

## Degradation of Humic Acids by the Litter-Decomposing Basidiomycete *Collybia dryophila*

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Received 26 December 2001/Accepted 11 April 2002

**The basidiomycete *Collybia dryophila* K209, which colonizes forest soil, was found to decompose a natural humic acid isolated from pine-forest litter (LHA) and a synthetic <sup>14</sup>C-labeled humic acid (<sup>14</sup>C-HA) prepared from [U-<sup>14</sup>C]catechol in liquid culture. Degradation resulted in the formation of polar, lower-molecular-mass fulvic acid (FA) and carbon dioxide. HA decomposition was considerably enhanced in the presence of Mn<sup>2+</sup> (200 μM), leading to 75% conversion of LHA and 50% mineralization of <sup>14</sup>C-HA (compared to 60% and 20%, respectively, in the absence of Mn<sup>2+</sup>). There was a strong indication that manganese peroxidase (MnP), the production of which was noticeably increased in Mn<sup>2+</sup>-supplemented cultures, was responsible for this effect. The enzyme was produced as a single protein with a pI of 4.7 and a molecular mass of 44 kDa. During solid-state cultivation, *C. dryophila* released substantial amounts of water-soluble FA (predominantly of 0.9 kDa molecular mass) from insoluble litter material. The results indicate that basidiomycetes such as *C. dryophila* which colonize forest litter and soil are involved in humus turnover by their recycling of high-molecular-mass humic substances. Extracellular MnP seems to be a key enzyme in the conversion process.**

Humic substances are the most widespread and ubiquitous natural nonliving organic materials in terrestrial and aquatic environments and represent the major fraction of soil organic matter (12, 33). In addition, they make up a substantial part of the fossil organic carbon incorporated into peat and low-rank coals (e.g., lignite and brown coal) (11). Humic substances comprise a physically and chemically heterogeneous mixture of biogenic, relatively high-molecular-mass compounds with mixed aliphatic and aromatic natures (35). Based on solubility in acids and alkalis, they can be divided into three main fractions: humic acid (HA), which is soluble in alkali and insoluble in acid; fulvic acid (FA), which is soluble in alkali and acid; and humin, which is insoluble in both alkali and acid (33). Humic substances are formed by secondary synthesis reactions (humification) during the decay process and transformation of biomolecules originating from dead organisms and microbial activity. Lignin and its transformation products, as well as polyphenols and derived polymers from lower plants and microorganisms, are important starting materials in this process and provide aromatic building blocks of high physicochemical stability (35).

Microbial degradation of humic substances—particularly of high-molecular-mass, aromatic moieties in HA and humin—is an important part of humus turnover and is therefore essential for maintaining the global carbon cycle (14). Despite this fact, little is known yet about the microorganisms which decompose and recycle humic matter. Since lignin, the complex aromatic polymer providing strength and rigidity to the cell walls and tissues of vascular plants, is a major parent material in the formation of humic substances (33), several authors studied

the decomposition of natural and synthetic HA by white-rot fungi (2, 4, 6, 19, 23, 38, 41), which are the most effective lignin degraders in nature (16). Some of these basidiomycetous fungi which colonize wood (e.g., *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Nematoloma frowardii*) were shown to disintegrate high-molecular-mass HA by forming lower-molecular-mass FA and carbon dioxide (CO<sub>2</sub>). Correlation was observed between the activity of extracellular peroxidases and HA degradation, and isolated manganese peroxidase (MnP) was even found to depolymerize and mineralize different HAs in vitro (5, 20, 40). However, since most white-rot fungi grow preferentially in compact wood (trunks, logs, branches, and stumps) and cannot compete in soil for a prolonged time (7, 28), it is doubtful whether they are involved to a large extent in humus decomposition under natural conditions (25). In the present study, therefore, we have focused on HA degradation by a true soil-colonizing basidiomycete, *Collybia dryophila*, which is a very common species in European and North American woodlands and grows in different layers of forest litter from both coniferous and deciduous trees (17, 24, 30).

### MATERIALS AND METHODS

**Soil-litter and litter HA (LHA).** Soil-litter samples (wet weight, 3 × 500 g) from a mixed pine-spruce forest (*Pinus sylvestris* and *Picea abies*) near Lahti (Finland) were collected at a depth of 5 cm. Samples were combined and sieved, using a <1-cm-pore-diameter sieve, to remove larger twigs, cones, stones, etc. The combined litter sample had a pH of 3.9 and a water content of 60% and was stored at 4°C.

LHA was extracted from litter (wet weight, 750 g) with 0.5 N NaOH (4 liters) by continuous agitation for 2 h (1). After sedimentation of the alkaline suspension, the dark-brown supernatant was filtered through Miracloth (Calbiochem, La Jolla, Calif.), centrifuged (8,500 × g), and filtered again (glass fiber filter GF52; Schleicher & Schuell, Dassel, Germany). LHA was precipitated by adjusting the pH level to 1.5 with 6 N HCl, and the suspension was left overnight at 4°C. After centrifugation, the LHA precipitate was washed with water and dried at 40°C.

