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Fate of Residual Lignin during Delignification of Kraft Pulp by *Trametes versicolor*

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The fungus *Trametes versicolor* can delignify and brighten kraft pulps. To better understand the mechanism of this biological bleaching and the by-products formed, I traced the transformation of pulp lignin during treatment with the fungus. Hardwood and softwood kraft pulps containing ^{14}C-labelled residual lignin were prepared by laboratory pulping of lignin-labelled aspen and spruce wood and then incubated with *T. versicolor*. After initially polymerizing the lignin, the fungus depolymerized it to alkali-extractable forms and then to soluble forms. Most of the labelled carbon accumulated in the water-soluble pool. The extractable and soluble products were oligomeric; single-ring aromatic products were not detected. The mineralization of the lignin carbon to CO_2 varied between experiments, up to 22% in the most vigorous cultures. The activities of the known enzymes laccase and manganese peroxidase did not account for all of the lignin degradation that took place in the *T. versicolor* cultures. This fungus may produce additional enzymes that could be useful in enzyme bleaching systems.

Trametes versicolor is a white rot fungus that can delignify kraft pulps (28). The lower lignin contents of pulps treated with this fungus lead to higher brightness and higher bleachability of the pulps. Although the application of this biological delignification process to pulp bleaching has been studied for several years, its mechanism—the chemical changes produced in the lignin and the enzymes that catalyze them—is not yet known in detail.

Biodegradation of native lignin, the model polymer dehydrogenative polymerizate, and lower-molecular-weight model compounds by *Phanerochaete chrysosporium* and other white rot fungi has been studied extensively (17). Kraft lignin, the lignin which is solubilized during kraft pulping, has also received some attention (21). White rot fungi are able to extensively mineralize (convert to CO_2) and solubilize these lignins (8, 11, 25, 26, 33). These fungi produce various combinations of the extracellular oxidative enzymes lignin peroxidase, manganese peroxidase (MnP), and laccase (13), which are thought to play important roles in lignin degradation (12, 35). Participation of MnP has been implicated in kraft pulp delignification by *T. versicolor* (22), and it has been determined that lignin peroxidase is not needed (2). MnP and laccase can cause substantial decreases in the kappa numbers and improvements in the bleachability of kraft pulps under appropriate conditions, but their activities do not fully explain the delignifying effects of *T. versicolor* cultures (3).

The structure of the residual lignin in kraft pulps is significantly different from the structure of the lignin in wood and even from the structure of dissolved kraft lignin. During kraft pulping, aryl ether bonds are depleted, condensed bonds are enriched, and links to polysaccharides are formed (3). Kraft pulps have much lower lignin contents than wood has, and the porosity of the polysaccharide matrix enclosing them is increased (31). Consequently, the mechanism of pulp delignification may differ from the mechanism of wood delignification.

This study traced the fate of ^{14}C-labelled lignin removed from hardwood kraft pulp (HWKP) and softwood kraft pulp (SWKP) during delignification, and the molecular sizes of intermediates and products were determined. Preliminary results showing that solubilization and mineralization of labelled lignin from HWKP occur have been published previously (27).

MATERIALS AND METHODS

The labelled pulps used in this study were prepared on two occasions, once in 1991 and once in 1994. These pulps are referred to below as HWKP*1, SWKP*1, and SWKP*2.

^{14}C labelling of wood. L-[U-^{14}C]phenylalanine (39 MBq; 17.6 GBq/mmol; catalog no. CFB.70; Amersham) was converted to [U-^{14}C]cinnamic acid with phenylalanine ammonia lyase (catalog no. P-1016; Sigma) (23). The labelled cinnamic acid was dissolved in 10 mM potassium phosphate buffer (pH 7) and was fed to cut stems of aspen (*Populus tremuloides* Michx.; 16 MBq; June 1991) and white spruce (*Picea mariana* L.; 15 MBq; July 1991). The labelled stems were kept in a fume hood with their cut ends immersed in water and with illumination for 10 h per day for 2 weeks, until the aspen leaves wilted and the white spruce needles began to fall off. The leaves or needles and small branches were removed and discarded, and the bark was peeled off the main branches and discarded. The wood from the main branches was cut into matchstick-sized pieces and sequentially extracted in a Soxhlet apparatus with water, benzene-ethanol (1:1), ethanol, and water again and then air dried.

In a separate experiment (July 1994), 32 MBq of [U-^{14}C]cinnamic acid was fed to a white spruce stem as described above. After debarking, the labelled wood was ground in a Wiley mill until it passed through a 10-mesh screen, and then it was extracted as described above.

Pulping of labelled wood. The extracted labelled wood pieces were placed, along with kraft white liquor (15% active alkali, 27% sulfidity) (5), inside Teflon-lined stainless steel bombs and heated in a computer-controlled oil bath to H-numbers (34) of 1,400 (aspen) and 1,600 (spruce). After cooling, the bombs were opened, and the pulps were separated from the black liquor by filtration, thoroughly washed with water, and freeze-dried. Portions (0.4 g) of the pulps were then sequentially extracted with 0.05% Tween 80 (2% consistency, 20 min at 120°C) and 0.01 M NaOH (2% consistency, 15 min at 120°C) and again freeze-dried. The specific radioactivity of the extracted aspen pulp (HWKP*1) was 57 kBq/g, and the specific radioactivity of the extracted spruce pulp (SWKP*1) was 72 kBq/g.

The ground labelled spruce wood was pulped by mixing it at a ratio of 4 ml/g with white liquor (16% active alkali, 14% sulfidity) in stainless steel bombs, heating the preparations in an oil bath from room temperature to 170°C over a 90-min period, and then keeping them at 170°C for an additional 70 min (H-number, 1,100). After cooling in cold water, the bombs were opened, and the pulp was recovered by filtration on a glass frit and washed with hot distilled water. This pulp (SWKP*2) had a specific radioactivity of 73 kBq/g.

Cellulase isolation of residual lignin. Crude cellulase (product code BR-1101; 154 filter paper units/ml) was purchased from Iogen Corporation, Ottawa, Canada, and was used without purification. Pulp (up to 2.5 g [oven dry weight]) was

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weighed into a 250-ml polypropylene centrifuge bottle with a conical bottom (catalog no. 25350; Corning) and suspended in 200 ml of 0.02% NaN_3. The pH of the suspension was adjusted to 4.7 with acetic acid. Concentrated Iogen cellulase solution (0.5 ml) was added, and after thorough mixing, the suspension was incubated at 37°C for 24 h with occasional shaking. Then the pH was adjusted to 2.0 with 2 M H_2SO_4, and the suspension was centrifuged for 10 min at 2,550 × *g* in a swinging bucket rotor. The supernatant was discarded, and the pellet was resuspended in 200 ml of 0.02% NaN_3, mixed with 0.5 ml of cellulase concentrate, adjusted to pH 4.7, and incubated at 37°C for another 48 h. Then 4 ml of 5 M NaOH was added to dissolve the lignin, and the mixture was incubated at 37°C for 1 h. After centrifugation in a swinging bucket rotor at 2,550 × *g* for 10 min, the supernatant was carefully decanted into a clean 250-ml centrifuge bottle. The pellet was discarded. The pH of the supernatant was adjusted to 2.0 with 2 M H_2SO_4. After another centrifugation, the supernatant was discarded, and the pellet was used as the lignin preparation.

