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H_2O_2-Dependent Decolorization of Poly R-481 by Particulate Fractions from Phanerochaete chrysosporium

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A cell-free preparation from Phanerochaete chrysosporium culture medium decolorized the polymeric dye Poly R-481. The majority of this decolorization activity sedimented when centrifuged at 150,000 X g, indicating that it was associated with a particulate body. The activity was sensitive to heat, azide and cyanide, was stimulated by exogenously added H_2O_2, and was optimal around pH 4. Electron micrographs of the sedimented culture medium fraction showed the presence of numerous particulate structures. A similar dye decolorization activity from sonicated mycelium also sedimented at 150,000 x g.

Several lines of evidence suggests an intimate association between the outer surface of P. chrysosporium hyphae and lignin during the degradation process. Binding of lignin to ligninolytic fungal mycelia is a long recognized phenomenon (1-3), and the stimulation of P. chrysosporium H_2O_2 production by lignocellulose (4) suggests the possibility that lignin binding may activate the degradation mechanism. Finally, electron microscopic studies of P. chrysosporium hyphae have shown an apparent correlation between the occurrence of periplasmic microbodies and the occurrence of ligninolytic activity (5). Because these microbodies were heavily

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stained by 3,3'-diaminobenzidine (DAB), it was suggested that extracellular H_2O_2 production may be localized in these structures.

A number of enzymes isolated from P. chrysosporium culture media have been found to oxidize some bonds in lignin and lignin model compounds, but only in the presence of H_2O_2 (6-9). However, the ligninolytic enzymes isolated from P. chrysosporium culture media by Gold and coworkers (6,7) and Tien and Kirk (10,11) did not sediment with the hyphal cells in a low-speed centrifugation, suggesting that these enzymes may be soluble and extracellular. In an effort to better characterize the cellular location of the lignin degrading activity of P. chrysosporium, we have examined the ability of several fractions isolated from hyphae and from cell-free culture medium to decolorize Poly R-481.[1] In this paper we report that the majority of the decolorization activity in P. chrysosporium culture medium is associated with a particulate fraction that sediments readily at 150,000 X g. Similarly, the decolorization activity present in a sonicated hyphal preparation was also found to be associated with a particulate fraction sedimenting at 150,000 x g.

MATERIALS AND METHODS

Growth conditions. P. chrysosporium was grown as previously described (14,15,18) in a low-nitrogen medium (0.6 mM NH_4NO_3 and 0.6 mM asparagine). Cultures were grown in a volume of 500 ml in 3-liter Fernbach flasks at 37°C. The mycelial mat was harvested 10 days after inoculation by filtration, and the filtrate was stored on ice pending further fractionation as described below. The mat was washed twice by filtration with 300 ml of deionized water.

Fractionation procedure. The filtered culture medium was centrifuged at 30,000 x g for 30 min to remove whole cells and heavier cellular debris. The pellet was discarded. The cell-free supernatant (S_{lo}^{filt}; see Fig.1) was further fractionated by centrifuging at

[1] Poly R-481 is a water-soluble, red dye comprised of anthrapyridone residues covalently linked to a polyvinyl backbone (11). It is a substrate for the lignin degrading system of P. chrysosporium (12,13).

150,000 x g for 20 min. The resulting translucent pellet (P^{filt}_{hi}) was resuspended in a volume of fresh basal salts (10 mM NaH_2PO_4, 10 mM KH_2PO_4, 10 mM 2,2-dimethylsuccinate, 5 mM $MgSO_4$ and 1 mM $CaCl_2$ at pH 4.5) equal to one-tenth the decanted supernatant. The decanted supernatant from the 150,000 x g centrifugation was concentrated 10-fold by ultrafiltration through a PM-10 membrane. This fraction was designated S^{filt}_{hi}.