**Synthetic <sup>14</sup>C-HA.** <sup>14</sup>C-labeled humic acid (<sup>14</sup>C-HA) was synthesized by spontaneous oxidative polymerization of [U-<sup>14</sup>C]catechol (specific radioactivity, 3.4

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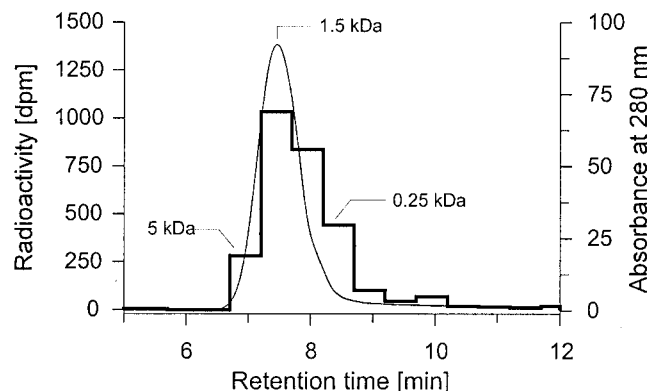


FIG. 1. HPSEC elution profile of a synthetic HA prepared from catechol, showing the distribution of molecular masses (thin line, spline curve) and radioactivity (bold line, step plot). Injection volume, 10  $\mu$ l of the tenfold diluted stock solution (about  $5 \times 10^3$  dpm).

mCi  $\text{mmol}^{-1} = 125.8$  MBq  $\text{mmol}^{-1}$ ; Sigma, Saint Louis, Mo.) in an alkaline solution (13, 40).  $^{14}\text{C}$ -catechol ( $1.38 \times 10^7$  dpm) and 11 mg of unlabeled catechol were dissolved in 1 ml of NaOH (0.1 N) which also contained  $\text{Mn}^{2+}$  (final concentration, 2 mM) to enhance the polymerization process (20). The reaction mixture was incubated with vigorous stirring at 25°C for 3 days.  $^{14}\text{C}$ -HA was precipitated by addition of 50  $\mu$ l of 6 N HCl, followed by centrifugation. The  $^{14}\text{C}$ -HA pellet was washed twice with water and resuspended in 1 ml of 0.1 N NaOH, forming a dark-brown solution with a radioactivity of  $4.75 \times 10^3$  dpm  $\mu\text{l}^{-1}$ ; i.e., about 35% of the original  $^{14}\text{C}$ -labeled catechol was incorporated in this preparation. The distribution of molecular mass and radioactivity of this preparation are shown in Fig. 1. A structural model of a similar humic substance, based on  $^{13}\text{C}$ -CP-TOSS nuclear magnetic resonance spectroscopic data, has been described recently (25).

**Organism and culture conditions.** *C. dryophila* (Bull.:Fr.) P. Kumm. (russet tough-shank) strain K209, which has been grouped into the litter-decomposing fungi according to the classification system of Dix and Webster (7), was isolated from basidiocarps collected in the geographical area described above. They grew in soil litter consisting of needles, twigs, dead moss material, and humus-rich soil particles. Isolates were recovered by placing small pieces of fruit body plectenchyma aseptically on 2% malt extract agar (MEA) plates containing chloramphenicol (50 mg  $\text{liter}^{-1}$ ) and benomyl (50 mg  $\text{liter}^{-1}$ ) to prevent growth of bacteria and molds, respectively. Stock cultures of *C. dryophila* were maintained on MEA slants at 4°C and are deposited at the fungal culture collection of the Department of Applied Chemistry and Microbiology at University of Helsinki (Finland).

Precultures were grown on MEA plates, and agar plugs (10 mm in diameter) were used to inoculate liquid- and solid-state cultures. The basal liquid medium consisted of 10 g of glucose  $\text{liter}^{-1}$ , 2 g of  $\text{KH}_2\text{PO}_4$   $\text{liter}^{-1}$ , 0.5 g of  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$   $\text{liter}^{-1}$ , 0.1 g of  $\text{CaCl}_2$   $\text{liter}^{-1}$ , 0.5 g of ammonium tartrate  $\text{liter}^{-1}$ , 2.2 g of 2,2-dimethylsuccinate  $\text{liter}^{-1}$ , and 0.1 g of yeast extract  $\text{liter}^{-1}$  (pH was adjusted to 5.0). Stationary culture cultivation was carried out in 1-liter Erlenmeyer flasks containing 150 ml of medium. In addition, certain cultures were supplemented with  $\text{MnCl}_2$  (200  $\mu\text{M}$ ) and/or LHA (250 mg  $\text{liter}^{-1}$  dissolved in 0.1 N NaOH). Flasks were inoculated with 10 agar plugs (10 mm in diameter) and incubated at 20°C in the dark for 6 weeks. Every 3 to 7 days, samples (1.5 ml) were taken to determine enzyme activities and the absorbance at 450 nm (the wavelength of the brown color of LHA) (38). After 6 weeks of incubation, LHA-containing flasks were mixed with 1.5 ml of 10 N NaOH and shaken for 30 min (150 rpm). Aliquots of 1 ml were centrifuged, and 6 N HCl (20  $\mu$ l) was added to the supernatant to precipitate LHA and separate it from FA. The FA was present in the yellowish acidic supernatant after further centrifugation, and the LHA pellet was resuspended and dissolved in 0.1 N NaOH. Both fractions were used for high-performance size exclusion chromatography (HPSEC) analyses. The dry mass of the fungal cultures was determined after filtration through glass-fiber filters and drying to constant weight at 70°C.