Incubation with the fungus. Portions (ca. 100 mg) of the labelled pulps HWKP*1 and SWKP*1 were accurately weighed into 500-ml Erlenmeyer flasks (three flasks for the aspen pulp and three flasks for the spruce pulp). To each flask containing aspen pulp 3.9 g (dry weight) of a commercial HWKP (kappa number, 12.7) was added. To each flask containing spruce pulp 3.9 g (dry weight) of a white spruce kraft pulp (kappa number, 27.6) prepared in the Paprican pilot plant was added. One 125-ml Erlenmeyer flask containing ca. 25 mg of labelled pulp and 975 mg (dry weight) of unlabelled pulp was also prepared for each pulp type. The pulp in each flask was suspended at 2% consistency in a defined medium containing 0.5 g of Tween 80 per liter (29), and the flasks were fitted with rubber stoppers traversed by stainless steel tubes for air inlet and outlet. Latex tubing stuffed with cotton wool was attached to the external ends of the aeration tubes, and the flasks were autoclaved for 20 min. After cooling, the flasks were placed overnight on a gyratory shaker operating at 225 rpm to thoroughly mix their contents. Then a 10-ml sample of the pulp suspension was aseptically pipetted from each flask, and the 500-ml flasks were each inoculated with 30 ml of a shake culture of *T. versicolor* 52J (1) that had been grown for 5 days in the defined medium; the 125-ml flasks served as noninoculated controls. The air inlet tube of each flask was connected to a source of humidified air, and the air outlets were connected to Pasteur pipettes with their tips immersed in 5 ml of 0.1 M NaOH in test tubes to trap CO_2. The airflow through each culture flask was adjusted to give vigorous bubbling but no splashing in the CO_2 traps. The flasks were incubated on the shaker with continuous airflow in a room with the temperature controlled at 27.5°C. Samples (10 ml) were aseptically removed from the inoculated flasks after 1, 2, 3, 4, and 5 days of incubation for the aspen pulp and after 1, 2, 4, 8, and 12 days for the spruce pulp. The CO_2 traps were replaced daily. At the end of incubation, the contents of the 125-ml flasks and the remaining contents of the 500-ml flasks were harvested and fractionated.

Pulp SWKP*2 was incubated with *T. versicolor* 52J for 14 days in a similar way, with these differences: the initial glucose concentration in the medium was increased to 15 g/liter, and the sulfate concentration was lowered to 0.2 mM; the cultures were incubated in 2-liter flasks containing 800 ml of pulp suspension; the unlabelled pulp was prepared from black spruce in the Paprican pilot plant and was washed with 0.1 M NaOH and water at room temperature before use; the labelled pulp SWKP*2 was added to each flask as 1.96 ml of a suspension containing 53.3 kBq; the flask headspaces were continuously flushed with O_2 during incubation; and the effluent gases were bubbled through 20 ml of 2 M NaOH, which was replaced every 2 days, to trap CO_2. Samples (25 ml) were aseptically removed every 2 days from each flask, and 40-ml samples were removed on days 0, 7, and 14 to measure the kappa number and brightness. The pulp was recovered by filtration on Whatman no. 1 filter paper in a Buchner funnel, washed with distilled water, and lyophilized.

Fractionation of labelled products. The contents of the CO_2 traps were washed into 20-ml scintillation vials with three 5-ml portions of EcoLite(+) scintillation fluid (ICN) and were counted with a Beckman model LS6800 liquid scintillation counter, after chemiluminescence was allowed to decay for 24 h in the dark. The $^{14}CO_2$ yields were corrected for the removal of radioactivity from the cultures by sampling. The pulp suspension samples were filtered on a stainless steel mesh with fines recycle. Subsamples (0.5 ml) of the filtrates were mixed with 5 ml of EcoLite(+) for counting. The pulp mats were thoroughly washed with water and then suspended at 2% consistency in 0.01 M NaOH and heated for 15 min at 120°C in an autoclave. After cooling, the pulp was again filtered on the stainless steel mesh with fines recycle, and 0.5 ml of the filtrate was mixed with 5 ml of EcoLite(+) for counting. The extracted pulp mat was thoroughly washed with deionized water and freeze-dried. The dried pulp samples were combusted in a model OX400 Biological Oxidizer (R. J. Harvey Instrument Corp.); the CO_2 produced was collected and counted in 15 ml of Carbon 14 Cocktail (R. J. Harvey Instrument Corp.)

The samples removed from the cultures containing SWKP*2 were fractionated as outlined in Fig. 1. The pulp suspensions were filtered on Whatman type GF/C filters with 0.65-μm pores. The radioactivity in the filtrate was measured by liquid scintillation counting of 5-ml samples mixed with 15 ml of Optiphase 3 (Wallac). One milliliter of the filtrate was used to measure laccase and MnP activities, and the remainder of the filtrate was freeze-dried. The pulp on the filter was washed with 100 ml of water, freeze-dried, and weighed. The pulp was extracted with 5 ml of 1.6% NaOH for 60 min at 60°C and then filtered with suction on a type GF/C filter. One milliliter of the alkali extract was mixed with 5 ml of OptiPhase 3 for counting, and the remainder was stored at 4°C. The extracted pulp was washed with 100 ml of water and then transferred to a 50-ml polypropylene centrifuge tube for cellulase digestion. The cellulase treatment was carried out as described above, except that the procedure was scaled down to a liquid volume of 40 ml. At the end of the second incubation with cellulase, the digest was acidified to pH 2 and centrifuged. The pellet was suspended in 5 ml of 0.1 M NaOH and incubated at 37°C for 1 h. After centrifugation, the supernatant was decanted and used as the cellulase lignin preparation. One milliliter of this solution was counted, and the remainder was saved for gel filtration analysis. Five-milliliter samples of the supernatants obtained from centrifugation of the acidified first- and second-stage cellulase digests were also counted. The pellet remaining after extraction of the cellulase lignin was washed with 10 ml of water and then transferred to a combustion boat lined with filter paper. The radioactivity in this residue was determined by burning the residue in a Harvey Biological Oxidizer and trapping and counting the $^{14}CO_2$ produced.

