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AN EXTRACELLULAR HAEM-PROTEIN FROM *CORIOLUS VERSICOLOR*

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Key Word Index—*Coriolus versicolor*, wood-rotting basidiomycete; haem-protein; lignin degradation; hydroxyl radicals

Abstract—A haem-containing protein has been isolated from the growth medium of *Coriolus versicolor*, a wood-rotting basidiomycete. The polypeptide was identified as a 'peroxidase-type' haem protein of MW 53 700, which appeared to be a glycoprotein and had a protoporphyrin IX prosthetic group with a mid-point redox potential of −121 mV. It also bound carbon monoxide suggesting it may act as an oxidase, and liberated hydroxyl radicals from hydrogen peroxide as measured by its ability to release ethylene from methional.

INTRODUCTION

Coriolus versicolor is a wood-rotting basidiomycete of the type causing white-rot decay. It has the ability to degrade the three major wood components—lignin, cellulose and hemicellulose—at approximately the same rate [1, 2]. The mode of attack on wood as shown by electron-microscopy studies is extracellular and must occur close to the tip of the hyphae, at least in the initial stages of breakdown, to account for the patterns of decay in wood [3, 4].

Previous studies have identified some of the extracellular enzymes of *C. versicolor* including a flavoprotein with alcohol oxidase activity towards primary but not secondary aromatic alcohols [5]; cellulolytic enzymes with activities which include a β-glucosidase and up to five cellulolytic components [6, 7]; a cellobiose:quinone oxidoreductase activity which catalysed the reduction of a quinone to the corresponding phenol, coupled to the conversion of cellobiose to cellobionic acid [8]; three phenol oxidases of the laccase type, all copper-containing proteins [9]; and laccases A and B, induced by 2,5-xylidine which were purified by Fähraeus and Reinhammer [10]. Many of these enzymes have been implicated as active ligninolytic enzymes, though few of them have been fully characterized as to their structures, prosthetic groups and activity towards lignin-type compounds. The white-rot fungus *Phanerochaete chrysosporium* (*Sporotrichum pulverulentum*) has been studied in more detail with respect to its physiological requirements for ligninolytic activity [11–15], and some interesting properties of an isolated extracellular enzyme from *S. pulverulentum* have been reported [16], namely a cellobiose oxidase which used molecular oxygen to oxidise cellodextrins to the corresponding aldonic acids. Characteristics of this enzyme show it to be a glycoprotein of MW 93 000, with a haem/flavin prosthetic group. Eriksson [17] has postulated that cellobiose oxidase may give rise to free radicals of oxygen.

In the last few years it has been suggested that lignin breakdown may be triggered by a non-enzymatic attack involving free radicals of oxygen [18] and recent experimental evidence [19, 20] that hydroxyl radicals ($OH^{\cdot}$) produced by cultures of *P. chrysosporium* will cause lignin breakdown have supported this hypothesis. The origin of the $OH^{\cdot}$ in these cultures is unknown, but in some biological systems it is known to arise from the interaction of superoxide (O_2^-) with hydrogen peroxide in the iron-catalysed Haber–Weiss reaction, or by a reaction similar to the Fenton reaction in which Fe(II) reacts with hydrogen peroxide. It is possible that an Fe(II)-containing protein may also catalyse the generation of hydroxyl radicals from hydrogen peroxide.

In this paper we report the purification of an extracellular protein from *C. versicolor* which has not previously been described from any white-rot fungus. It is a haem-containing polypeptide which has the ability to produce superoxide radicals from hydrogen peroxide, suggesting that it may be a potential source of oxygen radicals for lignin degradation.

RESULTS AND DISCUSSION

C. versicolor was grown in a glucose–amino acid medium with the production of laccase induced by the addition of 2,5-xylidine. The extracellular proteins were extracted from the growth medium by ammonium sulphate precipitation; 90 l. of growth medium containing 11 g of protein. As large quantities of material were available it was possible to isolate other proteins as well as laccase in reasonable amounts from this preparation.

