Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*

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Two H_2O_2-dependent oxidases found in the extracellular medium of the white rot fungus *Phanerochaete chrysosporium* were separated by chromatography on blue agarose. The first enzyme fraction to elute from the column generated ethylene from 2-keto-4-thiomethylbutyric acid (KTBA) in the presence of veratryl alcohol, and catalyzed the α,β cleavage of the diarylpropane 1-(3′,4′-diethoxyphenyl)-1,3-dihydroxy-(4″-methoxyphenyl)propane (I). During the diarylpropane cleavage, ^{18}O from $^{18}O_2$ was incorporated specifically into the α-position of the product 1-(4′-methoxyphenyl)-1,2-dihydroxyethane (III), suggesting that this enzyme is an H_2O_2-dependent oxygenase. The second enzyme which binds to blue agarose is an Mn^{2+}-dependent, lactate-activated peroxidase. The enzyme catalyzed the oxidation of phenol red, *o*-dianisidine, Poly R, and a variety of other dyes. It was also capable of decarboxylating vanillic acid.

Oxidase *Lignin degradation* *Basidiomycete* *Diarylpropane* *Hydrogen peroxide* *Ethylene*

1. INTRODUCTION

Recently, we reported on an H_2O_2-requiring enzyme preparation found in the extracellular medium of ligninolytic cultures of *Phanerochaete chrysosporium* [1,2]. This activity was not found in nonligninolytic cultures nor in clutures of a non-ligninolytic mutant [3] of this organism. This extracellular preparation is able to generate ethylene from KTBA [1,4], to oxidize a variety of lignin model compounds [1,5], to depolymerize lignin [1,5], and to decolorize the polymeric dye Poly R [2,6]. We describe here the separation of this crude preparation into two H_2O_2-requiring enzyme fractions by blue agarose chromatography. The first fraction, containing an apparent H_2O_2-requiring oxygenase, is responsible for the cleavage of KTBA and the oxidation of a variety of lignin model compounds, including the diarylpropane (I). The second enzyme is an Mn^{2+}-dependent, lactate-activated peroxidase.

2. MATERIALS AND METHODS

KTBA, phenol red and Poly R-481 were obtained from Sigma. COOH-labeled vanillic acid (4-hydroxy-3-[*carboxy*-^{14}C]methoxybenzoic acid) was obtained from Research Products International. Lignin model compounds were synthesized as in [7,8]. *P. chrysosporium* ME 446 was maintained on slants as in [9]. Stationary cultures in 2-l flasks containing 150 ml medium were inoculated, incubated at 28°C and purged with 100% O_2 every 3 days. After 6 days cultures were filtered through cheesecloth and the filtrate was centrifuged at

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Abbreviations: KTBA, 2-keto-4-thiomethylbutyric acid; TMS, trimethylsilyl

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25 000 × *g* for 30 min to remove mycelial and spore contaminants, as in [1]. Acetone (−10°C) was added to the crude enzyme fraction to 66% (v/v). The precipitated protein was centrifuged at 25 000 × *g* at −10°C for 15 min and resuspended in 20 mM Na–succinate (pH 4.5), one-fiftieth of the original volume. Insoluble material was removed by recentrifugation and the supernatant was dialyzed against 20 mM Na–succinate (pH 4.5).

The acetone concentrate (6 ml) was adsorbed to a column of reactive blue 2–cross-linked agarose (Sigma) (1.0 × 17 cm) equilibrated with 20 mM Na–succinate, 100 mM NaCl (pH 4.5). The column was eluted with 25 ml equilibration buffer after which it was eluted with 20 mM Na–succinate, 250 mM NaCl (pH 4.5). Fractions (2 ml) were collected. Enzyme peaks were pooled and 1 ml of each fraction was applied separately to a Sephadex G-100 column (1.7 × 50 cm) previously equilibrated with 20 mM Na–succinate, 100 mM NaCl (pH 4.5). Fractions were pooled and assayed for enzyme activity.

Ethylene generation from KTBA was measured as in [1] except that veratryl alcohol (3,4-dimethoxybenzyl alcohol) (1 mM) was added to all reactions. Reaction mixtures (2 ml) contained enzyme, glucose oxidase (0.02 units/ml), glucose (3 mM) and KTBA (1 mM) plus veratryl alcohol. After 1 h at 37°C, 1–2.5 ml of gas was removed from the headspace and ethylene was measured by GC. Peroxidase activity was measured by a modification of the procedure in [10]. Reaction mixtures (1 ml) consisted of phenol red (0.01%), lactate (25 mM), $MnSO_4$ (100 μM), egg albumin (0.1%), and H_2O_2 (100 μM) in 1.0 ml of 20 mM Na–succinate buffer (pH 4.5). Reactions were carried out at 30°C for 5 min and terminated with the addition of 2 N NaOH (40 μl). Absorbance was read at 610 nm.