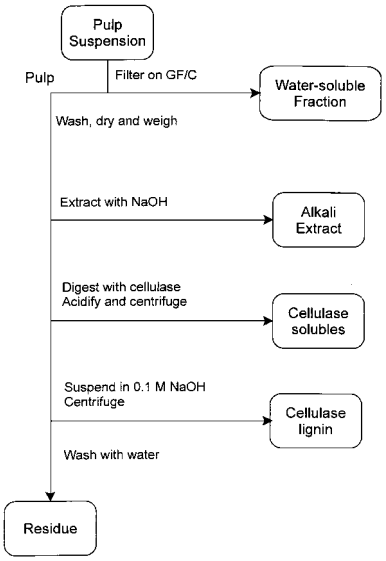

FIG. 1. Fractionation of samples removed from cultures containing SWKP*2.

Size exclusion chromatography. Samples (450 μl) of culture filtrates were mixed with 50 μl of 1 M NaOH; freeze-dried culture filtrates were redissolved in a minimum volume (0.5 to 1.5 ml) of 0.1 M NaOH; and alkali extracts and cellulase lignin solutions were used directly. All samples (500 μl) were mixed with 500 μl of ethanol in 1.5-ml microcentrifuge tubes and centrifuged for 2 min at full speed in a Beckman Microfuge 12 centrifuge. The molecular size distributions were analyzed by chromatography on Superose 12 (Pharmacia) as previously described (24). One-milliliter effluent fractions were collected in scintillation vials and counted after mixing with 5 ml of Optiphase 3. The radioactivity applied to the column was recovered quantitatively in the eluted fractions. The amount of radioactivity in each 1-ml fraction was converted to a percentage of the total radioactivity supplied to the culture by dividing by the amount of radioactivity applied to the column and multiplying by the percentage of the total radioactivity in the extract which the sample represented. Cytochrome *c* and veratryl alcohol were used as molecular size standards. The recorder tracings of the effluent A_{280} were digitized with the program Un-Scan-It (Silk Scientific) after scanning with a Hewlett-Packard ScanJet 4C scanner.

Analytical methods. Kappa numbers were determined by the micro kappa method (4). Pulp brightness (International Standards Organization) was measured on handsheets with a Gretag model SPM 50 spectral photometer at 457 nm. Laccase activity was measured with 2,2′-azinobis(3-ethylbenzthiazoline-5-sulfonate) (7), and MnP activity was measured by determining the rate of appearance of the Mn(III)-malonate complex (22). Klason lignin was measured by the method of Effland (10) with estimation of acid-soluble lignin from the A_{205} of the hydrolysis filtrates.

RESULTS

Lignin solubilization. When the labelled hardwood pulp HWKP*1 was incubated with *T. versicolor* for 5 days, a small fraction (1%) of the radioactivity was given off as $^{14}CO_2$ (Fig. 2). Fractionation of the pulp recovered from the fungal cultures showed that the radioactivity in the alkali extract in-

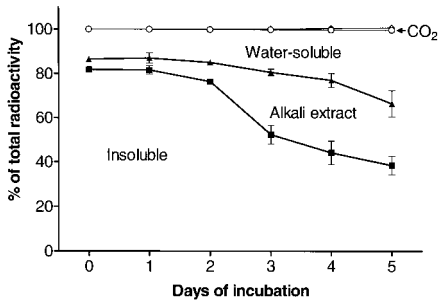

FIG. 2. Distribution of label from residual lignin during incubation of HWKP*1 with *T. versicolor* 52J. This is an area graph; the vertical distances between pairs of lines indicate the portion of the total radioactivity in each fraction. Data are the means of values from three replicates ± standard errors.

creased substantially during incubation, from 5% initially to 33% by day 4. The increase in extractable radioactivity was especially marked on the third day of incubation with the fungus. There was a more gradual increase in the radioactivity dissolved in the culture medium, from an initial value of 13.4% to 33.3% by day 5. The radioactivity that remained in the pulp after alkali extraction decreased from 82% before incubation to 38% after 5 days of fungal treatment.

When the labelled softwood pulp SWKP*1 was incubated with *T. versicolor* for 12 days, the release of $^{14}CO_2$ was very small (<0.5%) (Fig. 3). After a 2-day lag, the alkali-extractable ^{14}C increased from 7 to 42% of the total by day 8, at the expense of the insoluble fraction. On day 12, the alkali-extractable fraction was smaller than the alkali-extractable fraction at day 8. This decrease was consistent in all three replicates and was accompanied by an increase in the insoluble fraction of the radioactivity from 51% at day 8 to 67% at day 12. The radioactivity dissolved in the medium increased from 1.4% at day 0 to 8% at day 12.

Molecular size distribution of soluble materials and alkali extracts. The molecular sizes of the labelled materials solubilized and extracted from the pulps before and after fungal treatment were investigated by gel filtration. The water-soluble fraction obtained from incubation of the hardwood pulp HWKP*1 with the fungus produced a single broad peak that eluted before the molecular size standard veratryl alcohol (molecular mass, 168 Da) but after cytochrome *c* (12,384 Da) (Fig. 4). The apparent molecular mass for the apex of the radioactive peak was 1,550 Da, or 7.3 guaiacylglycerol units. The molecular size distribution of the soluble radioactive material

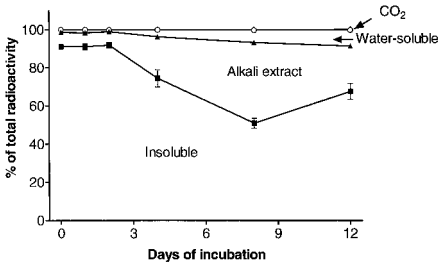

FIG. 3. Distribution of ^{14}C from residual lignin during incubation of SWKP*1 with *T. versicolor* 52J. The vertical distances between pairs of lines indicate the portion of the total radioactivity in each fraction. The amount of $^{14}CO_2$ produced was too small to produce a visible separation between the two top lines. Data are the means of values from three replicates ± standard errors.

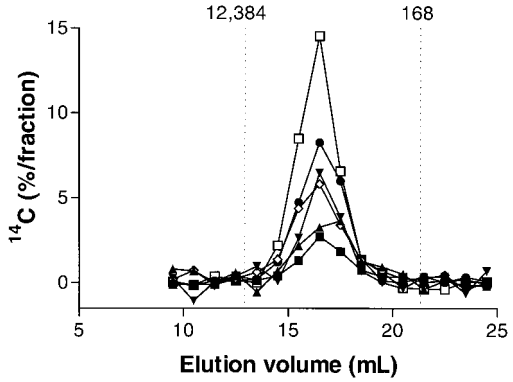

FIG. 4. Gel filtration of the labelled water-soluble fraction obtained from incubation of ^{14}C-lignin-labelled HWKP*1 with *T. versicolor* 52J. Symbols: □, day 5; ●, day 4; ◇, day 3; ▼, day 2; ▲, day 0; ■, control. Dashed lines show elution volumes of cytochrome *c* (12,384 Da) and veratryl alcohol (168 Da).

did not change as the amount of soluble material increased during the fungal incubation.

