Mineralization of Native Pesticidal Plant Cell-Wall Complexes by the White-Rot Fungus, *Phanerochaete chrysosporium*

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The white-rot fungus, *Phanerochaete chrysosporium*, has previously been demonstrated to mineralize soluble chloroaniline/lignin conjugates as well as the free chloroanilines (Arjmand, M.; Sandermann, H. *J. Agric. Food Chem.* **1985**, *33*, 1055−1060). It is now shown that the fungus also mineralizes 3,4-dichloroaniline bound to insoluble native wheat cell-walls, as well as two further insoluble cell-wall complexes, and the corresponding free herbicides (isoproturon and maleic hydrazide). The 3,4-dichloroaniline/wheat cell-wall complex has previously been shown to be digested only to a minor degree by monogastric and ruminant animal species (Sandermann, H., et al. *J. Agric. Food Chem.* **1992**, *40*, 2001−2007). The present results thus illustrate the unusual degradative power of *P. chrysosporium* from a new perspective.

Keywords: *Phanerochaete chrysosporium; mineralization; 3,4-dichloroaniline; maleic hydrazide; isoproturon; plant cell-wall bound complexes; lignin peroxidase*

INTRODUCTION

In 1985, we discovered that the lignin-degrading white-rot fungus, *Phanerochaete chrysosporium*, can also mineralize lignin−[^{14}C]chloroaniline copolymers and even the free [^{14}C]chloroanilines in the absence of lignin (Arjmand and Sandermann, 1985). Similar results were obtained with the chloroaniline−lignin complex solubilized by the Björkman procedure from native wheat cell-walls (Arjmand and Sandermann, 1986). The chemical nature of pesticidal complexes with plant cell-wall components, in particular lignin, has been reviewed (Lamoureux and Rusness, 1986; Sandermann et al., 1989). Links between the formation of soluble "phase II" pesticidal conjugates and of non-extractable residues have recently been described (Sandermann et al., 1997). The synthetic chloroaniline−lignin conjugates had high bioavailability in a monogastric animal species (rat) and a ruminant animal species (sheep) (Sandermann et al., 1990, 1992). In contrast, a native wheat cell-wall fraction with most of the bound [^{14}C]chloroaniline in lignin was hardly bioavailable (Sandermann et al., 1992). This raised the possibility that the initial results on the high mineralization capabilities of *P. chrysosporium* (Arjmand and Sandermann, 1985, 1986) were due to the soluble nature of the lignin complexes and free chloroanilines employed. The fungus is well known to mineralize the lignin component in native particulate plant cell-walls and in wood (Zeikus, 1981; Kirk et al., 1987). However, it has not yet been examined whether pesticidal residues that are tightly bound to native plant cell-walls can be mineralized. Several "bound" pesticidal residues have therefore been tested.

MATERIALS AND METHODS

Fungal Strain. *P. chrysosporium* (ATCC 34541) was maintained on malt extract−agar slants, as described previously (Arjmand and Sandermann, 1985).

Chemicals. All chemicals used in this study were of analytical grade and, unless otherwise mentioned, were used without prior purification. High-performance liquid chromatography (HPLC) solvents were from Riedel-de-Haën (HPLC-grade, Seelze, Germany). The purity of all radioactive stock solutions was controlled by HPLC prior to usage, and, if less than 98%, the compounds were purified by preparative HPLC. [UL-*ring*-^{14}C]-3,4-Dichloroaniline (DCA) was supplied by Sigma (Deisenhofen, Germany), [1-^{14}C]hexadecane and [UL-*ring*-^{14}C]-Toluene by Amersham (Braunschweig, Germany) and [2,3-^{14}C]-maleic hydrazide, choline salt, by Pathfinder (St. Louis, MO, U.S.A.). [UL-*ring*-^{14}C]Isoproturon (IPU) was purchased from the Institute of Isotopes of Hungarian Academy of Sciences (Budapest, Hungary).

Ligninase Assay. Total ligninase (EC 1.11.17) activity (Reddy, 1995; Tien and Kirk, 1984) was measured spectrophotometrically by following the rate of veratryl aldehyde production at 310 nm and 23 °C in plastic cuvettes (light path 1 cm) 30 s after mixing the solutions in the following order: 500 μL of 1 M sodium phosphate buffer (pH 3.2), 20 μL of 100 mM veratryl alcohol, 100 μL of fungal culture medium, 860 μL of water, and 20 μL of 27 mM H_2O_2. One enzyme unit was defined as the amount of enzyme releasing 1.0 μmol of veratryl aldehyde per min.