Model compound oxidations were carried out in 1 ml of 20 mM Na–tartrate (pH 3.0) containing 100 μM H_2O_2 or glucose/glucose oxidase as in [1] and enzyme. 1-(3′,4′-Diethoxyphenyl)-1,3-dihydroxy(4″-methoxyphenyl)propane (I) or veratryl alcohol were added to reaction mixtures to a final concentration of 0.02% (w/v). Substrates and products were extracted with ethyl acetate. The combined organic fraction was dried over anhydrous sodium sulfate and evaporated under a stream of nitrogen. Trimethylsilylation was carried out by adding bis(*N*,*O*-trimethylsilyl)trifluoroacetamide–pyridine (1 : 1) to the dry residue and heating carefully at 60°C for 3 min. GC and mass analyses were as performed as in [7,8,11]. [*carboxy*-^{14}C]Vanillic acid decarboxylation was measured by purging reaction mixtures with 100% O_2, trapping $^{14}CO_2$ in a basic scintillation fluid and counting radioactivity as in [3].

Diarylpropane (I) and veratryl alcohol oxidation by purified fractions were routinely monitored spectrophotometrically at 310 nm. Reaction mixtures (1 ml) consisted of substrate (3 mM), H_2O_2 (100 μM) and enzyme in Na–tartrate buffer (pH 3.0).

In experiments to measure the incorporation of ^{18}O, reaction vessels contained two compartments, one containing the enzyme and $H_2{}^{16}O_2$, the other the diarylpropane (I). Reaction vessels were evacuated, flushed with scrubbed argon, re-evacuated and finally equilibrated with $^{18}O_2$. Reactions were started by mixing the contents of the vessel and incubating at 37°C for 2 h. Extraction was as above and mass analyses were as in [7,8].

3. RESULTS AND DISCUSSION

Approx. 60% of the KTBA and phenol red oxidase activities were recovered in the acetone concentrate. The results in fig.1 indicate that the two enzyme fractions were separated by chromatography on blue agarose. KTBA cleavage activity eluted with 0.1 M NaCl while the phenol red peroxidase eluted only after the salt concentration of

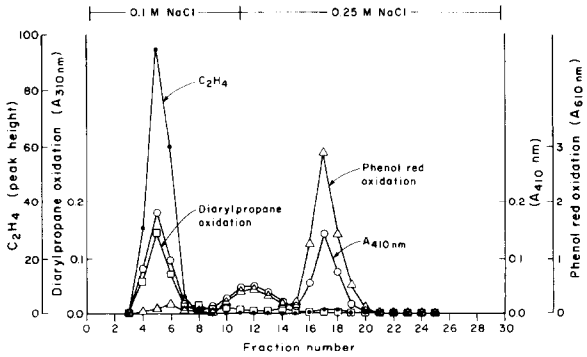

Fig.1. Separation of two extracellular oxidases by blue agarose chromatography. (□) Diarylpropane oxidation measured at 310 nm; (●) ethylene generation from KTBA; (△) phenol red oxidation; and (○) absorbance of the fractions at 410 nm. Experimental procedures and assays are described in the text.

the eluant was increased to 0.25 M. Absorbance of the fractions at 410 nm parallels the activity peaks, suggesting that both enzymes are heme proteins. Preliminary spectral evidence also indicates that both oxidases are heme proteins.

Fractions from each of the two major activity peaks were pooled and used for enzyme characterization and gel filtration experiments. Fig.2 shows a Sephadex G-100 gel filtration profile for each enzyme activity. Each enzyme elutes as a single symmetrical peak. The M_r of each enzyme as determined by the method in [12] was ~41 000. This indicates that these enzymes are not separable solely by the use of gel filtration. SDS–polyacrylamide gel electrophoresis indicates, however, that the dye peroxidase has an M_r of ~46 000.