The radioactive material in the alkali extracts obtained from the hardwood pulp HWKP*1 treated with *T. versicolor* produced a gel filtration profile similar to that of the soluble material (Fig. 5). The molecular size distribution did not change with the length of incubation. The smaller amount of alkali-extractable material initially present in the pulp eluted from the column at the same volume as the material from the treated pulp.

The amount of water-soluble radioactive material obtained from the softwood pulp SWKP*1 was too small to determine its gel filtration profile. The alkali extracts obtained from the softwood pulps had molecular size distributions similar to those of the extracts obtained from the hardwood pulps, but there was slightly more radioactivity on the high-molecular-size flank of the main peak (eluting at 12 to 16 ml) (Fig. 6). In these pulps as well, the shape of the molecular size distribution profile did not change during the fungal incubation, even though the amount of the alkali-extractable radioactivity increased and then decreased.

Extraction of cellulase lignin. Polysaccharide hydrolysis with cellulase has frequently been used to isolate the residual lignin from kraft pulps (14–16, 36, 37). Typically, the cellulase solution is replaced one or more times, the solubilized carbohy-

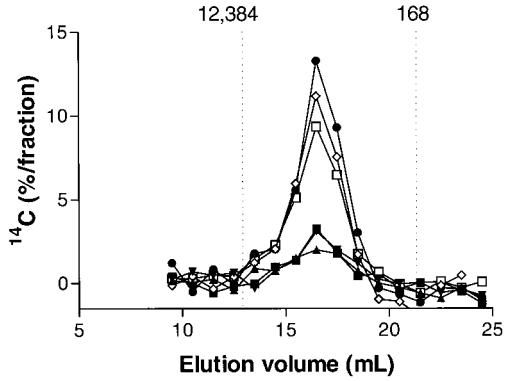

FIG. 5. Gel filtration of the labelled alkali extract obtained from incubation of ^{14}C-lignin-labelled HWKP*1 with *T. versicolor* 52J. Symbols: □, day 5; ●, day 4; ◇, day 3; ▼, day 2; ▲, day 0; ■, control. Dashed lines show elution volumes of cytochrome *c* (12,384 Da) and veratryl alcohol (168 Da).

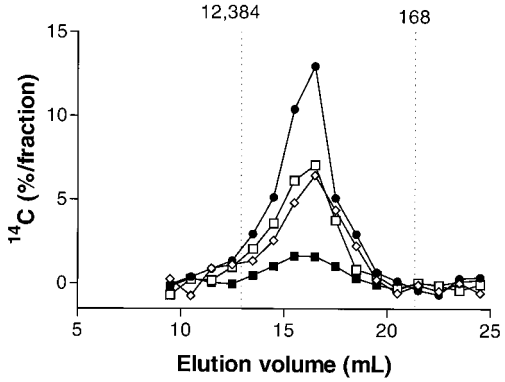

FIG. 6. Gel filtration of the labelled alkali extract obtained from incubation of ^{14}C-lignin-labelled SWKP*1 with *T. versicolor* 52J. Symbols: □, day 12; ●, day 8; ◇, day 4; ■, control. Dashed lines show elution volumes of cytochrome *c* (12,384 Da) and veratryl alcohol (168 Da).

drates are removed, and the insoluble lignin-enriched residue is treated with fresh enzyme. Sometimes the digest is acidified before the soluble and insoluble fractions are separated (37), and sometimes it is not (36). Hortling et al. (14) reported that 20 to 60% of the residual lignin in kraft pulps dissolved during the enzymatic hydrolysis. The residual lignin in the kraft pulps used in the present study also showed high solubility after cellulase hydrolysis; the enzyme digests at pH 4.8 had a marked yellow color, and 40 to 80% of the ^{14}C from labelled pulps stayed in the clear supernatant after centrifugation. Acidification of these digests precipitated a dark brown material and most of the ^{14}C from labelled pulps. This brown material readily dissolved in dilute alkali solutions. The pH dependence of the solubility of the cellulase-liberated residual lignin is illustrated in Fig. 7. The amount of soluble lignin, as determined by A_{280}, was large and roughly constant from pH 4 to 10 and appeared to increase slightly at pH 12. At pH 2, almost all of the lignin from the softwood pulp and most of the lignin from the hardwood pulp precipitated.

The standard procedure for cellulase digestion of pulps used in this study (see Materials and Methods) took this solubility of the lignin into account. The digests were acidified to pH 2 before replacement of the cellulase solution. At the end of the second digestion, the digests were made up to 0.1 M NaOH to maximize the extraction of lignin from the pulp. The extracted lignin was then precipitated by acidification to concentrate it

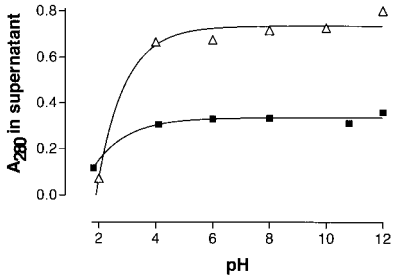

FIG. 7. Effect of pH on the solubility of lignin obtained from cellulase-digested kraft pulps. Samples of HWKP (■) and SWKP (△) were digested with cellulase at pH 4.8 and 40°C for 3 days, and then subsamples were adjusted to the pH values indicated and centrifuged. The supernatants were separated from the pellets and diluted 1:10 with 0.1 M NaOH before the absorbance was measured. The A_{280} contributed by the cellulase solution was negligible compared to the A_{280} contributed by the pulp.

TABLE 1. Recovery of Klason lignin in cellulase lignin preparations from kraft pulps

Prepn	Yield (%)	Klason lignin (% of pulp)		
		Acid insoluble	Acid soluble	Total
HWKP		1.59 ± 0.03[a]	0.99 ± 0.16	2.58 ± 0.16
Cellulase lignin from HWKP	2.9 ± 0.1	1.86 ± 0.02	0.72 ± 0.00	2.58 ± 0.02
SWKP		3.25 ± 0.06	0.85 ± 0.05	4.15 ± 0.08
Cellulase lignin from SWKP	5.8 ± 0.9	3.25 ± 0.06	0.40 ± 0.00	3.67 ± 0.06

[a] Data are means of values from three replicates ± standard errors.

and separate it from carbohydrates and other acid-soluble materials in the digest. After cellulase digestion and alkaline extraction of the lignin, a small amount of dark brown insoluble material always remained. Further treatment with cellulase did not dissolve this residue. The recovery of lignin from the pulp by the cellulase digestion-alkaline extraction procedure was determined by Klason lignin analysis (Table 1). Substantial amounts of the lignins were in the acid-soluble fraction, as estimated from the A_{205} values of the Klason filtrates. The apparent recovery of lignin from the hardwood pulp was 100%, and the apparent recovery from the softwood pulp was 88%. The Klason lignin contents of the material extracted by alkali from the digested hardwood pulp and of the extract from the softwood pulp were 89 and 63%, respectively.

