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Biodegradation of Natural and Synthetic Humic Acids by the White Rot Fungus *Phanerochaete chrysosporium*

ROLAND BLONDEAU

Laboratoire de Microbiologie, Université des Sciences et Techniques de Lille Flandres-Artois, 59655 Villeneuve d'Ascq Cédex, France

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Biodegradation of natural and synthetic (melanoidin) humic acids by *Phanerochaete chrysosporium* BKM-F 1767 was demonstrated by decolorization in batch culture, reduction in molecular weight, and $^{14}CO_2$ production from labeled melanoidin. This biodegradation occurred during secondary metabolism of the fungus in nitrogen-limited cultures; experimental results suggest that all or a part of the lignin-degrading system of BKM-F 1767 plays a part in biodegradation.

Humus is a complex mixture of organic compounds resulting from the decomposition of organic matter through enzymatic or auto-oxidative reactions (21, 27). These humic compounds are particularly stable, and their age in some soils is thousands of years according to radiocarbon dating (12). This stability and the environmental factors acting on the turnover of humus are poorly understood. A previous study, intended to isolate microorganisms capable of humic acid mineralization in batch culture, has shown little structural modifications of these compounds in the presence of heterotrophic bacteria (4).

Phanerochaete chrysosporium has been shown to produce a relatively nonspecific ligninolytic enzyme system which appears irrespective of the presence of lignin (13), and this fungus can oxidize a broad spectrum of chemical compounds (5). For these reasons, we investigated the mineralization of humic acids and its synthetic homologous melanoidins by *P. chrysosporium*. Melanoidins are used concurrently with natural humic acids for carrying out mineralization experiments using a labeled substrate. Humic acid and melanoidin are similar in elemental composition, spectroscopic characteristics, and environmental stability (1, 11, 16, 26).

P. chrysosporium DSM 1556 was obtained from Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany. *P. chrysosporium* BKM-F 1767 (ATCC 24725) was a gift from E. Odier, Centre de Biotechnologies Agro-Industrielles, Institut National de la Recherche Agronomique, Thiverval-Grignon, France. These strains were routinely maintained and checked for purity on malt agar slants or plates at room temperature. Subcultures were routinely made every 15 days. Experiments were conducted in aerobic batch culture with 500-ml serum flasks (each flask containing 50 ml of liquid basal medium) with cotton stoppers. The basal medium contained the mineral salts suggested by Kirk et al. (14), namely, thiamine (2 mg/liter), 56 mM glucose, and 2.4 mM nitrogen (NH_4NO_3 and L-asparagine, both 0.6 mM), and was buffered with 20 mM sodium tartrate (pH 4.5). In addition, veratryl alcohol (1.5 mM, Fluka) was incorporated for some assays. Humic acids and melanoidins were sterilized by filtration (pore size, 0.45 μm) and incorporated into previously autoclaved basal medium at final concentrations of 0.05 and 0.025%, respectively. Inocula consisted of conidial suspensions collected from 2- to 3-week-old slants and filtered through glass wool (final concentration was approximately 20×10^6 spores per culture flask). Cultures were incubated at 35°C.

Natural humic acids were extracted from a forest soil with 0.1 M PP_i solution and purified as previously described (3). The final mean elemental composition was 52.6% C, 5.1% H, and 3.2% N (ash-free). Melanoidins were synthetized at 68°C by mixing glucose and glycine (1 M in phosphate buffer [pH 7]). The polymers recovered after 170 h were dialyzed against distilled water with Spectra Por 6 tubes (Spectrum Medical Industries Inc.; molecular weight cutoff of 1,000) and freeze-dried. The final mean elemental composition was 52.4% C, 5.6% H, and 5.4% N (ash-free). For preparation of labeled melanoidin, [U-^{14}C]glucose (Commissariat à l'Energie Atomique, gif-sur-Yvette, France) (specific activity, 10 GBq/mmol; final concentration, 185 kBq/ml) was used.

Degradation of humic acids or melanoidins was monitored by following the decrease in A_{350} at pH 4.5. Results were expressed in comparison with noninoculated cultures incubated under the same conditions. Growth was assessed by the dry weight as determined after collection of the mycelia and drying at 105°C on weighed filters. The most prevalent molecular weights of humic acids and melanoidins were evaluated by using gel filtration on Sephacryl S400 superfine and Sephadex G-50 fine (Pharmacia), respectively (eluent, Tris hydrochloride [pH 9.0]; ionic strength, 0.05; column calibration with globular proteins). Lignin peroxidase activity was assessed as the initial rate of oxidation of veratryl alcohol to veratraldehyde at pH 3.0, determined by continuous spectrophotometry by the method of Tien and Kirk (28). One unit of enzyme oxidized 1 μmol of veratryl alcohol to veratraldehyde in 1 min at 35°C. For experiments with labeled melanoidin, $^{14}CO_2$ was trapped by placing a sterile cup containing 2 ml of KOH solution (0.5 N) in rubber-stoppered culture flasks. After incubation at 35°C for 4 hours, labeled CO_2 was assayed for radioactivity by liquid scintillation spectrometry. All studies were performed in triplicate.

