Short Communication

A Manganese(II)-Dependent Extracellular Peroxidase from the White-Rot Fungus *Trametes versicolor**

Tomas Johansson and Per Olof Nyman

Division of Biochemistry, Chemical Center, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden

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Ever since it became known that lignin and lignocellulose can be degraded by strongly oxidixing enzymes found in common wood-degrading organisms, primarily the white-rot basidiomycetes, efforts have been made to elucidate the properties and the roles of these enzymes. The most studied fungus, *Phanerochaete chrysosporium,* has been shown to produce a veratryl alcohol-oxidizing peroxidase called ligninase.[1,2] Furthermore, a Mn(II)-dependent peroxidase has been identified.[3,4] It has an oxidizing activity towards phenolic structures and is also supposed to be involved in the degradation of lignin. The Mn(II)-dependent enzyme oxidizes Mn(II) to Mn(III), which in turn is able to oxidize various substrates. It is a true peroxidase which can utilize hydrogen peroxide in oxidizing low-molecular-weight phenols, thereby forming phenoxy radicals.

The ligninase and the Mn(II)-dependent peroxidase from *P. chrysosporium* have been purified and are now under study in several laboratories. Both enzymes occur in multiple forms as glycosylated heme proteins. It is unclear whether this heterogeneity is due to varying degrees of glycosylation or other post-translational modifications, or is caused by multiplicities of structural genes.[5]

We have started investigations on another lignin-degrading fungus, namely the white-rot basidiomycete *Trametes (Coriolus) versicolor*. Our aim is to find out whether the principles and pathways for enzymatic lignin degradation now emerging from studies on *P. chrysosporium* are also valid for other lignin-degrading systems. *T. versicolor* has long been known to produce laccases and to be effective in degrading lignins and lignocelluloses.[6] It has recently been shown to produce ligninase.[7,8] Here, we present evidence for the existence of an inducible Mn(II)-dependent peroxidase excreted from *T. versicolor* and some information concerning the properties of this enzyme.

Experimental

The organism *Trametes versicolor* (L.ex Fr.) Pilát, strain PRL 572, was grown in a carbon-limited medium according to Dodson *et al.*,[7] slightly modified for increased peroxidase production by using: 10× [$FeSO_4$], 50× [$CaSO_4$, $MnSO_4$, $ZnSO_4$, $CuSO_4$, thiamin-HCl] and 10 mM phosphate, giving a pH around 5. Stationary cultures (300 ml of culture fluid in 1000 ml Erlenmeyer-flasks) were induced from the start by inclusion of 10 mM veratryl alcohol (final concn.), a concentration which had been found to increase peroxidase production. On the 11th day the medium was collected, filtered and dialysed against a 20 mM histidine buffer (pH 5.5) containing 10 mM NaCl. The dialysate (500 ml) was subjected to chromatography on a 50 ml Q-Sepharose column (Pharmacia) pre-equilibrated with 20 mM histidine buffer (pH 5.5) containing 10 mM NaCl.

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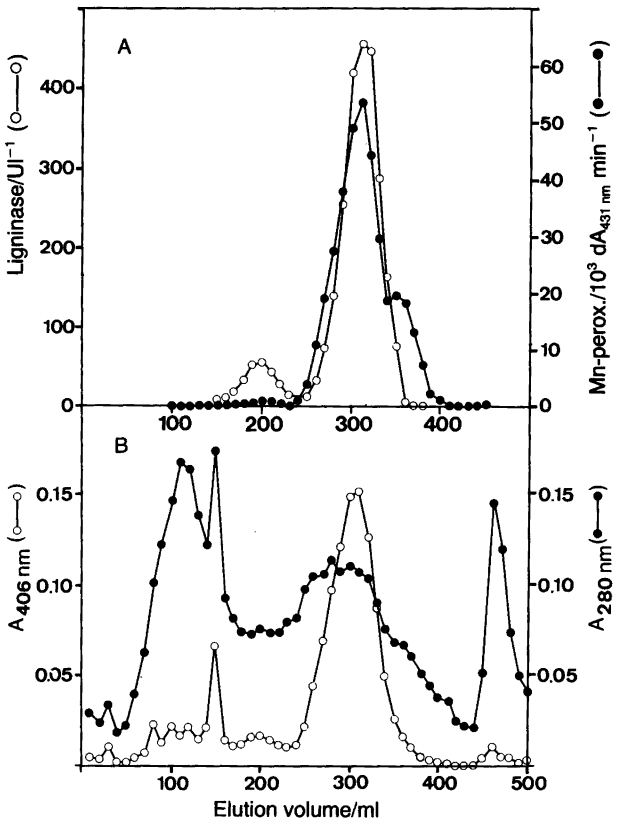