The washed mycelial mat was disrupted briefly (15 sec) in a Waring Blendor containing 50 ml of basal salts medium, and then sonicated (3 x 10 sec) with a Bronson model S125 probe sonifier. The disrupted mycelial mat was fractionated in a manner similar to that described above, except the fractions from the 150,000 x g centrifugation were not concentrated. The designations employed above were used to describe the corresponding fractions obtained from the mycelial mat, but are distinguished because they lack the superscript "filt".

Dye decolorization assay. A modification of the procedure of Glenn and Gold (12) was employed. In this method the change in absorbance at two wavelengths is measured and decolorization is monitored as a change in the resulting absorbance ratio. For the fairly transparent subcellular fractions used in this study, dye decolorization was measured at a single wavelength (513 nm) using a Bausch and Lomb Spectronic 2000 spectrophotometer. Assays were routinely conducted in disposable polystyrene cuvettes (Fisher), that contained 2 ml of the desired cell fraction and 0.0175 mg/ml of dye. The complete assay system also contained glucose (1 mM) and glucose oxidase (0.5 unit/ml) to generate H_2O_2. All data points represent the average of quadruplicate assays and are normalized to a common initial absorbance. Actual initial absorbances did not vary more than 10% from the normalized value.

Electron Microscopy. Fractions were suspended in 0.1 M Na_2HPO_4 buffer (pH 7.2) containing 5 mM $MgCl_2$ and were fixed with 1.5% glutaraldehyde for 30 min at room temperature. They were then washed with phosphate buffer and postfixed with 1% osmium tetroxide in phosphate buffer for 20 min at room temperature. The samples were again washed with phosphate buffer and dehydrated in a graded ethanol series. Samples prepared for scanning electron microscopy (SEM) were critical point dried and examined with an ISI model 55130 SEM. Samples prepared for transmission electron microscopy (TEM) were embedded in Poly-bed (Polysciences, Inc.), sectioned, and stained with uranyl acetate followed by lead citrate. They were examined with a Hitachi H-500 TEM.

Miscellaneous. Protein content was determined by the method of Lowry et al. (16). Carbohydrate content was determined by the method of Dubois et al. (17). Lipid content was determined by the method of Christie (18). Glucose oxidase, glutaraldehyde, Poly R-481 and OsO_4 were purchased from Sigma. All other chemicals were reagent grade.

RESULTS

Cultures exhibiting maximum dye decolorization activity were fractionated as shown in Fig.1 and the fractions indicated by brackets were assayed for their ability to decolorize Poly R-481. Filtered culture medium was centrifuged at 30,000 x g for 30 min

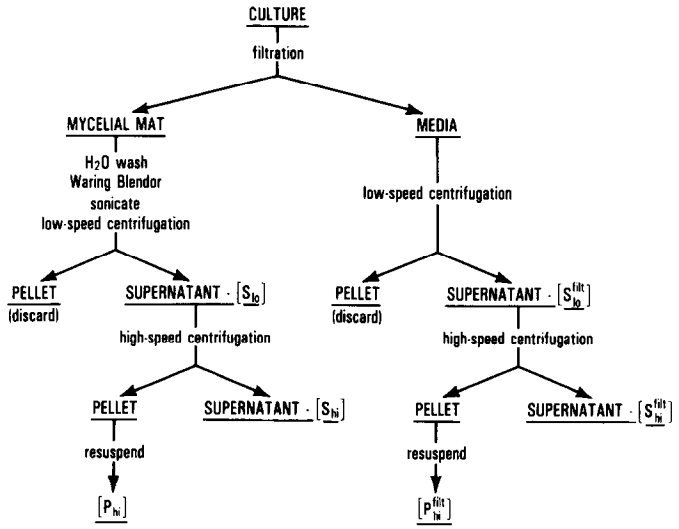

Figure 1. Schematic representation of procedure used to fractionate a 500-ml culture of Phanerochaete chrysosporium. The bracketed nomenclature indicates fractions which were examined for polymeric dye decolorization activity.

to remove whole cells and heavier cellular debris. The resulting supernatant (S_{lo}^{filt}) contained a small amount of decolorization activity (Table 1). When S_{lo}^{filt} was centrifuged at 150,000 x g for 20 min most of the decolorization activity was recovered in the