Production of MnP for further enzyme purification occurred in 1-liter tissue culture flasks containing 200 ml of basal medium plus 200  $\mu\text{M}$   $\text{MnCl}_2$  (34). Flasks were cultured at 24°C and harvested after 5 weeks.

Solid-state cultures consisted of sterilized litter (15 g; water content, 80%) in

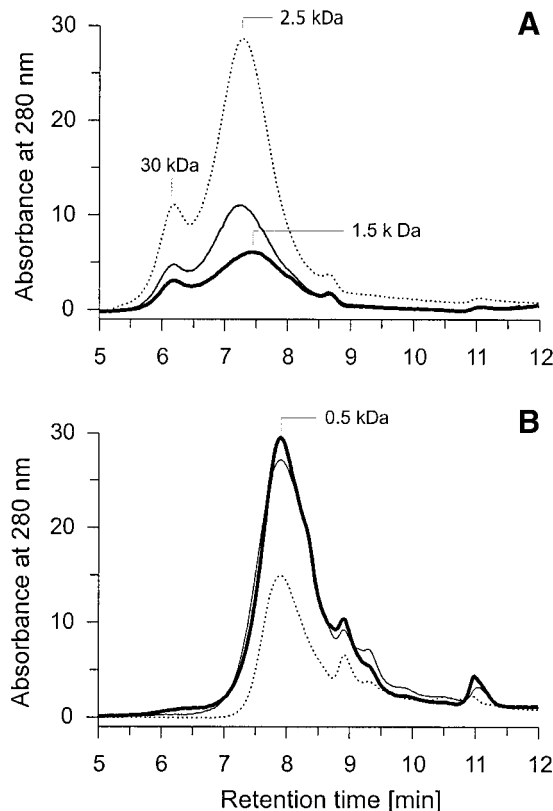


FIG. 2. HPSEC elution profiles of LHA recovered from liquid cultures of *C. dryophila* grown in the presence or absence of  $\text{Mn}^{2+}$  (A) and of the respective FA formed (B). Bold lines, fungal cultures supplemented with 200  $\mu\text{M}$   $\text{MnCl}_2$ ; thin lines, fungal cultures without  $\text{Mn}^{2+}$ ; dotted lines, controls without fungus.

100-ml Erlenmeyer flasks which were inoculated with 3 agar plugs (10 mm in diameter) of pregrown mycelium ( $11 \pm 2$  mg mycelial dry weight per plug). They were incubated at 20°C for 46 days and then extracted with 40 ml of distilled water by sonication (3 min) and shaking (30 min on a rotary shaker at 150 rpm). After centrifugation, samples were used for HPSEC, high-performance liquid chromatography (HPLC), and enzymatic measurements.

**Mineralization experiments with radioactive HA.** Mineralization studies using  $^{14}\text{C}$ -HA were carried out in liquid culture containing the basal medium mentioned above either in the presence of  $\text{Mn}^{2+}$  (200  $\mu\text{M}$ ) or in its absence.  $^{14}\text{C}$ -HA ( $2.5 \times 10^5$  dpm) dissolved in 0.1 N NaOH was added to 15-ml liquid cultures pregrown for 3 days (final  $^{14}\text{C}$ -HA concentration, 12.8 mg  $\text{liter}^{-1}$ ). Inoculated flasks (3 used in parallel; see Material and Methods) and uninoculated controls were sealed with rubber septa and aluminum caps. Incubation occurred at 24°C in the dark.  $^{14}\text{C}$ -labeled volatile compounds and  $^{14}\text{CO}_2$  were flushed out once a week with pure oxygen for 15 min and trapped by bubbling any gas released through two sequential flasks containing Opti-Fluor and Carbosorb/Opti-Fluor (Packard Instruments, Groningen, The Netherlands). After 6 weeks of incubation, residual  $^{14}\text{C}$ -HA was dissolved by addition of 15 ml 0.1 N NaOH followed by sonication (3 min) and vigorous shaking (5 min). After filtration and centrifugation, aliquots of 100  $\mu$ l were used to determine soluble radioactivity. The respective filters and the included fungal biomass were burned in a combustion chamber (Junitek, Turku, Finland) to confirm the identity of the bound  $^{14}\text{C}$  as evolving  $^{14}\text{CO}_2$ . A liquid scintillation counter (model no. 1411; Wallace, Turku, Finland) was used for all radioactivity measurements.

**HPSEC and HPLC.** An HPSEC method described previously (19, 22) was used for the determination of the molecular mass distribution of humic substances. Sodium polystyrene sulfonates (1.3 to 168 kDa) and biphenyl dicarboxylic acid (0.246 kDa) served as molecular mass standards.