The variety of polypeptides in this extract, on separation by polyacrylamide electrophoresis on sodium dodecyl sulphate (SDS) gels, are shown in Fig. 1 (track 1). The dominant band of MW 62 000 in the extract was a mixture of laccases A and B. Cultures not induced for laccase showed a similar pattern of polypeptide bands as the induced cultures, though the level of laccase was much lower (Fig. 1, track 2). There was some variation in the relative intensities of the bands which reflected the fact that laccase formed over 80% of the total protein in the induced cultures, so the amounts of other proteins in this extract loaded on to the gel were much less in comparison with those in the non-induced cultures. The protein extract from the induced culture was chromatographed on DEAE-Sephadex and separated into six fractions, by

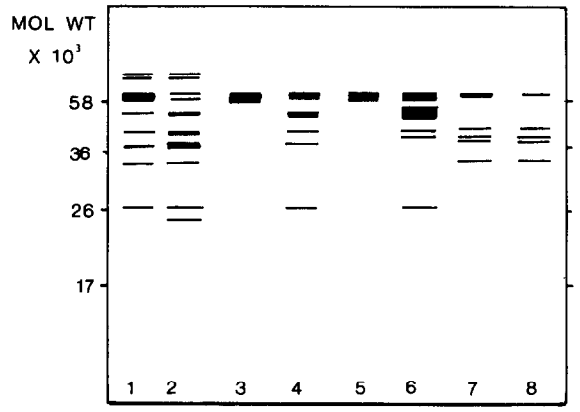

Fig 1. SDS-polyacrylamide gel electrophoresis of the extracellular proteins of a *C. versicolor* culture grown on glucose, before and after fractionation on DEAE-Sephadex. Track 1, total proteins from culture induced for laccase with 2,5-xylidine. Track 2, total proteins from non-induced culture Tracks 3–8 contain the following proteins from a laccase-induced culture after fractionation: 3. Fraction I (laccase B); 4. Fraction II; 5. Fraction III (laccase A); 6. Fraction IV (haem); 7. Fraction V; 8 Fraction VI.

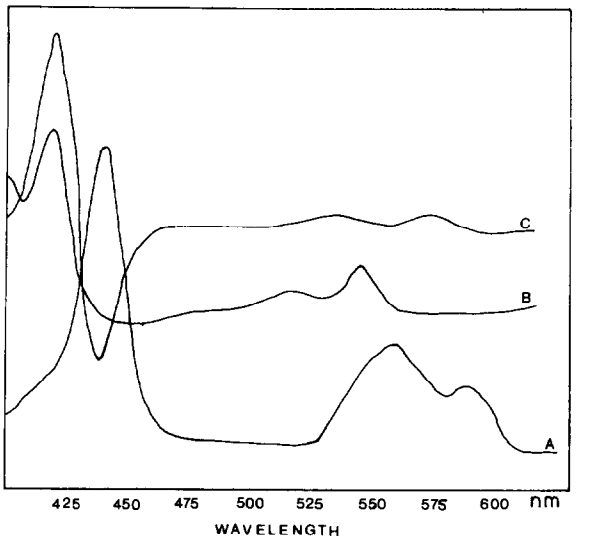

Fig. 2. Optical spectra of the haem protein from *C. versicolor*: A, redox difference spectrum at 77°K; B, pyridine-haemochromogen spectrum; C, CO-binding spectrum of the reduced haem protein.

eluting with an increasing gradient of phosphate buffer. Figure 1 shows the polypeptides in these fractions as separated by SDS-gel electrophoresis. Fraction 1 did not bind to the column, and was identified as laccase B. As the phosphate concentration was increased, a yellow band containing at least five proteins was eluted (Fig. 1, Fraction II) followed by laccase A (Fig. 1, Fraction III). When the phosphate concentration had reached 200 mM a brown protein band was eluted (Fig. 1, Fraction IV) which was separated into five polypeptides. Laccase A was identified as the highest MW component (62 000), but the dominant polypeptide in the fraction had a MW of 53 700. As the concentration of the phosphate buffer increased above 200 mM, a second brown band eluted (Fig. 1, Fraction V) followed by a second yellow band (Fig. 1, Fraction VI). Fractions V and VI contained smaller amounts of protein than the previous fractions, and traces of laccase were found in both fractions. Staining for glycoproteins on the gel showed that only laccase A and the dominant band at MW 53 700 in Fraction IV were positive. Fraction IV was then re-chromatographed on a second column of DEAE-Sephadex, after which virtually all traces of laccase A were removed from the fraction containing the MW 53 700 glycoprotein. Separation of this glycoprotein from other polypeptides in the fraction was achieved using Concanavalin A-Sepharose 4B, which tightly bound the glycoprotein but allowed all other proteins to pass through the column. The glycoprotein was eluted with α-methyl-D-mannopyranoside, giving a final yield of 22 mg obtained from 90 l. of growth medium.