The dye peroxidase displayed maximal activity at pH 4.5 and showed an absolute dependency for Mn^{2+}. Maximal stimulation occurs at 100 μM Mn^{2+}. The dye peroxidase also showed absolute dependency on lactate with optimal activity occurring at approx. 25 mM lactate. The enzyme was stimulated by increased protein concentration in reaction mixtures, e.g., 0.1% egg albumin stimulated activity approx. 2.5-fold. Optimal activity also occurred at approx. 100 μM H_2O_2. The peroxidase was inhibited completely by NaN_3, KCN and EDTA, each at 1 mM. The enzyme oxidized a variety of dyes including phenol red, *o*-dianisidine and Poly R [6] and was also capable of decarboxylating [*carboxy*-^{14}C]vanillic acid to yield $^{14}CO_2$.

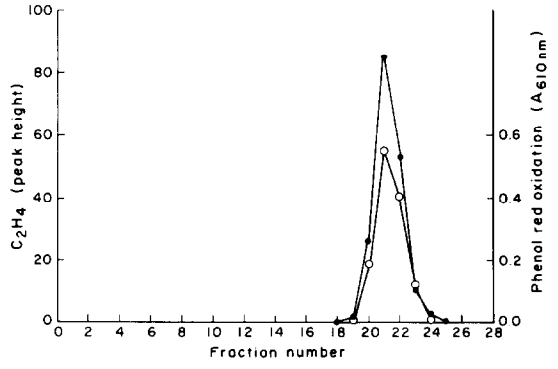

Fig.2. Gel filtration profiles of two oxidases on Sephadex G-100. (○) Phenol red oxidation; (●) ethylene generation. Each enzyme fraction was filtered separately and the profiles were superimposed in this figure. Experimental procedures and details are described in the text.

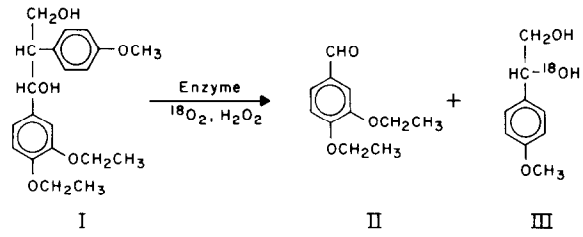

Fig.3. Incorporation of ^{18}O from $^{18}O_2$ into the diol product (III) during the α,β cleavage of the diarylpropane (I) by an oxygenase found in the extracellular medium of *P. chrysosporium*. Experimental details are described in the text.

However, the latter activity did not appear to be Mn^{2+} dependent. This is the first report of an Mn^{2+}-dependent peroxidase from *P. chrysosporium*. The exact role of the enzyme in lignin degradation is being investigated.

The second enzyme separated by blue agarose chromatography (fig.1) catalyzed, in the presence of H_2O_2, the cleavage of the diarylpropane (I) at the α,β bond to yield 3',4'-diethoxybenzaldehyde (II) and 1-(4'-methoxyphenyl)-1,2-dihydroxyethane (III) (fig.3). This reactivity and the similar M_r of ~41 000 suggest that this enzyme is probably the same as that recently reported [5]. This H_2O_2-requiring enzyme also cleaved KTBA in the presence of the secondary metabolite veratryl alcohol (1 mM) [13]. Finally, veratryl alcohol was also oxidized to veratrylaldehyde as measured at 310 nm and by thin-layer chromatography. Unlike KTBA cleavage, diarylpropane cleavage by this enzyme was not dependent on veratryl alcohol. The diarylpropane oxidase displayed maximal activity at pH 3.0 and approx. 50–100 μM H_2O_2. In the absence of H_2O_2, no products were formed. This enzyme was also completely inhibited by NaN_3, KCN, EDTA and thiourea, each at 1 mM.

As shown in fig.3, when the diarylpropane cleavage was performed in the presence of H_2O_2 + $^{18}O_2$, ^{18}O was incorporated into the diol (III). The mass spectrum of the TMS derivative of the diol product included the following ion peaks (*m/e*, rel. int.): 314, 0.02% (M^+); 299, 0.71% (M-CH_3); 211, 100% (M-CH_3-OTMS). Intensity of the ion peaks at 297 and 209 was less than 5% of those at 299 and 211. When the same experiment was conducted under $^{16}O_2$, the major fragment ions were found at *m/e*

297 and 209, the same as in the chemically synthesized standard. This indicates that one ^{18}O atom was incorporated specifically into the α-position of the diol (III) (β-position of the diarylpropane) as reported in [2], and that this oxygen atom is donated by O_2 rather than by H_2O_2. The results are the same as those obtained with ^{18}O incorporation into the diarylpropane (I) in intact cultures [14,15]. These results indicate that this enzyme is a novel H_2O_2-dependent oxygenase. Further characterization of these enzymes is in progress.

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