Elution profiles of aqueous litter extracts were recorded by HPLC using an Aqua-C<sub>18</sub> column (4.6 by 250 mm; Phenomenex, St. Torrance, Calif.) and an HP 1090 Liquid Chromatograph (Hewlett Packard, Waldbronn, Germany). Separa-

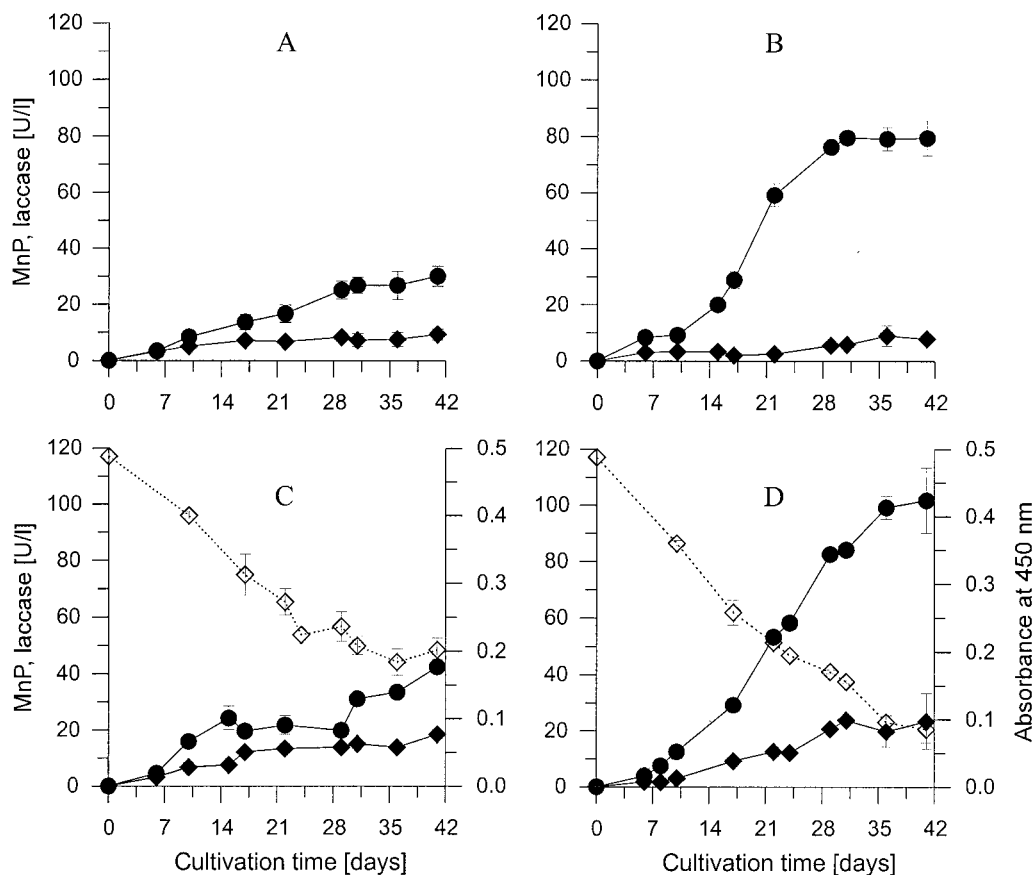


FIG. 3. Production of MnP (closed circles) and laccase (closed diamonds) by *C. dryophila* in liquid culture and decolorization of LHA (open diamonds). (A) Medium without  $\text{Mn}^{2+}$  and LHA. (B) Medium with  $200 \mu\text{M Mn}^{2+}$  ( $\text{MnCl}_2$ ) but without LHA. (C) Medium with  $250 \text{ mg of LHA liter}^{-1}$  but without  $\text{Mn}^{2+}$ . (D) Medium with  $200 \mu\text{M Mn}^{2+}$  and  $250 \text{ mg of LHA liter}^{-1}$ . Decolorization of LHA (panels C and D) was monitored by the decrease in absorbance at  $450 \text{ nm}$ . Data points represent means of three parallels with standard deviations.

tions were run at a constant temperature of  $40^\circ\text{C}$  by using a stepwise gradient of 0 to 100% acetonitrile (0 and 15 min, 0%; 40 min, 50%; 45 min and 47 min, 100%; 55 min, 0%) in 0.05% phosphoric acid over 55 min with a constant flow rate of  $0.75 \text{ ml min}^{-1}$ . Eluted substances were detected at  $254 \text{ nm}$ .

**Enzyme assays.** The activity of MnP was measured at  $270 \text{ nm}$  by monitoring the formation of  $\text{Mn}^{3+}$ -malonate complexes (37) or at  $420 \text{ nm}$  by monitoring the oxidation of ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate);  $\epsilon_{420}$ ,  $36 \text{ mM}^{-1} \text{ cm}^{-1}$ ] in the presence of HA, as described previously (21). Laccase activity was also determined by the oxidation of ABTS at  $420 \text{ nm}$ , but in the absence of  $\text{H}_2\text{O}_2$  and  $\text{Mn}^{2+}$  (8). Lignin peroxidase activity was measured at  $310 \text{ nm}$  using the veratryl alcohol method (26). Enzyme activities were expressed in units (U), i.e., micromoles of product formed per minute.

**Purification and characterization of MnP.** Culture fluid from tissue culture flasks (1.5 liter) supplemented with  $200 \mu\text{M Mn}^{2+}$  was harvested after 5 weeks of incubation and separated from the mycelium by filtration through a glass fiber filter (GF52; Schleicher & Schuell). The filtrate was concentrated at  $4^\circ\text{C}$  by two steps of ultrafiltration-diafiltration (Filtron Omega cassette, 8-kDa cutoff; Filtron Technology Corp., Northboro, Mass.; Amicon chamber, YM10 membrane, 10-kDa cutoff; Millipore, Bedford, Mass.). Extracellular proteins were separated by anion exchange chromatography on a MonoQ column (Amersham Pharmacia Biotech, Uppsala, Sweden) with a 0.02 to  $1 \text{ M NaAc}$  (pH 5.5) gradient. The elution of absorbing material from the column was simultaneously monitored at  $280$  and  $405 \text{ nm}$  to record total protein and heme, respectively. Fractions of  $0.5 \text{ ml}$  each were collected at a flow rate of  $0.5 \text{ ml min}^{-1}$ , and those containing MnP were pooled, concentrated, and diafiltrated (Filtron Microsep, 10-kDa cutoff) with double the amount of distilled water.