Distribution of ^{14}C from lignin-labelled SWKP. Another batch of labelled SWKP (SWKP*2) was prepared and incubated with *T. versicolor* for 14 days. After alkali extraction, the pulp was digested with cellulase, and the cellulase lignin was recovered. The delignification of this pulp was much more extensive than the delignification observed in the previous experiment; 22% of the label was mineralized to $^{14}CO_2$ and 61% was solubilized (Fig. 8A). The fraction of the label removed from the pulp by mineralization and solubilization agreed well with the decrease in kappa number of unlabelled kraft pulp in the cultures (Fig. 8B). The pulp brightness increased to 53 from an initial value of 32. The alkali-extractable radioactivity increased from 7% of the total initially to a maximum of 37% on day 6 and then decreased to 12% by day 14 (Fig. 8A). An unusually large portion (44%) of the radioactivity in pulp SWKP*2 remained in the insoluble residue after cellulase digestion and extraction of the cellulase lignin. Although this material was apparently inaccessible to cellulase, it was readily degraded by *T. versicolor* and almost disappeared by day 10. The cellulase lignin, which was soluble in alkali after cellulase digestion of the pulp, initially contained 42% of the total radioactivity, but this fraction decreased during the fungal incubation and was 2% at day 14. Some radioactivity (range, 2 to 13%) was solubilized during the cellulase digestion and was not precipitated by acidification. The amount of this material reached its maximum value after 4 days of incubation and then decreased as the radioactivity in the extracted pulp entering the cellulase digestion step decreased. Overall, as the fungal treatment progressed, label moved from the cellulase lignin and cellulase residue fractions into the alkali-extractable fraction and then into the soluble and CO_2 fractions (Fig. 8A).

Laccase and MnP activities were measured in the filtrates obtained from the pulp samples (Fig. 9). Both of these enzymes appeared early in the incubation period, and their maximum activities were reached by day 2. Laccase activity de-

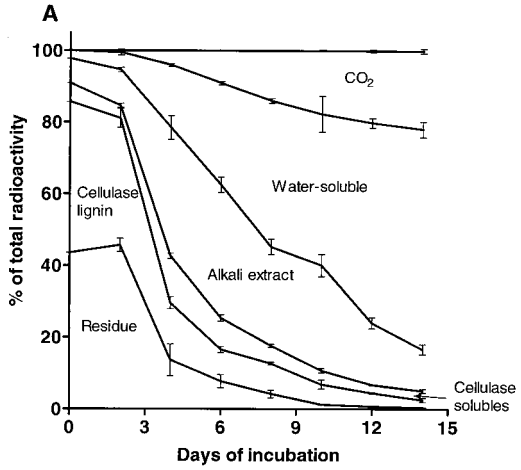

FIG. 8. Effect of *T. versicolor* 52J on the distribution of ^{14}C from lignin-labelled SWKP*2 and on the kappa number of the pulp. (A) The vertical distance between pairs of lines indicates the portion of the total radioactivity in each fraction. (B) Comparison between the fraction of ^{14}C remaining in the pulp and kappa number. Data are the means of values from three replicates ± standard errors.

clined thereafter and was negligible by day 6. MnP persisted longer but almost disappeared by day 10.

Molecular size distribution of soluble, extractable, and cellulase lignin fractions (i) Cellulase lignin. The cellulase lignin obtained from the untreated pulp SWKP*2 contained some

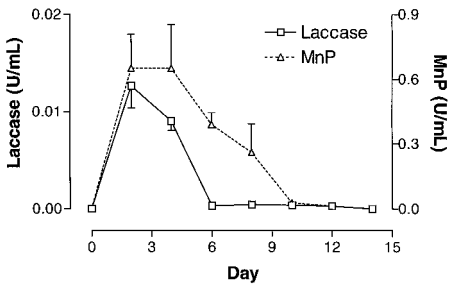

FIG. 9. Laccase and MnP activities in cultures of *T. versicolor* 52J delignifying SWKP*2. Data are means of values from three replicates; the bars indicate standard errors.

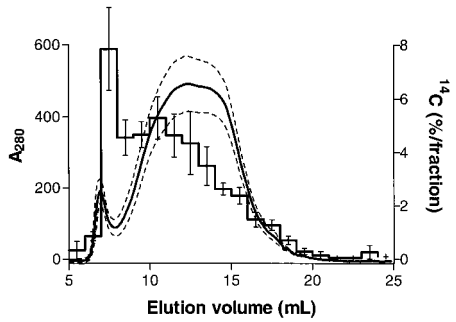

FIG. 10. Gel filtration profile of cellulase lignin extracted from SWKP*2 before incubation with the fungus. The smooth solid curve shows the A_{280} profile, and the stepped curve shows the ^{14}C profile. All of the data are means of values from three replicates ± standard errors; the dashed lines indicate the standard errors for A_{280}.

large-molecular-size material excluded from the gel filtration column (Fig. 10, peak at an elution volume of 7 ml); most of the 280-nm-absorbing material eluted in a broad peak at 8 to 19 ml. The radioactivity tended to elute earlier than the UV-absorbing material, suggesting that the molecular sizes of the labelled materials were larger than the molecular sizes of the bulk of the lignin.

After 2 days of incubation with the fungus, the amount of UV-absorbing material eluting between 10 and 19 ml had decreased, but the height of the excluded peak (6.5 to 8 ml) had increased slightly (Fig. 11). The ^{14}C-labelled materials also showed a small increase in the height of the excluded peak and a decrease in the amount eluting between 8 and 20 ml from day 0 to day 2. Between day 2 and day 12, the heights of the A_{280} curves decreased steadily; the absorbance in the larger-molecular-size portion of the chromatogram (6.5 to 13 ml) tended to decrease faster than the absorbance in the smaller-molecular-size region (13 to 19 ml). At day 14, the peak shifted toward earlier elution volumes (Fig. 11). The radioactivities in all fractions of the chromatogram decreased at about the same rate from day 2 to day 6. After that the amount of ^{14}C in the larger-molecular-size fractions (eluting at 6 to 13 ml) decreased faster than the amount in the smaller-molecular-size fractions.

(ii) Alkali extracts. The alkali extract obtained from pulp before incubation with the fungus produced a low broad A_{280} peak during gel filtration; the radioactivity in the extract was too low to measure its distribution after chromatography (Fig. 12). After 2 days of incubation with the fungus, the gel chromatogram had an asymmetrical A_{280} peak, with its maximum at an elution volume of 17 ml. This peak was higher and more symmetrical after 4 days of incubation and reached its maximum size on day 6. The ^{14}C in the extracts eluted from the column in a peak having the same shape and location as the A_{280} peak. The relative height of the radioactivity peak at day 2 was less than the relative height of the A_{280} peak, and the radioactivity showed a bigger increase than the UV absorbance between day 2 and day 4. The heights of both the A_{280} and ^{14}C peaks decreased slightly from day 6 to day 10 and more substantially between day 10 and day 12. The height of the radioactivity peak continued to decrease between days 12 and 14, but the A_{280} peak did not.

