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Depolymerization of Water Soluble Coal Polymer from Subbituminous Coal and Lignite by Lignin Peroxidase

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ABSTRACT

Coal polymers, water soluble at pH 3.5, were prepared from North Dakota lignite and German subbituminous coal in 35-61% yield. Gel permeation chromatography showed a major component of relatively narrow molecular weight range >75,000. The material did not dialyze through a 12,000-14,000 MW cutoff membrane under several conditions. Minor amounts of smaller fragments were present, but monomeric components were not detected. Incubation of soluble polymer with lignin peroxidase of *Phanerochaete chrysosporium* caused substantial disappearance of the high molecular weight polymer and formation of smaller amounts of both higher and lower molecular weight components, but not of monomeric compounds. Addition of veratryl alcohol enhanced depolymerization. Coal polymer competitively inhibited veratryl alcohol oxidation by lignin peroxidase.

Index Entries: Soluble coal polymer; lignin peroxidase; depolymerization; North Dakota lignite; German subbituminous coal.

INTRODUCTION

Solubilization of low-ranked coals and their partially oxidized derivatives by growing fungi and by fungal extracellular preparations has been reported by several investigators (*1–8*). Cohen and Gabrielle (*1*) found that growth of *Polyporus versicolor* (*Coriolis versicolor*) and *Poria monticola* on agar plus finely powdered lignite formed droplets of a water-soluble, dark

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brown or black liquid. Scott et al. (*2*), screened a number of organisms for growth on several coals and observed similar solubilizations in most tests. Solubilization increased with degree of oxidation of the coal, and the water soluble product contained fragments with molecular weights between 5×10^4 and 2×10^6 daltons. Wilson et al. (*6*) characterized the liquid material produced from oxidized lignite by *P. versicolor* as acid insoluble, alkali soluble, and having a molecular weight between up to 5×10^4 daltons with predominant components $> 1 \times 10^4$ daltons.

An extracellular fraction from *Coriolus versicolor* solubilized leonardite (an oxidized lignite) in vitro (*7*). The solubilizing activity was not associated with an effect of chelators or production of surface-active agents, but was considered to result from the activity of syringaldehyde oxidase (*8*). Laccase activity has also been implicated in the biosolubilization of coal (*8*).

These results indicate that fungi are capable of secreting extracellular enzymes that can modify the coal structure so as to render some of the material water soluble. However, it is difficult to determine from these studies whether depolymerization, defined as C–C bond breaking in the backbone of the polymer, also occurred.

The derivation of coal from ancient lignocellulosic materials and the resemblance of certain aspects of coal structure with that of lignin, suggests that some of the linkages in coal may be susceptible to attack by lignin peroxidase and by other, as yet undefined, lignin-degrading enzymes. If this process occurs to a significant extent, depolymerization to yield smaller fragments and even monomeric units would be anticipated.

Lignin peroxidase is unique among enzymes in its almost complete lack of specificity (*9–15*) and in the large number of seemingly different reaction types it catalyzes (*9–19*). However, its application to coal processing has not been considered because of the solid nature of coal, and the general requirement of enzymes for substrates in solution in order to obtain high reaction velocities.

To test the hypothesis that coal has numerous bonds susceptible to cleavage by lignin peroxidase, a water soluble coal polymer has been prepared from both lignite and subbituminous coal. Solubilization has been accomplished chemically, but the result superficially resembles the solubilization performed by a fungus growing on powdered coal. That is, the solubilization process is oxidative, as appears to be the case for fungal solubilization. In this report, data are presented on the nature of the water soluble coal polymer and on the ability of a lignin peroxidase preparation to perform the next step, depolymerization.

METHODS

Organism and Culture Conditions

The white rot fungus, *Phanerochaete chrysosporium* Burds ME-446 (ATCC 34541), was grown in the nitrogen-limited BIII medium buffered with 10

m*M* 2,2-dimethylsuccinic acid, pH 4.25, essentially as described by Kirk et al. (*20*). The culture sizes and corresponding Erlenmeyer flask volumes were: 10 mL in 125 mL; 300 mL in 1 L; 500 mL in 2 L. All cultures were grown at 39°C on a rotary shaker (200 rpm) and were sparged with 100% oxygen once daily. Inocula were grown in stationary 1-L flasks containing 50 mL of the medium described above. After 3–7 d, the cell mass was collected and homogenized in a Potter-Elvehjem homogenizer until a very fine homogeneous suspension was obtained. The suspension was diluted 1:10 in medium.