The molecular weight (MW) of *C. dryophila* MnP was determined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (27), using a 4% stacking gel, a 12% resolving gel, and the Bio-Rad

Mini Protean II PAGE chamber (Bio-Rad, Hercules, Calif.). Samples were boiled in the presence of SDS and  $\beta$ -mercaptoethanol for 3 min. After electrophoresis, gels were stained and protein bands were visualized with Coomassie brilliant blue R-250. For the determination of MW, a low-MW protein calibration kit was used (Amersham Pharmacia).

Analytical isoelectric focusing (IEF) was carried out with a Multiphor II electrophoresis system (Amersham Pharmacia). The IEF gel (7.5%) was prepared using ampholines of isoelectric points (pIs) 2.5 to 5.0 and 3.5 to 10.0 (Pharmacia). The pH gradient of the gel was measured with a surface electrode (Sentek, Braintree, United Kingdom) and a pH meter (Orion, Beverly, Mass.), and in addition, a protein standard kit (Amersham Pharmacia) was used. Gels were stained with phenol red according to the method of Pease et al. (29).

## RESULTS

**LHA decomposition and enzyme activities.** *C. dryophila* grew relatively slowly in liquid media, and after only 3 weeks, the whole surface was covered with a white mycelium layer. In the presence of both  $\text{Mn}^{2+}$  and LHA, biomass production was noticeably stimulated ( $2.18 \pm 0.07 \text{ g liter}^{-1}$ ) compared with that of nonsupplemented cultures ( $1.01 \pm 0.09 \text{ g liter}^{-1}$ ) and cultures containing either  $\text{Mn}^{2+}$  ( $1.09 \pm 0.08 \text{ g liter}^{-1}$ ) or LHA ( $1.25 \pm 0.05 \text{ g liter}^{-1}$ ). HPSEC elution profiles of LHA recovered from fungal cultures show that about 75% of the material was converted in  $\text{Mn}^{2+}$ -containing cultures, whereas only 60% was converted in the absence of  $\text{Mn}^{2+}$  (Fig. 2). Molecular mass

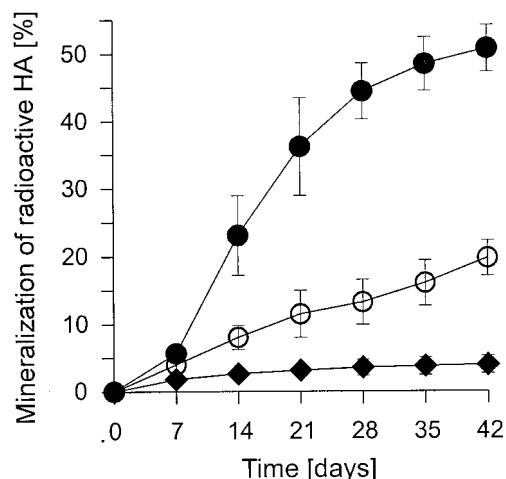


FIG. 4. Mineralization of  $^{14}\text{C}$ -HA by *C. dryophila* in liquid culture. Closed circles, fungus in  $\text{Mn}^{2+}$ -supplemented medium; open circles, fungus in  $\text{Mn}^{2+}$ -free medium; closed diamonds, control without fungus. Data points represent means of three parallels with standard deviations.

distribution of residual LHA was changed towards lower masses only in the presence of  $\text{Mn}^{2+}$  (from 2.5 to 1.5 kDa for the predominant molecular mass) (Fig. 2A). Part of LHA was transformed into lower-molecular mass FA ( $\sim 0.5$  kDa) which was, in contrast to the original LHA, not precipitable with HCl (Fig. 2B). The largest amount of FA was found in cultures supplemented with  $\text{Mn}^{2+}$ , though the difference from  $\text{Mn}^{2+}$ -free cultures was slight. In both cases, the FA concentration was about twice as high as in the uninoculated controls.

Analysis of extracellular enzymatic activities revealed that *C. dryophila* produced MnP and laccase (Fig. 3). In the absence of  $\text{Mn}^{2+}$  and LHA, the fungus secreted only small amounts of both enzymes (a maximum of 9 U of laccase liter<sup>-1</sup> and 30 U of MnP liter<sup>-1</sup>) (Fig. 3A).  $\text{Mn}^{2+}$  stimulated the production of MnP considerably (a maximum of 80 U liter<sup>-1</sup>), whereas that of laccase was not influenced (Fig. 3B). The presence of LHA in the medium resulted in an increase of both laccase (a maximum of 18 U liter<sup>-1</sup>) and MnP (a maximum of 43 U liter<sup>-1</sup>) activities (Fig. 3C), although the latter was only half as much as that for  $\text{Mn}^{2+}$ -supplemented cultures. The highest levels of activity of MnP and laccase were observed in cultures containing both  $\text{Mn}^{2+}$  and LHA, reaching maximum levels of 102 and 23 U liter<sup>-1</sup>, respectively. The decolorization (bleaching) of LHA medium was most prominent in the presence of  $\text{Mn}^{2+}$

and agreed with the results of the HPSEC analysis (Fig. 3C and D).