Fig. 1. Anion-exchange chromatography of medium from a culture of *T. versicolor*. A: Distribution of enzyme activities. The ligninase was found to oxidize phenol red with or without veratryl alcohol or Mn(II) added. The Mn(II)-peroxidase curve is therefore an accumulated profile contributed to by Mn(II)-dependent peroxidase as well as ligninase. A unique contribution by Mn(II)-peroxidase at an elution volume of 360 ml (Pool B) was selected for further purification, resulting in the isolation of two isozymes (see Fig. 3). B: Distributions of protein ($A_{280\,nm}$) and heme ($A_{406\,nm}$).

The column was eluted with the same buffer using a linear gradient of from 10 to 250 mM NaCl and employing a total volume of 300 ml. The elution rate was 4.5 ml min^{-1} and 10 ml fractions were collected. Fractions corresponding to the peak of Mn(II)-dependent peroxidase activity (see Fig. 1) were pooled (Pool B), dialysed against the same buffer and separated further on a Mono Q HR 5/5 column connected to a FPLC apparatus (Pharmacia). In this way, two enzyme fractions were isolated (fractions I and II). After dialysis, they were concentrated using a Speed Vac concentrator (Savant) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3) using a gradient gel (12.6–20 %) (Pharmacia) which was subsequently stained with Coomassie Brilliant Blue.

Ligninase activity was assayed spectrophotometrically by monitoring the change in absorbance at 310 nm corresponding to the oxidation of veratryl alcohol, as described by Kirk *et al.*[9] but using 0.1 M Na-tartrate buffer, pH 2.9. Mn(II)-dependent peroxidase activity was measured as phenol red oxidation in the presence and absence of $MnSO_4$.[4] For testing Mn(II)-dependence, a constant amount of enzyme (100 µl) and increasing amounts of $MnSO_4$ were added up to a final reaction volume of 1 ml. Final concentrations in the reaction mixture were 0.0025 % phenol red, 6.25 mM lactate, 0.025 % bovine serum albumin and 5 mM 2,2-dimethylsuccinate buffer, pH 4.5. The reaction was started by adding 9 µl of 2 mM H_2O_2 and run at room temperature. For testing H_2O_2-dependence the reaction was carried out in the presence of 0.30 mM $MnSO_4$. We found that the reaction could be conveniently followed spectrophotometrically at 431 nm, monitoring the decrease in substrate concentration.

Results and discussion

Evidence has been obtained for the production of a Mn(II)-dependent peroxidase by the white-rot basidiomycete *T. versicolor*, this enzyme possibly

playing a role in lignin degradation. Stationary cultures of the organism in a carbon-limited medium, induced by the addition of veratryl alcohol, were found to excrete the enzyme almost in parallel with the veratryl alcohol-oxidizing peroxidase (ligninase), but in smaller amounts. This pattern of induced peroxidase production resembles that described for another white-rot basidiomycete, *P. chrysosporium*.[3]

The enzyme shows properties of a peroxidase, oxidizing phenol red in the presence of H_2O_2 and Mn(II). The dependence of the reaction rate upon the concentrations of these reactants is shown in Fig. 2. The concentrations for optimal rate were found to be 0.30 mM for $MnSO_4$ and 0.020 mM for H_2O_2. These concentrations differ from those given for the Mn(II)-dependent peroxidase from *P. chrysosporium*, where corresponding values of 0.100 mM and 0.100 mM have been reported.[4]