(iii) Water-soluble materials. Gel filtration of the dissolved materials from the pulp suspension after 2 days of incubation with the fungus partially resolved two peaks of UV absorbance, one centered at 17 ml and a higher one centered at 20 ml (Fig. 13). The soluble radioactivity eluted in a discrete peak cen-

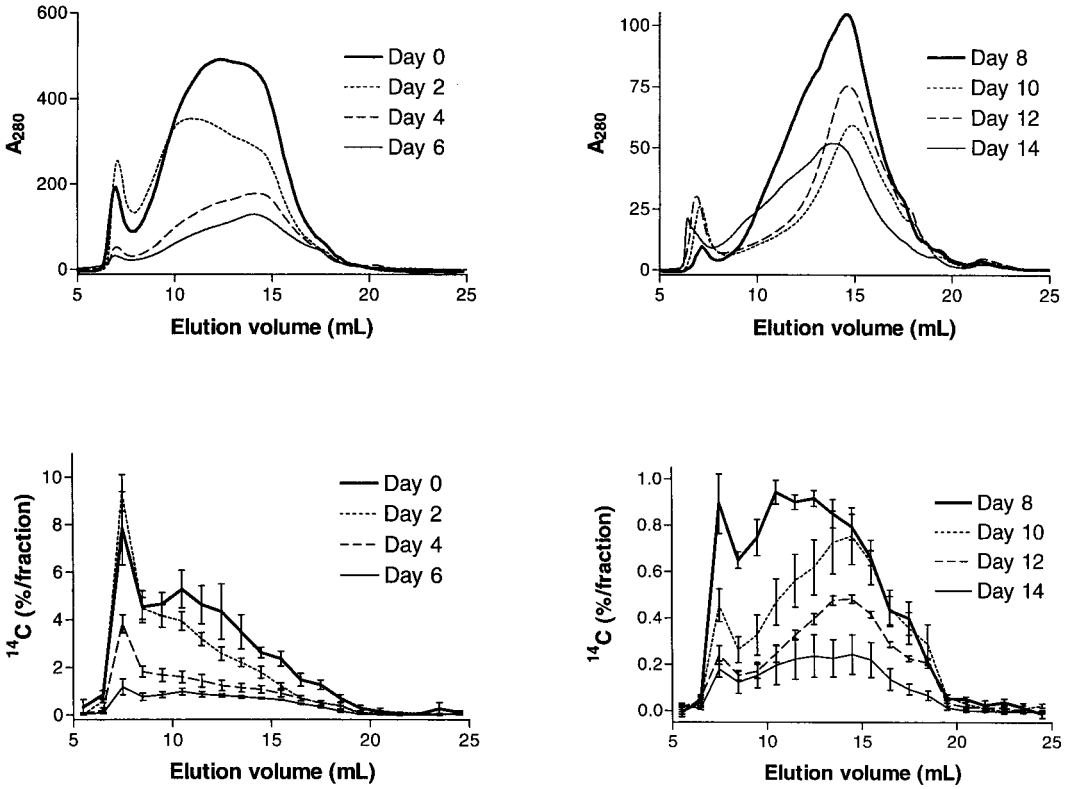

FIG. 11. Gel filtration profiles of cellulase lignin prepared from SWKP*2 after 0 to 14 days of incubation with *T. versicolor*. Data are the means of values from three replicates; the bars indicate standard errors. Note that the panels on the right have expanded scales.

tered at 17.5 ml. After 4 days of incubation with the fungus, the height of the A_{280} peak at 17 ml had tripled, but the height of the later peak had only increased by 20% and had shifted toward slightly earlier elution; a shoulder at 21 ml had disappeared. During further incubation with the fungus, the heights of the A_{280} and ^{14}C peaks centered at 17 ml continued to increase with no shift in elution volume.

DISCUSSION

The experiments reported here were carried out over several years with labelled pulps prepared at different times and in slightly different ways. The delignifying activities of the fungal cultures also varied from experiment to experiment. The results obtained for mineralization of ^{14}C show this variability clearly. Incubation of HWKP*1 with *T. versicolor* for 5 days yielded only 1% $^{14}CO_2$, whereas a similar experiment performed with an earlier preparation of labelled HWKP yielded 10% $^{14}CO_2$ (27). Treatment of SWKP*1 for 14 days released less than 0.5% of the label as $^{14}CO_2$, but incubation of SWKP*2 with the fungus for the same period released 22% $^{14}CO_2$. The experiment performed with SWKP*2 also revealed more solubilization of the label than the experiment performed with SWKP*1 did. It appears that the delignifying activity of the *T. versicolor* culture used in the experiment with HWKP*1 and SWKP*1 was less vigorous than normal; visual observation of the pulp brightness at the end of the incubation period supported this conclusion. In contrast, the culture used to treat SWKP*2 was more vigorous than usual; the pulp brightness reached 53, and the final kappa number, 7, was at the low end of the range observed in previous work (29). This variability in the activity of the fungus, as well as the variability in the properties of the labelled pulps, must be taken into account when the significance of the results is assessed. Only effects that were seen consistently can be relied upon.

One consistent result of all of the experiments was solubilization of the labelled lignin and accumulation of soluble labelled products in the culture medium. Soluble materials that absorbed light at 280 nm also accumulated in the cultures (Fig. 13). Gel filtration indicated that these soluble products had larger molecular sizes than single-ring aromatic compounds like veratryl alcohol. The peak elution volumes of the soluble products obtained from HWKP*1 and SWKP*2 both indicated that oligomers containing about seven phenylpropane units were present. Because the gel filtration column was calibrated with peptides and not lignin molecules and because the structures of the soluble products are unknown, molecular size estimates are very uncertain. It is clear, nonetheless, that single-ring lignin degradation products did not accumulate in the delignifying cultures.