The red–brown appearance of this protein suggested that it might contain a haem group, and this was confirmed by measuring its absorption spectrum. In Fig. 2, spectrum A shows the reduced minus oxidized spectrum measured at 77°K with dithionite as the reductant. The strong Soret band at 442 nm and α and β bands at 588 nm and 560 nm respectively characterized the spectrum. On treatment with alkaline pyridine, the pyridine–haemochromogen showed a reduced minus oxidized spectrum at 410, 523 and 556 nm (Fig. 2, spectrum B), which was consistent with the haem being protoporphyrin IX. Alteration of the spectrum occurred in the presence of carbon monoxide (Fig. 2, spectrum C), showing that carbon monoxide could bind to the iron in the haem group, suggesting that the haem would be able to bind oxygen and so function as an oxidase. The protein was tested for catalase activity, by measuring the release of oxygen from hydrogen peroxide, and was found to exhibit only a very transient activity that ceased soon after the peroxide was added. The electron paramagnetic resonance spectrum showed the haem prosthetic group to be typical of a peroxidase/catalase type haem protein (Fig. 3). However hydrogen peroxide had no effect on the absorption spectrum of the protein indicating that it did not bind to form a hydrogen peroxide complex, but it caused denaturation of the haem centre. Staining of the protein on the gel for peroxidase activity was strongly positive with both tetramethylbenzidine and 3-amino-9-ethylcarbazole. Although the protein showed some similarities to catalase or peroxidase it also had characteristics suggesting that it was neither of these enzymes. This was emphasized by its reduction by dithionite requiring only 10 min for complete reduction, and a mid-point potential measured at −121 mV, which was higher than that of catalase.

The reaction between hydrogen peroxide and the protein suggested that free radicals of oxygen might be released. Oxygen radical release could be measured using the reaction of methional and hydroxyl radicals to produce ethylene [21]. Figure 4 shows the rate of reaction (expressed as mol of ethylene) to be dependent on the concentration of protein in the reaction mixture in the presence of hydrogen peroxide. Table 1 shows the production of ethylene to be dependent on the presence of

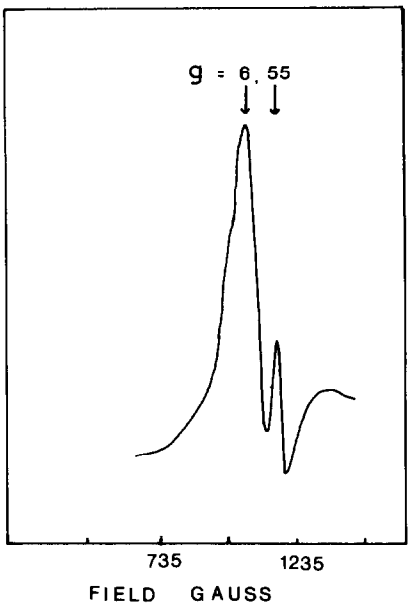

Fig. 3. The $g = 6$ region of the EPR spectrum of the haem protein.

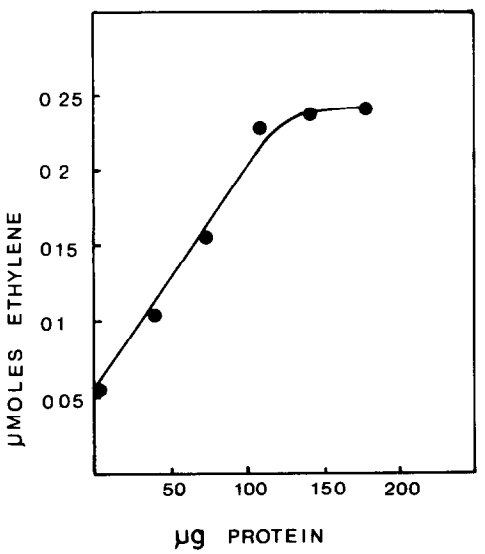

Fig. 4. Hydroxyl radical production by the haem protein from hydrogen peroxide. The reaction mixture contained 2×10^{-3} M methional, 1 ml of 6% hydrogen peroxide with 0–175 μg protein. Reaction time was 2 hr.

hydrogen peroxide and methional.