TABLE 1. Activity of P. chrysosporium culture media fractions

FRACTION*	ACTIVITY† (ΔA/hr)	PROTEIN CONCENTRATION (mg/ml)	PROTEIN RECOVERED‡ (%)	RELATIVE ACTIVITY
S_{lo}^{filt}	0.0043	0.077	100	1
P_{hi}^{filt}	0.0225	0.019	2.5	21.1
S_{hi}^{filt}	0.0103	0.197	25.6	0.9

*S_{lo}^{filt} was assayed as obtained. P_{hi}^{filt} and S_{hi}^{filt} were concentrated 10-fold. P_{hi}^{filt} was concentrated by resuspension in one-tenth the original volume of S_{lo}^{filt}. S_{hi}^{filt} was concentrated by ultrafiltration through a PM-10 membrane.

†Calculated from initial rate of absorbance decrease observed within the first 10-20 min of Poly R-481 decolorization.

‡The discrepancy in the amount of protein recovered results from the loss of a low-molecular-weight polypeptide fraction through the PM-10 membrane during ultrafiltration of S_{hi}^{filt}. This low-molecular-weight fraction exhibited negligible decolorization activity.

pellet fraction (P_{hi}^{filt}). Only 2.5% of the protein originally present in S_{lo}^{filt} was present in P_{hi}^{filt}. Much more protein was retained in the supernatant fraction (S_{hi}^{filt}), but this fraction exhibited less decolorization activity (Table 1).

Characterization of the P_{hi}^{filt} decolorization activity indicated that it was sensitive to heat (100°C for 10 min), azide (1 mM) and cyanide (1 mM). The optimum pH for activity was around pH 4. Exogenously added H_2O_2 greatly stimulated decolorization. Mn^{++} and lactate also stimulated activity. These activity characteristics are similar to those described for a Mn^{++}-dependent peroxidase isolated by Kuwahara et al. (7) and characterized by Glenn and Gold (13). Indeed, the activity observed in P_{hi}^{filt} is probably mediated by this enzyme because dye peroxidase activity can be isolated from P_{hi}^{filt} by the previously reported method (7).

The data in Table 1 show that the relative activity of P_{hi}^{filt} is more than 20-fold greater than that of S_{hi}^{filt}, indicating that the extracellular dye decolorization activity in P. chrysosporium culture medium resides primarily in a particulate material sedimenting at 150,000 x g. Scanning electron microscopy of P_{hi}^{filt} (Fig.2A) revealed numerous, generally spherical or oblong structures (0.1 to 0.3 μ in diameter) associated with an apparently amorphous mass. Transmission electron micrographs (Figs.2B and C) showed these structures are enclosed and exhibit a heavily-stained interior surrounded by a rather thick, lighter-stained envelope. Preliminary chemical determinations indicated the weight ratio of carbohydrate to protein to lipid in P_{hi}^{filt} was about 7:2:1. Gel electrophoresis analysis of P_{hi}^{filt} showed at least two prominent polypeptides that migrate near a 45 kD standard (not shown), the approximate molecular weight of previously described lignin degrading and dye peroxidase enzymes (7,9,10,13). P_{hi}^{filt} also appeared to contain several other polypeptides.