Determination of Protein Concentration. The determination of total protein was performed using the Bio-Rad protein assay kit (Bio-Rad, Munich, Germany) according to Bradford (1976) using bovine serum albumin as the standard.

HPLC Analysis. Sample extracts were analyzed on a Waters-Millipore (Eschborn, Germany) HPLC system composed of two 510 pumps, a 996 PDA detector, a 470 fluorescence detector, a Ramona (Raytest, Straubenhardt, Germany) radioactivity detector, and a 717 plus autosampler. The analytical column was a VYDAC 210 TP 54 (Sep/a/ra/tions Group, Hesperia, California) operated under the following conditions: eluent A, acetonitrile; eluent B, water acidified with 1 mL of H_3PO_4/L. Gradient: 0−3 min, 0% A; 3−28 min, linear to 100% A, at a flow rate of 1.5 mL/min.

Liquid Scintillation Counting (LSC). Aqueous solutions (maximum volume, 2 mL) were measured in 10 mL of Hydroluma (Baker, Deventer, Netherlands). Organic solutions (maximum volume, 2 mL) were measured in 10 mL of fluid consisting of 750 mL of toluene, 250 mL of methanol, and 5 g of 2,5-diphenyloxazole (Zinsser, Frankfurt, Germany). Samples were internally standardized with [^{14}C]toluene and corrected for background radioactivity. Radioactivity of lyophilized

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fungal biomass samples was determined after catalytic combustion as previously described (Komossa et al., 1992).

Preparation of ^{14}C-Labeled Non-Extractable Residues (NERs). NERs were generally prepared by exhaustive extraction of homogenized plant material with methanol, dichloromethane, and aqueous solvents, usually followed by extraction with 1% (w/v) sodium dodecyl sulfate (Komossa and Sandermann, 1995). The [UL-*ring*-^{14}C]isoproturon wheat NER with a specific activity of 86.83 kBq/g has been previously described (Bohnenkämper et al., 1994). By a sequential solubilization procedure (Langebartels and Harms, 1985), the bound radioactivity was distributed as follows: 65% in hemicellulose, 9% in lignin, and 5% in pectins (Bohnenkämper et al., 1994). The preparation and characterization of a soybean−[2,3-^{14}C]maleic hydrazide NER, specific activity 38.83 kBq/g, has been previously described (Komossa and Sandermann, 1995). The bound radioactivity was distributed as follows: 14% in hemicellulose, 31% in lignin, and 4% in pectins. Radioactivity in the previously reported (Sandermann et al., 1992) wheat [U-*ring*-^{14}C]-3,4-dichloroaniline−lignin metabolite fraction (NER) of 190.18 kBq/g was distributed as follows: 2% in hemicellulose, 85% in lignin and 4% in pectins [sequential solubilization procedure of Langebartels and Harms (1985)].

Culture Conditions. Low-nitrogen (low-N) media as well as inoculum were prepared as described (Kirk et al., 1978; Arjmand and Sandermann, 1985). The growth medium contained 0.1% (w/v) Tween 80. At least 5×10^6 spores, obtained from malt−agar slant cultures of *P. chrysosporium*, were used per flask. Typically, three culture flasks with fungus and one or two control flasks without fungus were employed per treatment group. Each culture flask was aerated on the first day and shaken at 125 rpm at 39 °C for 2 days. On the third day (when fungal pellets had formed), the culture volume was either reduced to 40 mL (DCA, experiment 1) or left unchanged (all other experiments). The radioactive bound residues of 3,4-dichloroaniline, maleic hydrazide, or isoproturon or the free radioactive herbicides were added. Concentrations and amount of radioactivity of the incubated free and NER herbicides are given in the legend of Figure 1. Each culture flask was flushed with pure oxygen at 3-day intervals and further incubated either at 60 rpm at 30 °C (DCA, experiment 1) or at 125 rpm at 39 °C (all other experiments) until cessation of the experiment.