**Mineralization of  $^{14}\text{C}$ -HA.** Figure 4 shows the time courses of  $^{14}\text{CO}_2$  evolution from  $^{14}\text{C}$ -HA by liquid cultures of *C. dryophila*. In the absence of  $\text{Mn}^{2+}$ , the fungus mineralized about 20% of the  $^{14}\text{C}$ -HA, while 35% was incorporated into the biomass or closely bound to the cell surface (Table 1). The presence of  $\text{Mn}^{2+}$  in the medium caused a noticeable stimulation of  $^{14}\text{CO}_2$  production (an increase of about 50%) and reduced the amount of  $^{14}\text{C}$  associated with the fungal mycelium. Substantial  $^{14}\text{CO}_2$  formation started after 1 week of incubation and reached an average rate of approximately 2.2% of  $^{14}\text{CO}_2$  per day during the following 2 weeks. Interestingly, at the same time MnP production had begun to increase in the LHA experiment (compare Fig. 3D). After 3 weeks,  $^{14}\text{C}$ -HA decomposition declined, thus reaching a mineralization rate of only 0.3%  $^{14}\text{CO}_2$  per day during the last week of incubation. In contrast,  $^{14}\text{CO}_2$  release by  $\text{Mn}^{2+}$ -free cultures was almost linear throughout the experiment.

In both the presence and absence of  $\text{Mn}^{2+}$ , only negligible amounts of  $^{14}\text{C}$ -labeled volatile organic compounds were formed (Table 1). A small part of  $^{14}\text{C}$ -HA (approximately 3.5%) was also converted to  $^{14}\text{CO}_2$  by the controls, particularly in the beginning of the experiment, indicating spontaneous oxidation reactions.

**Purification of MnP.** To produce larger amounts of MnP for subsequent purification, *C. dryophila* was cultured in 1-liter tissue culture flasks, which ensured a favorable oxygen supply. Using the  $\text{Mn}^{2+}$ -supplemented medium, the fungus produced considerably more MnP (269 U liter<sup>-1</sup>) under these conditions than in the Erlenmeyer flasks. After two steps of ultrafiltration-diafiltration, the concentrated culture fluid (988  $\mu\text{g}$  of protein ml<sup>-1</sup>) was fractionated by fast protein liquid chromatography (FPLC) (Fig. 5). The elution profile showed one large heme peak (405 nm), with high MnP activity corresponding to a smaller protein peak (280 nm; 41  $\mu\text{g}$  of protein ml<sup>-1</sup>). The pooled FPLC peak of MnP was analyzed by IEF and denaturing SDS-PAGE. IEF showed one enzyme band with a pI of 4.7 (stained with phenol red). SDS-PAGE followed by Coomassie staining also gave a single band with a molecular mass of 44 kDa (see insets in Fig. 5).

**Effect of *C. dryophila* on solid litter.** *C. dryophila* grew well on sterilized soil litter, and after one week, the whole surface of the solid material was covered with white mycelium. At the end of incubation on day 46, the material was thoroughly grown through by the fungus and showed a noticeably lighter color than the uninoculated controls. The already low pH of the

TABLE 1. Balance of radioactive carbon from a  $^{14}\text{C}$ -labeled synthetic humic acid ( $^{14}\text{C}$ -HA;  $2.5 \times 10^5$  dpm), added to stationary liquid cultures of *Collybia dryophila* K209, after 42 days growth<sup>a</sup>

Sample	% of $^{14}\text{C}$ in:				
	$^{14}\text{CO}_2$	$^{14}\text{C}$ -labeled volatile compounds	$^{14}\text{C}$ -labeled NaOH-soluble material	Residual $^{14}\text{C}$ after combustion	Total $^{14}\text{C}$
<i>C. dryophila</i>	18.9 $\pm$ 3.1	0.8 $\pm$ 0.3	47.1 $\pm$ 6.3	35.3 $\pm$ 2.8	102.1
<i>C. dryophila</i> + $\text{Mn}^{2+b}$	49.4 $\pm$ 2.8	1.4 $\pm$ 1.1	34.2 $\pm$ 4.3	12.9 $\pm$ 2.2	97.9
Control without fungus	3.7 $\pm$ 0.4	<0.1	97.8 $\pm$ 0.9	1.9 $\pm$ 0.3	103.6

<sup>a</sup> Values given are the means  $\pm$  standard deviations of three experiments.

<sup>b</sup> Concentration, 200  $\mu\text{M}$   $\text{MnCl}_2$ .