When *P. chrysosporium* strains were incubated in culture medium with humic acids or melanoidins as the sole carbon source, no growth was observed. With the addition of glucose, growth of the two strains was similar but decolorization of humic acids or melanoidins was only observed with BKM-F 1767. This decolorization started after an initial lag of approximatively 4 days and continued through the 15 days of the experiment (Fig. 1). Maximal production of $^{14}CO_2$ from labeled melanoidin by BKM-F 1767 was achieved after 5 days. A second activity period always occurred around days 11 to 12 and then declined (Fig. 2). *P. chrysosporium*

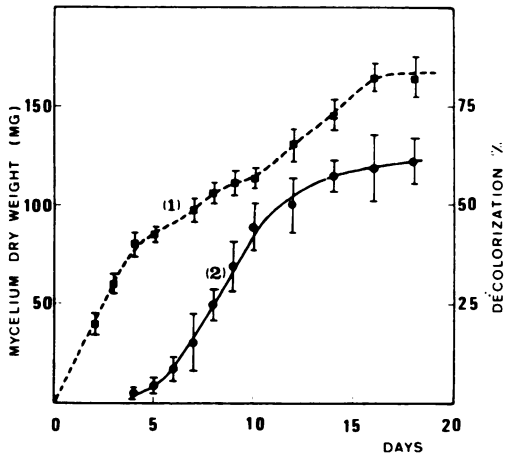

FIG. 1. Growth (curve 1) and decolorization of humic acids (curve 2) by *P. chrysosporium* BKM-F 1767. Each culture flask contained 0.05% humic acid in basal medium. Bars indicate standard errors (n = 3). MG, Milligrams.

DSM 1556 growing in medium containing melanoidin released only very small amounts of $^{14}CO_2$. However, the same pattern of two peaks was observed.

The decolorizing activity of the fungus and the mineralization of labeled melanoidin were completely inhibited by shaking of the cultures, despite growth of the fungal mycelium. Absence of decolorization also resulted from increasing the nitrogen concentration up to fourfold in cultures. With a twofold increase in nitrogen (4.8 mM), humic acid decolorization was found to be lowered and labeled melanoidin inoculated with BKM-F 1767 released approximately fourfold less $^{14}CO_2$ than in standard medium. In contrast, the presence of veratryl alcohol resulted in increased decolorizing activity and production of $^{14}CO_2$. For example, 17.3% ± 2.8% of the radioactivity of labeled melanoidin was lost with BKM-F 1767 after 15 days, compared with 23.5% ± 1.9% when 1.5 mM veratryl alcohol was added to the basal medium.

The decolorizing system of BKM-F 1767 was extracellular, and humic acid or melanoidin was not necessary for its induction. In preliminary experiments, humic acid was added to the basal growth medium inoculated with the fungus after a 7-day incubation period at 35°C. The decolorization occurred as early as the next day after this addition (23.1% of humic acid decolorization after 24 h compared with that of a control containing 9.6 mM nitrogen). In addition, the culture filtrate of the fungus recovered after 7 days of incubation without melanoidin exhibited high mineralization activities using labeled melanoidin (Table 1).

TABLE 1. Effect of *P. chrysosporium* BKM-F 1767 culture filtrate on a labeled melanoidin solution[a]

Prior incubation	Mineralizing activity[b]	
	Decolorization %	$^{14}CO_2$ (dpm/flask)
Basal medium	10.9 ± 0.9	566 ± 88
Basal medium + veratryl alcohol	23.4 ± 1.2	1,172 ± 132

[a] Five milliliters of a membrane-sterilized 7-day-old culture filtrate from a prior incubation in basal medium without melanoidin was added to a rubber-sealed flask containing sterile [^{14}C]melanoidin and KOH as described in the text.

[b] Mean ± standard deviation of three replicates of the percent decolorization and amount of $^{14}CO_2$ evolved at 35°C 24 h after addition of the filtrate.

Gel filtration chromatography profiles showed that humic acids showed a reduction in molecular weight after incubation for 15 days with strain BKM-F 1767 (Fig. 3). The predominant molecular weight was reduced from approximately 23 × 10^3 (control) to approximately 12 × 10^3. The size changes were particularly evident in the high-molecular-weight (5 × 10^4 to 5 × 10^5) range. However, no accumulation of low-molecular-weight compounds was noticed. A similar change of the molecular polydispersion of melanoidins was shown by gel filtration on Sephadex G-50, with a decrease from approximately 8,950 to 5,500 daltons after incubation for 15 days in the presence of the fungus.

No lignin peroxidase activity of *P. chrysosporium* DSM 1556 was observed in growth medium whatever the carbon source. In contrast, strain BKM-F 1767 exhibited significant peroxidase activity, particularly when veratryl alcohol was added, but the higher activity was obtained in the absence of humic acids or melanoidins (Table 2).