Product Analysis. The pressure exit of the fungal culture flask was connected to an impinger trap containing 20 mL of kerosene (Fluka, Neu-Ulm, Germany), followed by another trap containing 20 mL of 2-methoxyethanol/ethanolamine 2:1 (v/v, Fluka). Immediately following oxygen flushing, as explained above in culture conditions, 1 or 2 mL of liquid medium was withdrawn and used for the analysis of metabolites by HPLC as well as for the determinations of ligninase activity and of radioactivity. After removal of medium, the flasks were again sealed and reoxygenated for 5−20 min. Samples of 1 or 2 mL from kerosene traps and $^{14}CO_2$ traps were mixed with scintillation fluid.

Workup Procedure. At the end of the incubation, the medium and the fungal biomass in the incubation flasks were vacuum filtered through tared Schleicher & Schuell # 604 filter paper into a tared vacuum flask. The growth medium was either directly analyzed by HPLC or extracted as follows. The medium (70−90 mL) was extracted with 3×50 mL CH_2Cl_2. The aqueous phase was acidified to a pH of ca. 3 with 1 M HCl and was extracted with another 3×50 mL of CH_2Cl_2. The aqueous phase was then adjusted to pH ca. 10 with 1 M NaOH and extracted with an additional 3×50 mL of CH_2Cl_2. The combined organic CH_2Cl_2 phase extracts were pooled and dried for 30 min with anhydrous Na_2SO_4 (ca. 10 g/L of CH_2Cl_2), filtered, and concentrated to an oil at 35 °C on a rotary evaporator. The weighed lyophilized fungal biomass was treated three times with 1 mL of acetone/CH_2Cl_2 3:1 (v/v) in a closed vessel for 5 min. The solution was carefully decanted after each washing. The combined organic extracts were pooled and concentrated under a stream of nitrogen. After the solution was re-dissolved to a known volume with CH_3CN/CH_2Cl_2 1:1 (v/v), the amount of radioactivity was determined and the extract was analyzed by HPLC.