Incubation with *T. versicolor* also consistently increased the amounts of ^{14}C and UV-absorbing material that could be extracted from the pulps with alkali. Fungal treatment has been shown to increase the effectiveness of alkali extraction to lower pulp kappa numbers (30). The alkali-extractable lignin seemed to be an intermediate between the initial insoluble form and the ultimate soluble products, since the amount of extractable label increased early in the fungal treatment and later decreased as the soluble label accumulated. The apparent reconversion of alkali-extractable lignin to the insoluble form between days 8 and 12 of treatment of SWKP*1 (Fig. 2) is anomalous, and I can offer no explanation for it. On the gel filtration column, the ^{14}C and A_{280} peaks from the alkali ex-

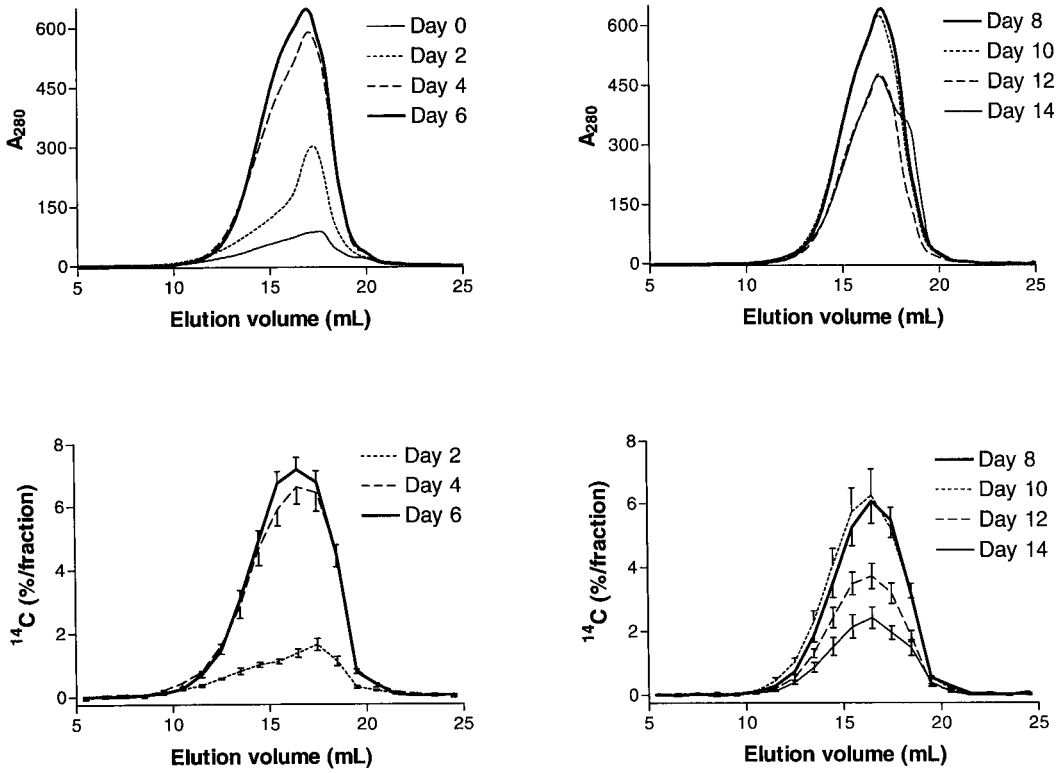

FIG. 12. Gel filtration profiles of alkali extracts from SWKP*2 after 0 to 14 days of incubation with *T. versicolor*. Data shown are the means of values from three replicates; the bars indicate standard errors.

tracts eluted slightly earlier than the peaks from the water-soluble materials, although the elution patterns for the two fractions overlapped greatly (Fig. 14). This suggests that the alkali extracts included some larger molecules than the water-soluble fractions did. The apparent molecular size distribution of the alkali extracts did not change much from day 4 to day 14 of treatment of SWKP*2, from day 4 to day 12 of treatment of SWKP*1, or from day 2 to day 5 of treatment of HWKP*1.

Cellulase digestion makes much of the residual lignin in kraft pulps extractable and hence available for characterization. This technique was used only with pulp SWKP*2. With this pulp, the ^{14}C and A_{280} gel filtration profiles for the cellulase lignin extract differed; a larger portion of the ^{14}C than of the A_{280} eluted in the large-molecular-size excluded peak. Almost all of the A_{280} came from the unlabelled pilot plant pulp in the mixture, whereas the ^{14}C came from the laboratory pulp. Because the labelled wood was milled to a powder before pulping, the pulping reagents should have penetrated the wood particles faster than they penetrated the chips used in the pilot plant digestion. To compensate for this effect, the cooking time for the pulping of the radioactive wood powder was shorter than the regular cooking time for chips. However, the amount of compensation needed was not known exactly, and SWKP*2 could have been under- or overcooked. The labelled pulp was too scarce and too valuable to measure its kappa number. In this case the A_{280} behavior is probably more representative of normal residual lignin.

Residual lignins isolated by cellulase digestion of kraft pulps are contaminated with proteins (15). The protein contamination can be decreased by reprecipitating the lignins from solutions in dimethylacetamide and in alkali (15). Such lignin purification steps were not used in this study because they might have lowered the yield of cellulase lignin, especially the smaller-molecular-size portion. Contaminating proteins may have made minor contributions to the yields of cellulase lignin reported in Table 1 and to the A_{280} profiles in Fig. 10 and 11. Because the specific absorptivity at 280 nm of proteins is only about one-tenth the specific absorptivity at 280 nm of lignin, the effect of protein contamination on the A_{280} results should have been negligible.

The cellulase lignin apparently underwent net polymerization during the first 2 days of fungal treatment. The amounts of both ^{14}C and A_{280} in the large-molecular-size excluded peak increased, and the amounts eluting later decreased (Fig. 11). Polymerization in the early stages of degradation of dehydrogenative polymerizate by white rot fungi has been observed previously (9, 25) and attributed to oxidative coupling catalyzed by phenol-oxidizing enzymes (25). Methanol is released from demethoxylation of phenolic rings in the residual lignin within the first few days of pulp treatment by *T. versicolor*, catalyzed by MnP (22). The polymerization and demethoxylation of the lignin probably both result from oxidation of the phenolic rings in the lignin by MnP and laccase. After the second day of treatment, both the large-molecular-size and the smaller-molecular-size parts of the cellulase lignin gradually disappeared. The large-molecular-size fraction of the cellulase lignin disappeared at least as fast as the smaller molecules, and between days 6 and 12, the large-molecular-size peak disappeared faster than the smaller-molecular-size material. Both the ^{14}C and the A_{280} measures of lignin followed this pattern.