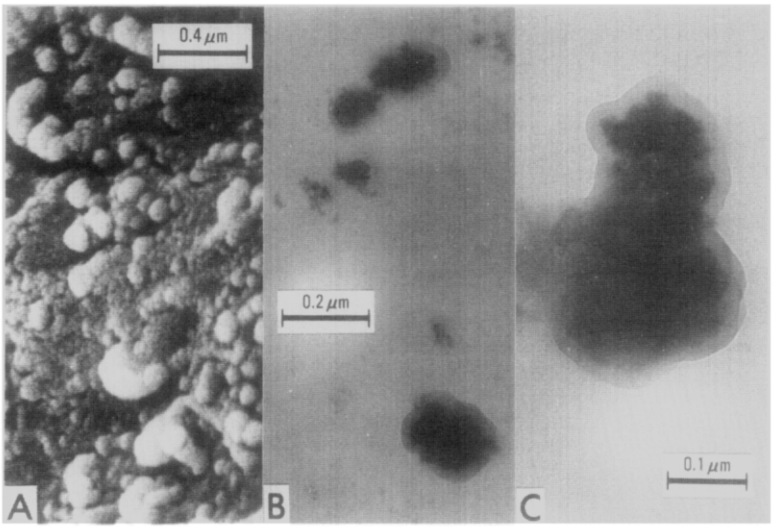

Figure 2. Scanning and transmission electron micrographs of P_{hi}^{filt}. A: SEM, magnification = 50,000 x; B: TEM, magnification = 100,000 x; C: TEM, magnification = 180,000 x.

The activity profile of an analogous fractionation of sonicated mycelium are presented in Table 2. The data indicate that considerable decolorization activity originally associated with the mycelial mat was at least in part released by sonication into the basal salt medium (S_{lo}). Like the activity in filtered culture media (S_{lo}^{filt}), most of the activity in S_{lo} sedimented at 150,000 x g (P_{hi}), indicating that, it too was associated with particulate matter.

TABLE 2. Fractionation of decolorization activity from sonicated P. chrysosporium mycelia

FRACTION	ACTIVITY* (ΔA/hr)	PROTEIN CONCENTRATION (mg/ml)	PROTEIN RECOVERED (%)	RELATIVE ACTIVITY
S_{lo}	0.0173	0.092	100	1
P_{hi}	0.0148	0.009	9.8	8.7
S_{hi}	0.0041	0.085	92.4	0.3

*Calculated from initial rate of absorbance decrease observed within the first 10-20 min of Poly R-481 decolorization.

DISCUSSION

The data in Tables 1 and 2 indicate that dye decolorization activity from P. chrysosporium is associated with particulate material. Scanning and transmission electron micrographs of the sedimented fraction from cell-free culture media (P_{hi}^{filt}) revealed numerous, generally sperical bodies associated with an apparently amorphous mass (Fig.2). These characteristics coupled with the high proportion of carbohydrate found in P_{hi}^{filt} suggest the possibility that the decolorization activity sediments as a large carbohydrate-protein complex similar to particulate cellulase activity from Ruminococcus albus, a cell wall degrading rumen bacterium (R. J. Stack, submitted for publication; see also Ref. 19).

Extracellular cellulase activity has been reported to be associated with particulate structures in at least three bacteria (19,20,21). Recently, Lamed et al. (21) proposed the term "cellulosome" to describe a "discrete cell surface organelle which displays separate antigenic, cellulose binding, and multiple types of cellulolytic activities" found in Clostridium thermocellum. Forney et al. (5) reported the appearance of DAB-stained, "microbody-like" structures in the periplasm of P. chrysosporium in conjunction with the onset of ligninolytic activity, and proposed that extracellular H_2O_2 production was localized in these structures. Interestingly these DAB-stained structures resemble in both size and shape the structures we found to be prevalent in the electron micrographs taken of fraction P_{hi}^{filt} (Fig.2).

Localization of lignin degrading activity in a particulate body, whether it be a periplasmic or extracellular organelle, the cytoplasmic membrane, an amorphous polysaccharide capsule, part of the cell wall, or a multienzyme complex, may offer some advantages to the fungus. For instance, such an association could protect the lignin degrading enzymes from proteolytic attack and nonspecific oxidation. An organized particulate structure could also adhere

to an insoluble substrate such as lignocellulose, increasing reaction efficiency and possibly enhancing synergistic interaction between several ligninolytic enzymes or enzyme systems. Paszczynski et al. (22) have proposed a model in which several ligninolytic enzymes act in conjunction with the dye peroxidase to ultimately degrade lignin. However, the validity of this model and elucidation of the role of the structure found in P_{hi}^{filt} in lignin degradation await further study.

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