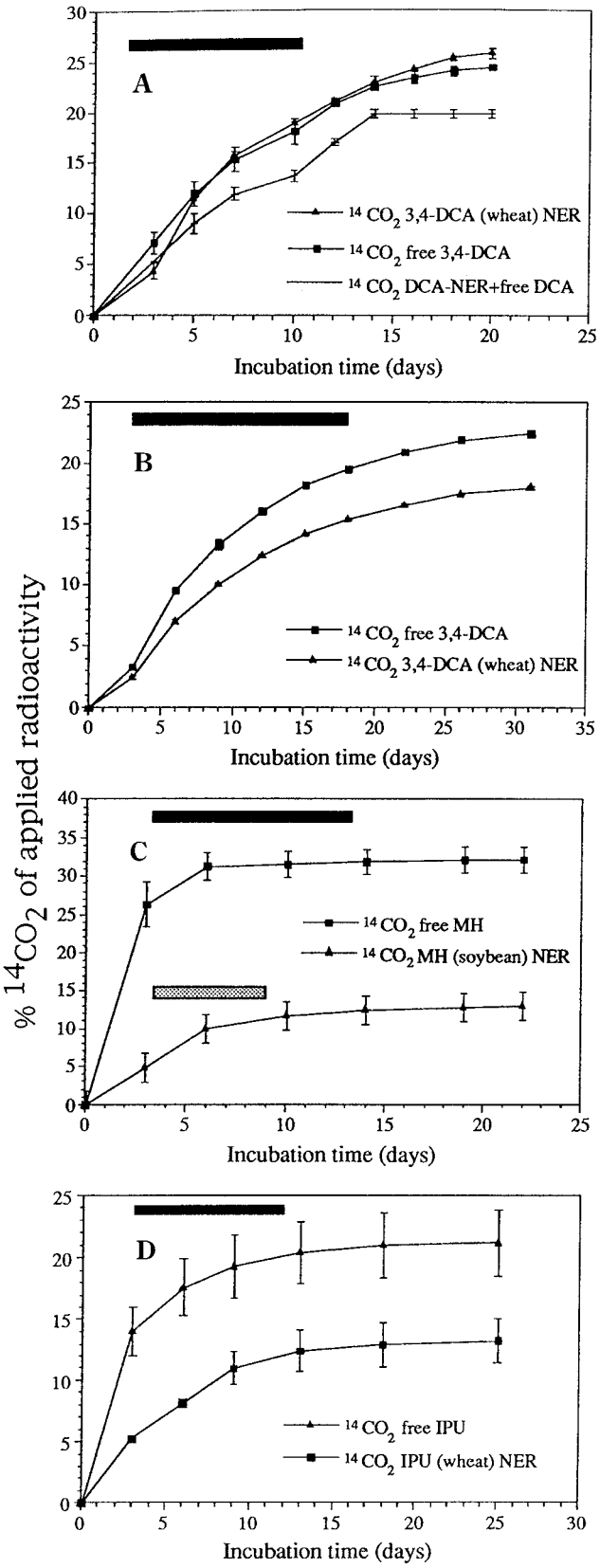

Figure 1. Mineralization of free and bound herbicidal substrates by *P. chrysosporium*. All incubations were conducted according to the standard procedures of Materials and Methods. The horizontal bars represent the phase of ligninase activity. (A) [UL-*ring*-^{14}C]-3,4-DCA (12.5 kBq, 0.75 μM); wheat NER (12.5 kBq, 65,3 mg); non-radioactive 3,4-DCA (0.62 μM) plus wheat NER (6.25 kBq, 32.6 mg). (B) [UL-*ring*-^{14}C]-3,4-DCA (11.7 kBq, 0.70 μM); wheat NER (13.33 kBq, 60.5 mg). (C) [2,3-^{14}C]MH (9.25 kBq, 0.73 μM); soybean NER (8.67 kBq, 237 mg). (D) [UL-*ring*-^{14}C]IPU (40.47 kBq, 0.61 μM); wheat NER (16.67 kBq, 190 mg). The symbols used are explained on the individual panels.

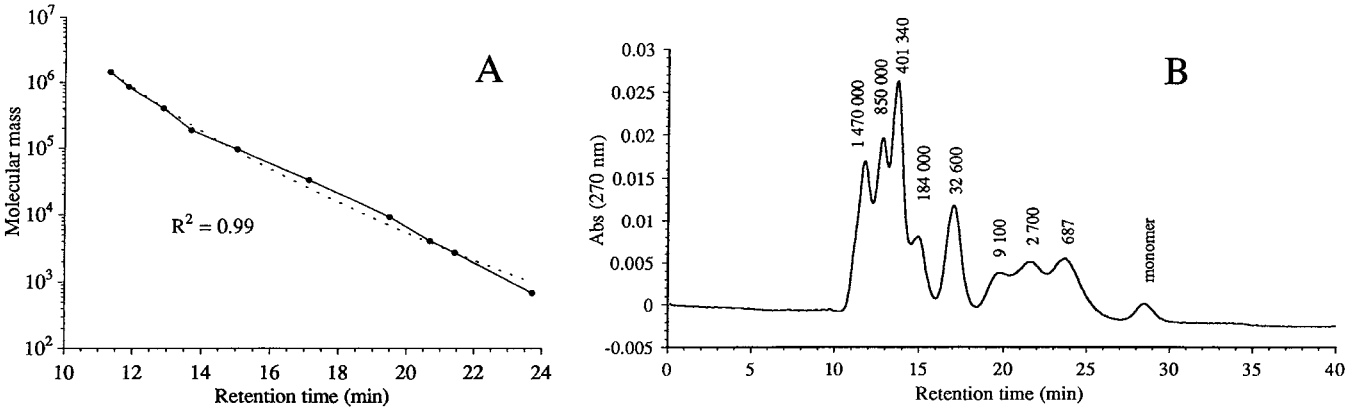

Figure 2. Molecular mass determination. (A) Calibration of the GPC system as a function of retention time. Molecular masses of the standards ranged from 1.6×10^6 to 687 Da. DMF degassed at 50 °C served as the mobile phase at a flow rate of 1 mL/min. (B) Chromatogram representing a typical example for the resolution of nine polystyrene standards.