The Mn(II)-dependent peroxidase was isolated from the growth medium of *T. versicolor* by repeated chromatographic steps. The first of these steps is illustrated in Fig. 1 as an elution profile from a Q-Sepharose column. The two peaks of ligninase activity (at 200 and 300 ml of effluent volume, respectively) were investigated by SDS-PAGE (Fig. 3). The material in the first peak shows a molecular mass significantly lower than for the two isozyme bands in the second peak. Besides the two peaks of lingninase activity, there is a shoulder of activity which could be separated into two isozymes of Mn(II)-dependent peroxidase. These isozymes seem to differ in charge but not in molecular mass (Fig. 3). With the procedure used, both isozymes appear to be obtained in, or close to, a state of homogeneity sufficient for characterization of molecular properties.

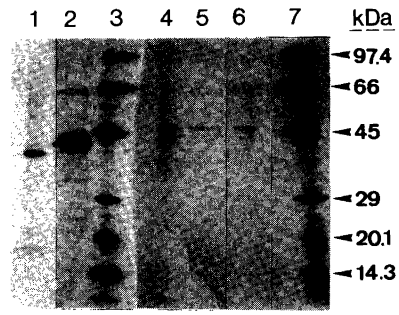

Fig. 3. Different Mn(II)-dependent peroxidase-containing fractions analyzed by SDS-PAGE. **Lanes 3 and 7:** Molecular mass standard. **Lane 1:** Top fraction from the first ligninase peak in Fig. 1 (around 200 ml effluent volume). **Lane 2:** Top fraction from the second ligninase peak in Fig. 1 (around 300 ml effluent volume), showing two isozyme bands (cf. Ref. 8). **Lane 4:** Pool B [pooled fractions of Mn(II)-dependent peroxidase peak at 360 ml effluent volume in Fig. 1]. By rechromatography of pool B two enzyme fractions were obtained (I and II). **Lane 5:** Rechromatographed Pool B, fraction I, **Lane 6:** Rechromatographed Pool B, fraction II.

Besides the Mn(II)-dependent peroxidase representing the shoulder of activity at 360 ml of effluent volume (Fig. 1), a smaller contribution of Mn(II)-dependent peroxidase, not further in-

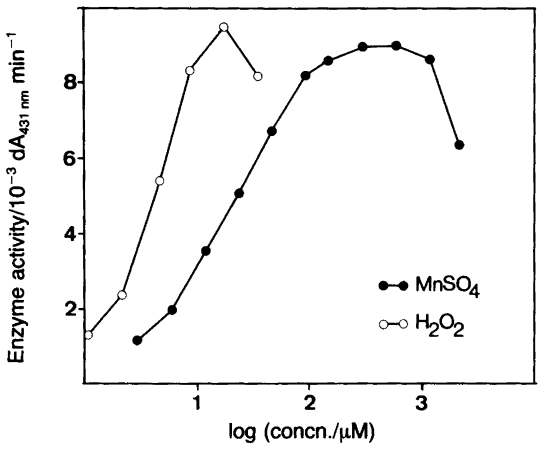

Fig. 2. Manganese and H_2O_2-dependences of the oxidation of phenol red catalyzed by a peroxidase preparation from *T. versicolor*. The experiment was carried out with a partially purified preparation of Mn(II)-dependent peroxidase (Pool B from the chromatography in Fig. 1).

vestigated, appears to occur at around 270 ml of effluent volume.

The molecular mass of the two isozymes of Mn(II)-dependent peroxidase isolated from *T. versicolor* was estimated from electrophoretic mobility (Fig. 3) to be around 49 000 Da, comparable to the value of 45 000–47 000 Da reported for the corresponding enzyme from *P. chrysosporium*.[3]

As far as we know, this is the first description of a procedure for production and purification of a Mn(II)-dependent peroxidase from *T. versicolor*. The results obtained will be utilized in further studies of the enzyme and its role in the ligninolytic pathways of *T. versicolor*.

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