Experiments carried out in the presence of OH·-scavenging agents such as mannitol or thiourea, resulted in the expected suppression of ethylene production. When superoxide dismutase was included in the reaction mixture, there was also a marked decrease in ethylene production. This observation tends to suggest that superoxide radicals are involved in the sequence of reactions leading to ethylene production, possibly as precursors of hydroxyl radicals. It is unclear from these experiments whether ethylene is formed as a result of the direct release

Table 1. Effect of OH·-scavenging agents on the production of ethylene gas from methional and hydrogen peroxide by haem-protein from *C. versicolor*

Treatment	Ethylene production (nmol/ml)	Percent of complete
Complete	45.8	100
$-H_2O_2$	4.6	10
− methional	0.1	0.2
+ 100 mM mannitol	21.6	47
+ 10 mM thiourea	22.6	49
+ 0.2 mg superoxide dismutase	16	35

Complete system contained 100 μg of methional, 1 ml of 6% hydrogen peroxide and 50 μg of haem-protein

of hydroxyl radicals from the reaction between the haem protein and hydrogen peroxide or from superoxide or hydroxyl radicals arising from the breakdown of the iron moiety of the protein which then reacts with hydrogen peroxide in a Fenton-type reaction. However the dependence of ethylene production on the concentration of the protein, and its suppression by radical scavengers provides evidence that this haem protein is capable of producing oxygen radicals in the presence of hydrogen peroxide, and must therefore be considered as a potentially lignin olytic enzyme.

EXPERIMENTAL

Stock cultures. Coriolus versicolor strain 28A PRL (Building Research Establishment, Princes Risborough Laboratory, Aylesbury, Bucks, UK) was grown on malt agar plates at 25°

Large-scale cultures for enzyme production. Coriolus versicolor was grown in 100 l. cultures on a glucose–amino acid medium [10] for 5 days. For the production of laccase, 2,5-xylidine was added as inducer by one addition in 50% EtOH at a concn in the growth medium of 2×10^{-4} M, on the fifth day of growth, and the culture harvested 19 hr later.

Isolation of proteins After filtration to remove the fungal hyphae, the extracellular proteins were extracted from the growth medium by pptn with $(NH_4)_2SO_4$ to 80% satn. The ppt included a large amount of a mucilaginous material produced by the fungus, entrapping the proteins, which were released by successive freezing and thawing of the ppt.

The proteins were redissolved in 50 mM KPi buffer at pH 6, and dialysed overnight against the same buffer, before pptn for a second time with 80% $(NH_4)_2SO_4$. This ppt was redissolved in the KPi buffer and dialysed as previously A third $(NH_4)_2SO_4$ pptn up to 30% satn removed some non-proteinaceous material and was followed by satn up to 65% with $(NH_4)_2SO_4$. This ppt was collected, redissolved in the KPi buffer before dialysis overnight, against the same buffer, and used as the crude protein extract The protein components of this extract were separated by ion exchange on a DEAE-A50 Sephadex column The column (40 × 3 cm) was equilibrated with 50 mM KPi buffer pH 6, and the crude protein extract was added Fraction I (mainly laccase B) was eluted unretarded with 50 mM KPi buffer, followed by a slightly retarded yellow fraction (Fraction II). Laccase A (Fraction III) was then eluted as a blue band with 0.1 M KPi buffer, and increasing the buffer concn to 0.2 M a brown band was eluted (designated IV). At 0 5 M KPi buffer a second brown band (designated V) was separated, closely followed by a second

yellow band (designated VI).

Fraction IV was rechromatographed on a second column of DEAE-Sephadex (20 × 2 cm) with a gradient elution (between 0.1 and 0 5 M) of KPi buffer, pH 6.