Gel Permeation Chromatography (GPC). For the determination of molecular weight profiles, samples were analyzed by GPC on a Nucleogel highly crossed-linked polystyrene−divinylbenzene matrix column system (Macherey Nagel, Düren, Germany) consisting of a pre-column (GPC-5P, 5 μm spheres), column 1 (GPC 105−5, 4000×10^3 Da cutoff, 5 μm spheres, 10^5 Å pore size), and column 2 (GPC 103−5, 60×10^3 Da cutoff, 5 μm spheres, 10^3 Å pore size). The GPC system was run using HPLC-grade degassed (30 min, 20 Torr) *N*,*N*-dimethylformamide (DMF; Aldrich, Steinheim, Germany) at 50 °C and a flow rate of 1 mL/min. Commercially available polystyrene standards (Aldrich) with molecular mass values of ca 1.47×10^6 Da down to the monomer were used for calibration. Detection was at 270 nm. The PDA detector was set to a wavelength region of 250−400 nm at 1.2 nm resolution, and 0.05 spectra/s were recorded. Each standard was dissolved in DMF (ca. 2 mg/mL) and injected three times. The separation of a mixture of standards as well as the derived calibration curve for all the individual standards are depicted in Figure 2. For GPC sample preparation, aqueous medium samples were frozen in liquid nitrogen, lyophilized in either a Speed Vac (Uniequip, Martinsried, Germany) or a freeze-dryer (Leybold-Heraeus, Hanau, Germany) and mixed with a small volume of DMF/LiCl (5% w/v, Aldrich) to afford a typical suspension of 2 mg of substrate per mL of DMF. A spatula tip of anhydrous Na_2SO_4 (Fluka, Neu-Ulm, Germany) was added, and the heterogeneous slurry was stored in dark glass vials for at least 24 h. Samples in organic solvents were treated similarly but without prior lyophilization. Before analysis, the samples were centrifuged for 10 min at 13 000 rpm in a bench-top centrifuge (Hettich, Tuttlingen, Germany).

RESULTS AND DISCUSSION

Free and Bound 3,4-Dichloroaniline (DCA). The mineralization of free [^{14}C]DCA and wheat non-extractable residue (NER) in low-N cultures was carried out under two different experimental conditions (see Materials and Methods). In the first experimental setup (Figure 1A), maximal ligninase activity in low-N medium exceeded 25 units per liter of medium on day 6 and declined between days 10 and 13 to below 5 units per liter. The mineralization of free and wheat cell-wall bound 3,4-dichloroaniline by *P. chrysosporium* was found to be high in low-N cultures. It increased continuously with increasing duration of incubation. At the end of the fermentation period, about 26% of applied radioactivity from bound [^{14}C]DCA and 24% from free [^{14}C]DCA was recovered as $^{14}CO_2$. The mineralization of bound [^{14}C]DCA supplemented with free nonlabeled DCA reached approximately 20% of the applied radioactivity at the end of the fermentation period. In contrast, no mineralization of [^{14}C]DCA could be detected in control cultures without fungus. Although specific ligninase activity declined, mineralization continued up to the end of the incubation period (20 days).

In the second experimental setup (Figure 1B), ligninase activity rose to a peak on day 12 (ca. 45 units per liter) and declined thereafter. Even after ligninase activity became undetectable, mineralization continued. The accumulated percent mineralization was 22.4% for free DCA and 18.0% for the NER. As in the first experiment, the fungal incubations led to approximately four times more radioactivity (38%) released from the bound metabolite fractions into the medium than determined in the control without fungus (10%). The latter release could be due to the slight acidity (pH 4.3) of the growth medium. However, we did not attempt to identify the medium component leading to release of radioactivity from the pre-extracted residue. Neither GC nor TLC showed any evidence of free DCA in the medium from the fungal experiment. The GPC profile of the lyophilized medium (column system cutoff = 4×10^6 Da; calibrated to 1.5×10^6 Da) from Figure 3A showed a single broad peak corresponding to ca. 600 000 Da down to 5000 Da. Thus, both experiments have shown that *P. chrysosporium* mineralized the cell-wall integrated 3,4-dichloroaniline (and free 3,4-dichloroaniline) in low-N cultures with comparable efficiency. In contrast, the same 3,4-dichloroaniline/wheat cell-wall preparation as used here had shown very little bioavailability in rats and lambs (Sandermann et al., 1992).

Free and Bound Maleic Hydrazide (MH). CO_2 production from free [^{14}C]MH amounted to approximately 25% after the third incubation day (Figure 1C). This was followed by release of another ca. 6% during the next three days. An additional ca. 2% was produced during the last 14 incubation days. The CO_2 production for the MH (soybean) NER was linear for the first six incubation days, ranging from ca. 7 to 11%, followed by a near-linear increase to 11−14% over the remaining 16 incubation days.