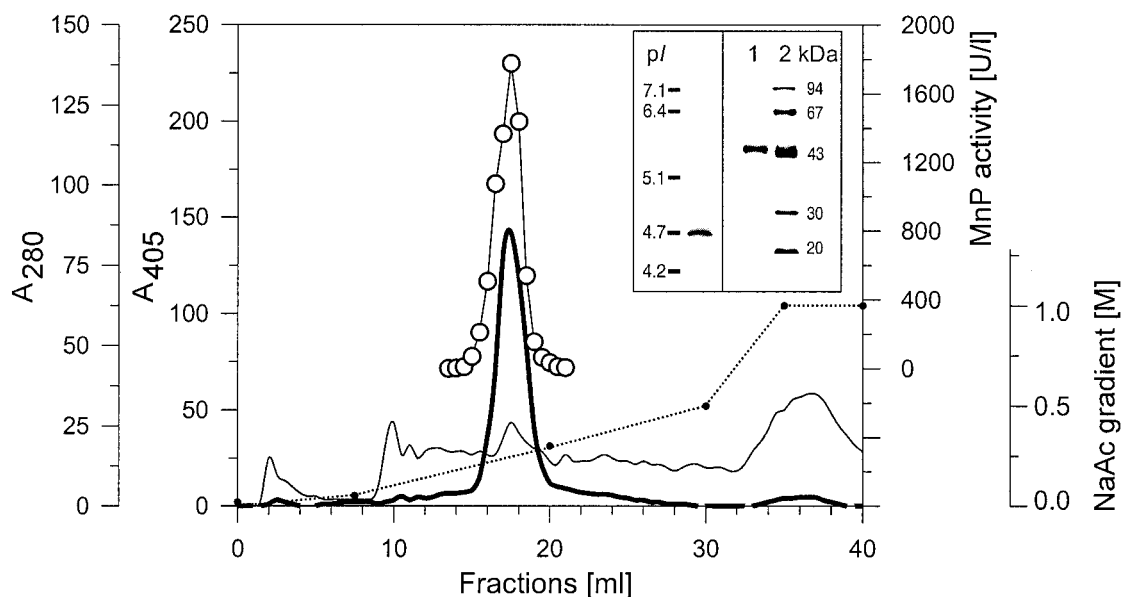


FIG. 5. FPLC protein profile of 5-week-old concentrated culture filtrate from *C. dryophila* grown in the presence of  $Mn^{2+}$  (200  $\mu M$ ). The sodium acetate (NaAc) gradient is represented by a dotted line. Absorbance levels at 405 nm (bold line) and 280 nm (thin line) represent the elution of heme-containing protein and total protein, respectively, along with the MnP activity levels (open circles). Inset: IEF (left) and SDS-PAGE (right) analyses of the pooled MnP peak (lane 1) and molecular mass markers (lane 2).

original litter samples dropped further during the growth of *C. dryophila* (from 3.9 to 3.1). HPSEC elution profiles of aqueous litter extracts revealed that, in comparison to that of the uninoculated litter, the amount of water-soluble material was considerably increased as the result of fungal activity (Fig. 6A). The major part of these yellowish compounds in the extract showed physicochemical characteristics of FAs, and only a small fraction (<5%), representing polar HA, could be precipitated with HCl. The predominant molecular mass of FA was around 0.9 kDa, which was slightly higher than that of FA in the uninoculated litter (0.7 kDa). HPLC analysis using a reversed-phase  $C_{18}$  column gave similar results. Considerably more water-soluble material which absorbed at 280 nm, including highly polar fragments which were eluted with pure water from the column, was detected in litter extracts overgrown by the fungus (Fig. 6B). Enzymatic activities detected in the aqueous litter extracts were relatively low, probably due to the interference of soluble litter products with the assays (MnP: maximum level, 12 U liter $^{-1}$  of litter extract; laccase: maximum level, 29 U liter $^{-1}$  of litter extract).

## DISCUSSION

The litter-decomposing *C. dryophila* was found to be capable of decomposing both natural HA from soil litter and synthetic  $^{14}C$ -HA prepared from catechol in liquid culture. The conversion process resulted in the formation of lower-molecular-mass FA and carbon dioxide. HA decomposition was considerably enhanced in the presence of  $Mn^{2+}$ , which stimulated the production of MnP. *C. dryophila* grew well on sterilized solid litter and released substantial amounts of FA from the water-insoluble material during growth.

Decolorization (bleaching) of different high-molecular-mass

HAs, indicating their conversion into low-molecular-mass FA, has been reported for a number of white-rot fungi under co-metabolic conditions (i.e., in the presence of an assimilable carbon source such as glucose). Among 20 species of basidiomycetes tested for their ability to decolorize an HA from podzol soil, four white-rot fungi (*Trametes versicolor*, *T. suaveolens*, *T. rubescens*, and *Hypholoma fasciculare*) were found to attack the dissolved humic material and bleach it completely (23). In another screening test using HA from podzol, black peat, and brown coal, the white-rot fungus *Bjerkandera (Gloeoporus) adusta* turned out to be particularly active (41). The fungus not only bleached HAs but also changed their physicochemical properties towards higher polarity. In connection with the investigation of brown coal bioconversion, further HA-attacking white-rot fungi were discovered, e.g., *Phanerochaete chrysosporium*, *Trametes versicolor*, and two unidentified species isolated from coal mining areas (31, 38). Indications were found that lignin peroxidase, laccase, and above all, MnP were involved in the bleaching and depolymerization process (10, 32, 38, 39). Our findings show that *C. dryophila* also produced at least two ligninolytic enzymes, MnP and laccase (with MnP predominant), during LHA conversion.