Our findings indicate that *P. chrysosporium* cannot utilize humic substances as the sole carbon supply in contrast to

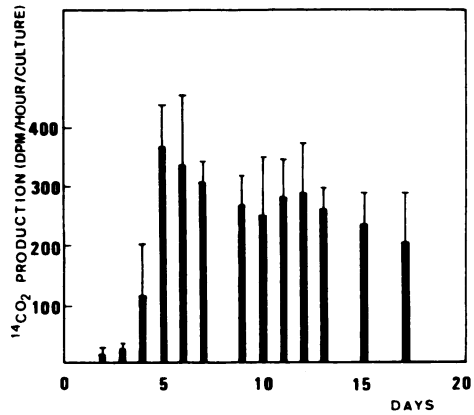

FIG. 2. Mineralization of [^{14}C]melanoidin in growth cultures of *P. chrysosporium* BKM-F 1767. The initial concentration of melanoidin was 0.025% in basal medium ($^{14}CO_2$ data represent mean values for three replicates. Bars indicate standard errors).

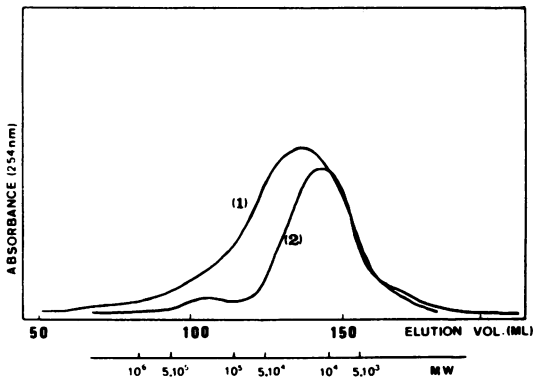

FIG. 3. Chromatography profiles of humic acids after gel filtration on Sephacryl S400. The humic acids were recovered from a 15-day culture of *P. chrysosporium* BKM-F 1767 grown with 9.6 mM nitrogen (control [curve 1]) or grown in a nitrogen-limited culture (2.4 mM nitrogen [curve 2]).

TABLE 2. Lignin peroxidase activity of *P. chrysosporium* BKM-F 1767[a]

Culture conditions	Enzyme activity[b]
Basal medium + veratryl alcohol	0.038 ± 0.004
Basal medium + veratryl alcohol + humic acids	0.026 ± 0.005
Basal medium + veratryl alcohol + melanoidins	0.023 ± 0.005

[a] *P. chrysosporium* BKM-F 1767 was grown for 8 days in basal medium supplemented with 1.5 mM veratryl alcohol, 0.5% humic acids, or 0.25% melanoidins.

[b] Results in units of lignin peroxidase per milliliter. Each result is the mean ± standard deviation of three replicates.

some reports about other fungi (6, 7, 19, 20). Moreover, the decolorization of humic acids by strain BKM-F 1767 was demonstrated under special experimental conditions and such decolorizing reactions of humic solutions by various fungi have already been demonstrated (2, 10, 17, 18), but this bleaching alone cannot prove a real biodegradation. Indeed, *P. chrysosporium* grown in low ionic strength also decolorized the humic acids, but this decolorization was a result of the binding of humic acid on fungus mycelia caused by the reduction of charge on these molecules induced by acidification of the medium (results not shown).

Veratryl alcohol has been shown to enhance lignin peroxidase activity of *P. chrysosporium* (8, 15) and its mediator role in oxidation reactions catalyzed by this enzyme has been proposed (9). In our experiments, a relationship between lignin peroxidase activity of the two *P. chrysosporium* strains used and biodegradation activities of the humic compounds has been shown. These results suggest that the lignin peroxidase system was at least partly responsible for the humus degradation. In fact, in the presence of H_2O_2, this nonspecific lignin-degrading system is able to catalyze a wide variety of reactions, including benzylic oxidation, carbon-carbon bond cleavage, hydroxylation and O demethylation (22, 23). The kinetics of $^{14}CO_2$ production from labeled melanoidin strongly suggests that the degradation process involved several mechanisms.

The oxidation of purified humic acid by a peroxidase producing nonidentified fungus isolated from the soil has been already considered (17). The determination of enzymatic activities in crude cultures is rather difficult because natural or synthetic humic acids are known to inhibit peroxidase activity by competitive as well as noncompetitive interactions (24, 25, 29). These inhibitory interactions may explain the lower activity of lignin peroxidase observed when humic acids or melanoidins are present in the test medium.

This report shows that humic acids are attacked by the peroxidase system of *P. chrysosporium* and that this process is very effective in causing molecular weight range change. More extensive study of the enzymatic specificities can provide useful information on the chemical structure of humic compounds. Humus mineralization in nature remains nevertheless questionable because *P. chrysosporium*, like other white rot fungi, promotes humus production.

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