The dynamics of the various pools measured in this study suggest that there is a sequential transformation from the cellulase lignin and insoluble residue form to the alkali-extractable form to the soluble form and finally to CO_2. Thus, the

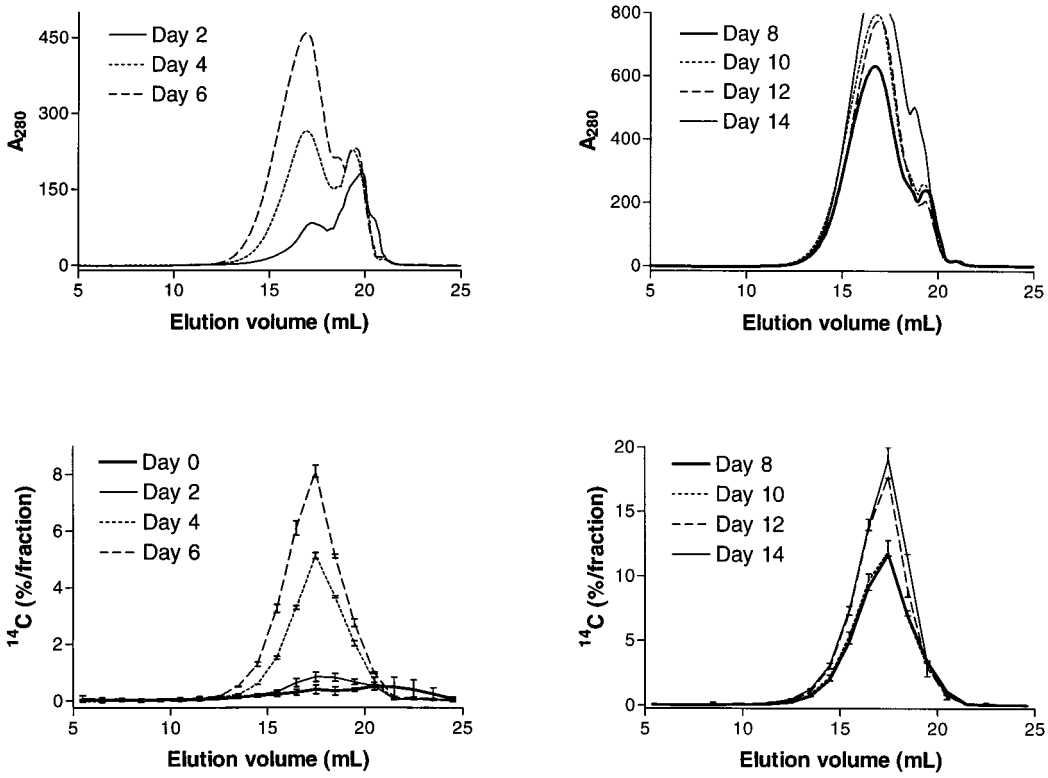

FIG. 13. Gel filtration profiles of soluble materials in SWKP*2 suspensions after 0 to 14 days of incubation with *T. versicolor*. Data shown are the means of values from three replicates; the bars indicate standard errors. Note that the panels on the left have expanded scales.

amounts of the cellulase lignin and insoluble residue, initially the most abundant forms, decreased throughout the treatment. The nearly complete disappearance of these fractions indicates that almost all of the lignin in the pulp was accessible to the fungus. The alkali-extractable fraction first increased and later decreased, behavior typical of an intermediate pool. The abundance of the soluble fraction increased throughout the treatment, and this fraction contained most of the label at the end of the experiment. Carbon dioxide is clearly the ultimate product of oxidative biodegradation; it is not clear whether all of the lignin-derived carbon would have been mineralized to CO_2 if the incubation with the fungus had been long enough. Possibly some molecules in the soluble pool are recalcitrant to further metabolism. The gel filtration results suggest that the transition from the cellulase lignin form to the alkali-extractable form involves considerable fragmentation of the lignin molecules. Smaller decreases in average molecular size accompany the transition from the alkali-extractable form to the soluble form.

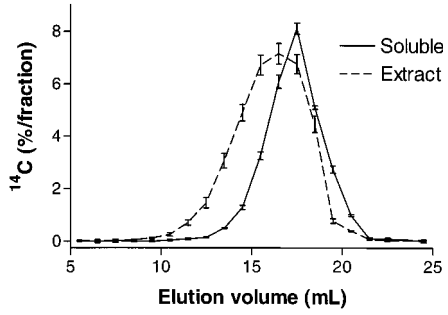

FIG. 14. Comparison between gel filtration profiles of labelled materials in the water-soluble fraction and the alkali extract from SWKP*2 treated for 6 days with *T. versicolor*. Data are means of values from three replicates; the bars indicate standard errors.

Lignin degradation products in the size range of the single-ring aromatic molecules from which lignin is polymerized were noticeably absent from both the alkali-extractable and soluble fractions. Possibly such fragments were rapidly taken up and metabolized by the fungus; if that is true, the 22% yield of $^{14}CO_2$ is an upper limit for the production of small fragments from lignin.

The soluble lignin degradation products had apparent molecular sizes in the size range of phenylpropane oligomers. The molecular size distribution did not change with time of incubation; there was no evidence of net polymerization or depolymerization. If these soluble products contained phenolic hydroxyls, oxidative polymerization, catalyzed by the laccase and MnP present in the cultures during the first two-thirds of the incubation period, would be expected. The resistance of the soluble products to polymerization suggests that they did not contain free phenols. Whether the phenolic rings were removed by reactions like alkyl-phenyl cleavage or destroyed by ring opening or the phenolic groups were tied up by intramolecular ether formation, by glycosylation (19), or by other derivatization reactions could not be determined from the information available. A soluble lignin derivative produced from bagasse by *Lentinus edodes* was characterized previously; this material has few phenolic hydroxyls but many carboxyls and is highly condensed (32).

Various explanations for the resistance of residual lignin to removal from kraft pulps have been offered (14–16, 36). If the polysaccharides are removed by enzymatic hydrolysis, the re-

sidual lignin becomes soluble in dilute alkali, showing that it is not inherently insoluble. Either chemical attachment of the lignin to polysaccharides or physical entrapment of the lignin molecules in the polysaccharide matrix is possible. Treatment of kraft pulps with xylanase or leaching at a high pH makes removal of a part of the residual lignin easier; removal of hemicelluloses which physically obstruct the diffusion of lignin molecules out of the fiber walls is implicated in both cases (20). In the case of pulp delignification by *T. versicolor*, the observed depolymerization of the lignin should facilitate its diffusion out of the polysaccharide network of the fiber walls and could also liberate lignin fragments from lignin-carbohydrate complexes. The enzymes responsible for lignin depolymerization and solubilization are not known with certainty. The combination of laccase with the synthetic mediator 2,2′-azinobis(3-ethylbenzthiozoline-5-sulfonate) has been shown to solubilize ^{14}C from lignin-labelled HWKP (5). Laccase and MnP were detected in the cultures during the early stages of delignification, but lignin solubilization continued after these enzyme activities had disappeared (Fig. 9). MnP, or laccase with a mediator, can delignify kraft pulps, but delignification to the extent achieved by living *T. versicolor* cultures requires repeated treatments interspersed by alkaline extractions, which seem to reactivate the lignin for enzyme attack (6, 18). These differences between the effects of known enzymes and the effects of whole cultures of *T. versicolor* provide circumstantial evidence that additional, unknown enzymes participate in pulp delignification. Chemical characterization of the soluble and alkali-extractable products of pulp delignification should allow more definitive identification of the delignifying enzymes.

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