Ligninase activity (Figure 1C) started on the third incubation day and had disappeared in the NER flasks by the ninth day of incubation. In all cases, it could be shown with HPLC-PDA analysis that free MH and MH glucoside (a known plant metabolite; Komossa and Sandermann, 1995) were absent from the fungal medium. The GPC molecular mass profile of the lyophilized medium DMF extract after incubation of the soybean NER is shown in Figure 3B. Elution of copolymers started at a molecular mass value of ca. 1 600 000 Da and continued to ca. 400 Da. As was typical for all incubations (free and NER), the most intensive peak appeared at ca. 21 min corresponding

Table 1. Distribution and Balance of Applied Radioactivity after Incubation of Substrates in Low-Nitrogen Cultures of *P. chrysosporium*[a]

	3,4-dichloroaniline				maleic hydrazide				isoproturon			
	whole culture		sterile control		whole culture		sterile control		whole culture		sterile control	
	free	bound	free	bound	free	bound	free	bound	free	bound	free	bound
number of flasks	3	3	2	2	4	3	2	1	2	2	2	2
$^{14}CO_2$	22.4 ± 0.3	18.0 ± 0.1	0.06	0.06	31.7 ± 2.7	11.0 ± 4.1	0.05	0.07	21.6	13.5	0.03	0.04
volatiles	0.04 ± 0.01	< 0.01	0.1	0.1	0.06 ± 0.02	0.04 ± 0.01	< 0.01	< 0.01	0.06	0.06	< 0.01	0.05
medium at end	29.9 ± 0.3	35.0 ± 1.1	80.7	10.2	5.4±1.1	21.9 ± 4.7	70.89	4.1	35.8	39.1	100.5	8.7
cell extracts	6.1 ± 0.2	nd	na	na	nd	nd	na	nd	10.6	nd	na	nd
cell residues	16.3 ± 0.1	34.8 ± 0.5	na	80.00	8.5	59.0 ± 11.0	na	64.2	0.8	35.32	na	87.8
org wash	1.4 ± 0.1	1.2 ± 0.1	1.5	1.1	0.7	3.6	nd	nd	0.7	1.7	0.7	1.71
removed samples	7.7 ± 0.1	8.0 ± 0.1	na	4.5	0.6	1.5	na	nd	0.8	3.5	na	3.5
sum	83.8 ± 2.7	97.0 ± 4.2	82.4	95.7	46.9 ± 5.5	97.1 ± 8.3	70.9	68.4	70.3	93.2	101.2	101.8

[a] Entries (% of applied radioactivity) are the mean of n flasks ± standard deviation (when $n > 2$); na, not applicable. nd, not determined; org. wash, washing incubation flasks after rinsing with water; removed samples, sum of all small residues and biomass removed through sampling during an incubation. No attempt was made to determine the reason for the very low radioactivity recovery in the free maleic hydrazide experiment.