The first experiments for monitoring the cometabolic decomposition of  $^{14}C$ -HA by white-rot fungi were performed with *P. chrysosporium*. The fungus mineralized about 35% of a  $^{14}C$ -HA extracted from a U- $^{14}C$ -wheat straw compost in liquid culture within 18 days (15). The same fungal species was later used to degrade a synthetic  $^{14}C$ -melanoidine prepared from  $^{14}C$ -glucose and glycine by the Maillard reaction method (2). Within an incubation period of 15 days, *P. chrysosporium* released ca. 17% of the  $^{14}C$ -labeled material as  $^{14}CO_2$ . The mineralization process was stimulated in the presence of veratryl alcohol (23.5%  $^{14}CO_2$ ), which was proposed to enhance

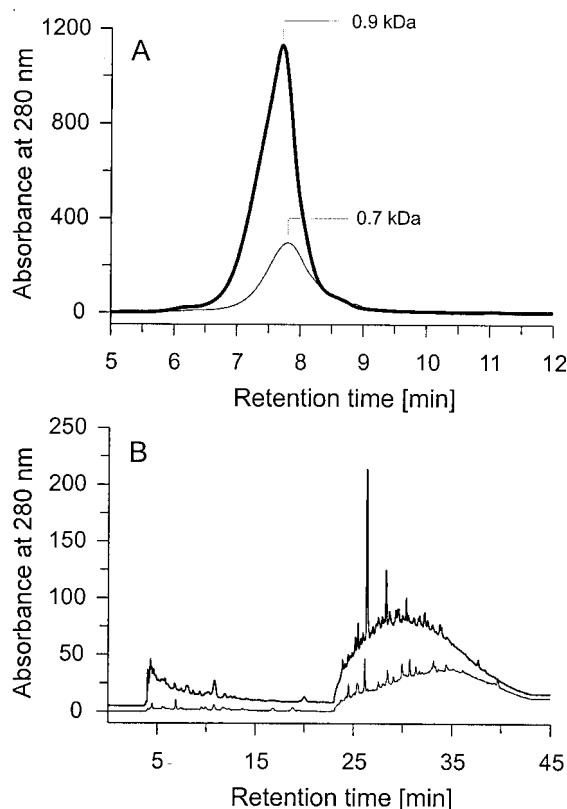


FIG. 6. HPSEC (A) and reversed phase  $C_{18}$ -HPLC (B) elution profiles of aqueous litter extracts. Thin lines represent controls without fungus; bold lines represent litter samples which were inoculated with *C. dryophila* for 46 days.

LiP activity and thereby melanoidine degradation. In the same study, the author also reported the decolorization and depolymerization of natural HA isolated from forest soil by *P. chrysosporium* (2). Later, *T. versicolor* was found to mineralize  $^{14}C$ -melanoidine more effectively than *P. chrysosporium*, and in addition to that of LiP, high levels of MnP were detected in HA- and melanoidine-containing culture filtrates (4). Culture filtrate and purified MnP of *T. versicolor* was shown to produce  $^{14}CO_2$  from  $^{14}C$ -melanoidine in a cell-free reaction system (5). This remarkable observation was later confirmed using a more recalcitrant  $^{14}C$ -HA, prepared as in the present study from  $^{14}C$ -catechol, and MnP from the agaric white-rot fungus *N. frowardii* (20). Our finding that  $Mn^{2+}$  in the medium stimulated the MnP level and the  $^{14}CO_2$  production from  $^{14}C$ -HA indicates that this enzymatic system is also involved in the mineralization process in *C. dryophila*. The molecular mass value (44 kDa) and pI (4.7) of the purified *C. dryophila* MnP are in the typical range for MnPs from white-rot fungi and other litter decomposers (16, 34). Unlike most other MnPs, which produce sets of isoforms that differ, that of *C. dryophila* showed only a single peak in the FPLC elution profile and one sharp band in the IEF analysis.

The finding that the pH of litter samples inoculated with *C. dryophila* decreased during the fungal growth is in concordance with those of earlier studies, which showed the secretion of oxalic and other organic acids by various basidiomycetous

fungi (17, 18, 36). Organic acids not only support MnP activity but also have an effect of their own in the mobilization of humic material that might subsequently undergo enhanced depolymerization (3, 9). Thus, FA and other polar fragments which form from litter as a consequence of *C. dryophila* treatment might be the result of concerted action by enzymatic and nonenzymatic agents. In this context, it cannot be ruled out that fungal metabolites also account for the variety of water-soluble products found in the litter extracts, although such compounds were not detected in the respective liquid cultures.

*C. dryophila*, representing a cosmopolitan species that belongs to the litter-decomposing basidiomycetes, possesses a ligninolytic enzyme system, based on MnP, which is similar to those of certain wood-degrading white-rot fungi (e.g., *Ceriporiopsis subvermispora*, *Dichomitus squalens*, and *Phanerochaete sordida*) (16) and which enables the fungus to disintegrate high-molecular-mass humic substances. In conclusion, we propose that litter-decomposing basidiomycetes play an important role in the recycling of organic carbon bound in the soil organic matter.

#### ACKNOWLEDGMENTS

This work was supported financially by the Finnish Graduate School in Environmental Science and Technology (EnSTe) and the Academy of Finland (Finnish Biodiversity Research Program), Biodiversity and Humus in Forest and Lake Ecosystems project grant 39590, the Academy of Finland project grant 39906 (to A.H.), and Bioconversion of Recalcitrant Soil Organic Matter by Litter-Decomposing Basidiomycetous Fungi (Academy of Finland) project grant 52063 (to M.H.).

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