Fractionation on Concanavalin-A Sepharose. A column (11 × 1 cm) of Concanavalin-A Sepharose (Pharmacia) was washed in 10 mM KPi buffer pH 6.5. The fraction containing the haem protein was added to the column after dialysis overnight against the washing buffer On washing with 10 mM KPi buffer, a tightly bound band remained at the top of the column, red–brown in colour, while a yellow band passed through without binding. The brown band was desorbed by elution with 10% (w/v) of α-methyl-D-mannopyranoside in 10 mM KPi buffer pH 6.5. This fraction was dialysed overnight against H_2O, and concd by freeze drying

Protein measurement. Protein determinations were made by the method of ref [22], using bovine serum albumin as a standard *A* of proteins at 280 nm, was used to determine the positions of protein-containing fractions in column effluents.

Assay for polyphenol oxidase (laccase). 10 μl of test soln was added to 1 ml of 0.1 M catechol dissolved in 100 mM NaOAc buffer at pH 5. The increase in *A* at 440 nm was proportional to polyphenol oxidase activity One unit of polyphenoloxidase caused a change in OD of 1 0 per min at 25°

Sample preparation for electrophoresis. Protein samples were adjusted to contain between 0 5 and 0.1 mg protein per ml, they were then mixed with an equal vol of a soln containing 90 mM Tris–HCl (pH 6.8), 18% glycerol, 0.0018% v/v bromophenol blue, 10% v/v mercaptoethanol and 0.4% SDS. The mixture was then heated at 100° for 3 min and finally cooled to 4°.

Electrophoresis The polypeptides were resolved on 10% linear polyacrylamide gels containing 0 1% SDS under a 3% polyacrylamide stacking gel containing 0.1% SDS The gels were polymerized according to the method of ref. [23]. The gels were cast in a slab 13 × 16 cm and 1.5 mm thick. The samples were applied as 100 μl aliquots, containing 10–50 μg of proteins, into wells 0.75 cm wide The running buffer contained 0.1% SDS, 0.3% Tris and 1.4% glycine. The polypeptides were then separated using a constant current of 35 mA per slab at 4° The process was stopped after *ca* 3.5 hr when the bromophenol blue marker reached the bottom of the resolving gel. The gel was then removed and stained in Coomassie blue for 2 hr at 50° and destained in several changes of 10% HOAc for 48 hr

Stain for glycoproteins. Gels were stained with periodic acid and Schiff's reagent [24].

Stain for peroxidases. Gels were immersed in 3-amino-9-ethylcarbazole reagent [24], when peroxidase areas stained red–brown

Stain for haem proteins. Samples for electrophoresis were prepared as above but contained 0 4% lithium dodecyl sulphate (LDS) in place of 0.4% SDS, without heating, and resolved on polyacrylamide gels polymerized without SDS. The upper gel running buffer contained 0 1% LDS, 0.6% Tris and 2 8% glycine, while the lower gel buffer contained 0.6% Tris and 2.8% glycine Gels were stained in 3,3',5,5'-tetramethylbenzidine [25]

Measurement of OH· formation. The OH· is known to react with methional to produce ethylene gas [21] which was measured using a Pye Unicam 104 gas chromatograph equipped with a Porapak R column (1 m × 5 mm) and a flame-ionization detector. Carrier gas was N_2 at 1 ml/sec and the chromatograph oven temp. was 45° The reaction mixture contained 2×10^{-3} M methional, 1 ml of 6% H_2O_2, with 0–200 μg of protein, in a 10 ml glass vial fitted with a Suba-seal stopper Radical scavenging agents were added as indicated in Table 1 The amount of ethylene gas present in 0.5 ml samples of head space in each vial was measured.

Optical spectra were obtained using an Aminco DW-2 dual wavelength spectrophotometer run in the split beam mode Samples were reduced by the addition of 1–2 mg of Na dithionite. Pyridine–haemochromogen spectra were obtained by adding 2 mg protein to 5 ml of alkaline pyridine (0.3 M KOH–pyridine, 3:2) This soln was divided between the blank and sample cuvettes and 1–2 mg of Na dithionite added to the sample cuvette. After 90 sec the difference spectrum was recorded.

Low-temp EPR spectra were obtained using a Jeol FE1-X band EPR spectrometer

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