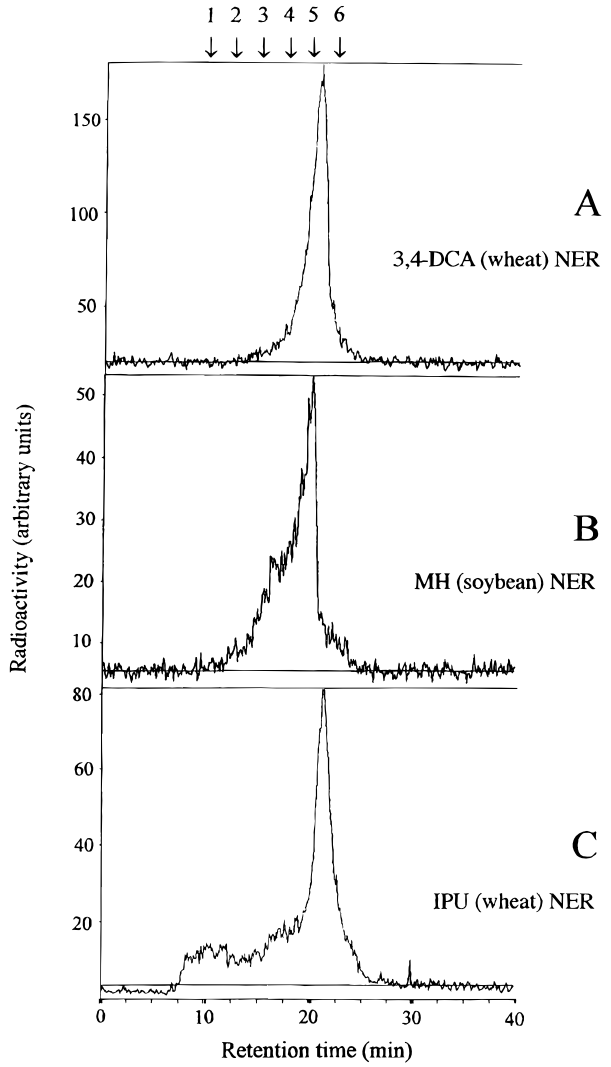

Figure 3. Molecular mass profiles of lyophilized medium samples obtained after incubation of the investigated NERs by *P. chrysosporium*. Samples were prepared and analyzed according to Materials and Methods. The initial amounts were as follows (cf. Figure 1): (A) 3,4-DCA wheat NER (13.33 kBq, 60.5 mg); (B) MH soybean NER (8.67 kBq, 237 mg); (C) IPU wheat NER (16.67 kBq, 190 mg). The numbers above the peaks are based on the following molecular mass standards (Da) of Figure 2A: 1 = 1 447 000; 2 = 401 340; 3 = 95 800; 4 = 32 600; 5 = 9100; 6 = 2700.

to a molecular mass of ca. 4000 Da, in both the UV channel (280 nm) and the ^{14}C channel. The extents of solubilization, based on medium radioactivity values at the end of the incubation, were 25% and 3% for the fungal incubation and the control, respectively.

Free and Bound Isoproturon (IPU). The fungal incubations with isoproturon and wheat cell-wall bound (NER) isoproturon are shown in Figure 1D. The mineralization of free isoproturon showed a release of 14% $^{14}CO_2$ within the first three days, followed by a further 5% over the following three days and 3% over the remaining 19 incubation days. This release preceded the onset of ligninase activity on the third day. As in all other incubations, no ligninase activity was observed at the time of substrate addition and the mineralization of the free or bound herbicides was not accelerated after the onset of ligninase activity. This may indicate that mineralization and ligninase induction were independent processes.

Mineralization of the NER amounted to 11% over the first 10 incubation days. This was followed by a further 2% over the following 15 days. A significant portion (25%) was solubilized in the growth medium, as determined 30−60 min after addition when ligninase activity was not yet detectable. In the sterile control flasks, only 4% of the starting herbicidal NERs was solubilized during the first three days and 6% over the next 22 days. No ligninase activity was observed in the sterile controls. Of all the investigated NER materials, the IPU (wheat) NER showed the largest overall solubilization, namely 40% and 8% for the fungal culture and the sterile control, respectively (Figure 3C). The molecular mass profile of the IPU (wheat) NER had a high-molecular mass shoulder and an overall distribution from 1 500 000 Da down to ca. 5000 Da. These observations may be related to the high proportion of initial ^{14}C label in the hemicellulose fraction (Bohnenkämper et al., 1994), whereas in the other two cases the lignin component of the residue was more significantly labeled (Sandermann et al., 1992; Komossa and Sandermann, 1995).

Conclusions. The mass balance analysis of the various experiments is summarized in Table 1. In spite of some variability, it is evident that the observed mineralization was caused by the fungus and was largely independent of ligninase induction. The latter observation (see also Sandermann et al. (1993)] is in contrast to the involvement of ligninase and manganese peroxidase in the degradation of other xenobiotic compounds (Lamar, 1992; Michel et al., 1991; Valli et al., 1992a,b). There are previous reports where ligninase and manganese peroxidase of *P. chrysosporium* appeared to be unimportant for mineralization viz. DDT (Köhler et al., 1988), phenanthrene (Sutherland et al.,

1991), 2,4-D (Yadav and Reddy, 1993), 2,4,5-T (Yadav and Reddy, 1992, 1993b), and benzene, toluene, ethyl benzene, and xylene (BTEX) compounds (Yadav and Reddy, 1993a). In the present study, mineralization seemed to be independent of the matrix to which the radioactively labeled herbicide was bound. Herbicidal substances bound preferentially to lignin (DCA, MH) were mineralized as well as herbicide bound preferentially to hemicellulose (IPU). The high metabolic capability of the white-rot fungus is further illustrated by the fact that the present DCA−wheat cell-wall preparation had remained largely undigested in monogastric and ruminant animals (Sandermann et al., 1992). In contrast, soluble synthetic DCA−lignin polymers were digested to about 30% by rats and lambs (Sandermann et al., 1990, 1992). In summary, the unusually high capacity of *P. chrysosporium* for solubilizing and mineralizing xenobiotics from the plant matrix is documented here from a new perspective.

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