2-Methoxybenzyl	–	–
3-Methoxybenzyl	19	16
4-Methoxybenzyl	200	266
2,3-Dimethoxybenzyl	–	–
2,4-Dimethoxybenzyl	50	75
2,5-Dimethoxybenzyl	–	–
3,5-Dimethoxybenzyl	7	8
3,4,5-Trimethoxybenzyl	–	–
3-Hydroxy-4-methoxybenzyl	62	71
4-Hydroxy-3-methoxybenzyl	–	–
2-Hydroxybenzyl	–	–
3-Hydroxybenzyl	–	–
4-Hydroxybenzyl	–	–
Benzyl	30	25
Coniferyl	–	–
Cinnamyl	77	55
3,4-Dimethoxyphenethyl	–	–
3-(3,4-Dimethoxyphenyl)-1-propyl	–	–
4-Biphenylmethyl	–	–
3-Phenoxybenzyl	35	18
Allyl	–	–
Crotyl	–	–
Ethyl	–	–

of the maximum activity being observed between pH 4 and 7.

The Lineweaver–Bank plots for the two enzymes are shown in Fig. 6. The K_m and turnover number for veratryl alcohol were respectively 0.41 mM and 4930 min^{-1} for VAO I and 0.46 mM and 3860 min^{-1} for VAO II.

Measurement of O_2 consumption, with and without catalase, indicates that 1 mol of O_2 oxidizes 1 mol of veratryl alcohol to give 1 mol of veratraldehyde and 1 mol of H_2O_2. The presence of superoxide radicals ($O_2^{\cdot-}$) was tested with cytochrome *c* at pH 7.5 (McCord & Fridovich, 1969). The VAO enzymes were unable to reduce cytochrome *c* in the presence of veratryl alcohol and O_2, indicating that $O_2^{\cdot-}$) was not produced during the reaction.

Substrate specificity of the enzyme

The relative activity of the two enzymes with different substrates was determined by measuring the O_2 consumption and was expressed as a percentage of the activity with veratryl alcohol (Table 3). All substrates that can be oxidized by one form of the enzyme can also be oxidized by the other, but sometimes at a different rate compared with veratryl alcohol. The highest activity was found with 4-methoxybenzyl alcohol. All compounds with a hydroxy moiety in position 4 were not oxidized by the enzymes. For the saturated aromatic alcohol, only the α-alcohols were oxidized but not the β- and γ-alcohols. However, in the case of cinnamyl alcohol, which is an αβ-unsaturated aromatic alcohol, the alcohol in the γ-position was rapidly oxidized by the enzymes. Non-aromatic alcohols and αβ-unsaturated aliphatic alcohols were not oxidized by the enzymes.

DISCUSSION

Veratryl alcohol is biosynthesized from glucose by white-rot fungi such as *Phanerochaete chrysosporium* and *Coriolus versicolor*. It has been proposed that it plays a key role in electron transfer between lignin peroxidase and lignin (Harvey *et al.*, 1986). The rapid oxidation of veratryl alcohol by cultures of *Pleurotus* has also been noted (Waldner *et al.*, 1986). We propose here that in

Pleurotus the major route to oxidation is through catalysis by VAO. As such, the veratryl alcohol is subject to a two-electron oxidation with concomitant reduction of O_2 to H_2O_2, instead of peroxidase-catalysed one-electron oxidation to a radical cation followed by proton loss. VAO may masquerade as lignin peroxidase in assays with veratryl alcohol, since the oxidase functions quite well at pH 3 in the presence of H_2O_2 (Fig. 5). Thus the absolute requirement for H_2O_2 in assays of lignin peroxidase must always be verified.

The two forms of the enzyme isolated here are so similar that they can probably be classified as isoenzymes. Their separation on ion-exchange chromatography may be due to a difference in one or a few charged amino acid residues, such as aspartic acid. Their substrate specificity and kinetic constants are similar. Primary aromatic alcohols only are oxidized, but the substitution pattern on the ring plays a key role. Thus cinnamyl alcohol is oxidized but coniferyl alcohol is not. Increasing hydroxy-group substitution in the ring prevents oxidation, whereas methoxy-group substitution increases oxidation rates.

The physiological significance of extracellular VAO is still unclear. The enzyme is produced during primary metabolism and has a fairly specific substrate range. Lignin biodegradation by *Pleurotus sajor-caju* also occurs during growth (Fig. 1), as observed for *Pleurotus ostreatus* (Leatham & Kirk, 1983), and unlike *Phanerochaete chrysosporium* or *Coriolus versicolor*. Laccases are produced by *Pleurotus sajor-caju* in agitated mycological broth (Fig. 1), but not lignin peroxidase. If VAO plays a role in lignin biodegradation, it could be either through two-electron redox cycling of veratryl alcohol or lignin fragments, with H_2O_2 production from O_2, or, in combination with laccase, as a dehydrogenase of aromatic compounds.

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