Assay of Peroxidase

Lignin peroxidase activity was determined spectrophotometrically at 310 nm as the H_2O_2-dependent oxidation of veratryl alcohol to veratraldehyde as reported by Tien and Kirk (*9,11*) with minor modifications. One unit of peroxidase oxidizes 1 μmol of veratryl alcohol/min at 20°C.

Manganese peroxidase was assayed spectrophotometrically at 258 nm as the rate of production of manganic pyrophosphate ($\epsilon_M = 6200$) (*21*) as reported by Glenn and Gold (*22*).

Preparation of Lignin Peroxidase

The following procedure was developed in order to avoid the ultrafiltration of culture medium to concentrate the extracellular peroxidase (*9–11*). Mycelium-free broth (500 mL) from 5-d cultures was filtered through glass wool and diluted 1:1 with equilibration buffer (10 m*M* potassium phosphate buffer, pH 7.0). A 1.0×50 cm column of the Macrosorb Kax.DEAE (6% crosslinked) (Sterling Organics US, NY) was equilibrated with the same buffer and the diluted broth pumped through the column at 3.0 mL/min. The column was washed with two bed volumes of equilibration buffer followed by a gradient of 500 mL of 0 to 0.2 *M* NaCl-10 m*M* potassium phosphate buffer. One bed volume of 0.2 *M* NaCl-10 m*M* potassium phosphate buffer then eluted the activity in 55 mL. This procedure concentrated the activity 10-fold, recovered 88% of the activity, increased the specific activity twofold, and removed the slime that pervades culture supernatants. The fraction was then concentrated an additional 10-fold by pressure filtration on an Amicon YM-10 membrane (American Corporation, Danvers, MA) with 10,000 dalton pore size. Manganese peroxidase activity (0.11 U/mL) of the partially purified preparation was present at 1% of the lignin peroxidase activity (12 U/mL).

Gel Permeation Chromatography

Molecular weight distribution was determined by conventional gel permeation chromatography on columns of Sephadex G-75 or G-200 (15×230 cm) equilibrated with 0.02 *M* KPO_4 buffer, pH 7, containing 0.5% Tween 80. 0.4 mL of coal Fraction IIIb (North Dakota lignite) diluted 1:1 with 0.04 *M* KPO_4 buffer – 1% Tween 80, pH 7 was run over the column

at 5.2 mL/h. Absorbance of the effluent at 254 nm was recorded. Excluded (V_o) and internal (V_i) volumes were determined with Blue Dextran (MW $>2\times10^6$ daltons) and $K_3Fe(CN)_6$, (MW = 422 daltons), respectively.

Gel permeation high performance liquid chromatography (HPLC) was also used to determine molecular weight distribution of water soluble coal preparations before and after incubation with lignin peroxidase. 100 μL of 1:10 dilution of coal fraction (usually 25–30 μg of Fraction IIIb; *see* Results—Preparation of Water Soluble Coal Polymer), was injected onto a SynChropak GPC 300 column (Synchrom Inc., Lafayette, IN) and chromatographed with equilibration buffer (0.2 *M* phosphate – 0.5% Tween 80, pH 7) at a rate of 0.24 mL/min. Absorbance of the effluent was recorded at 254 nm. The SynChropak column separates components over a range of 10^4–10^6 daltons. Thyroglobulin (MW = 2×10^6 daltons) and veratryl alcohol (MW = 168 daltons) were used as V_o and V_i markers.

Determinations

The carbon content of fractions was determined by the dichromate-sulfuric acid method by Johnson (*23*). The values are expressed as percent carbon relative to the carbon content of the nitric acid-treated coal. Ash content values of 11% for North Dakota lignite (*2*) and of 21.9% for German subbituminous coal (*24*) have been assumed in these analyses.

RESULTS

Preparation of Water Soluble Coal Polymer

In order to provide the best conditions for rapid enzymatic depolymerization of coal, a soluble form of coal polymer is required. For this purpose, two similar procedures have been developed which yield appreciable amounts of polymeric coal substrate that is soluble in water at pH 3.5 where lignin peroxidase is optimally active.

Treatment of low-ranked coals with limited amounts of nitric acid at low temperatures has long been known to oxidize some of the methylene groups to carboxylic groups, thereby, rendering a portion of the coal soluble in water and more so in alkali (*25–28*). In addition, at low temperature there is limited aromatic ring nitration and decarboxylation.

In procedure 1, ground nitric acid-treated North Dakota lignite was suspended in 1 *N* NaOH as described below. In procedure 2 (below), used with both lignite and subbituminous coal, less NaOH was added to the water slurry of coal to give a pH of 10–11. Neither procedure has been optimized to give the highest yields of soluble coal polymer.

North Dakota Lignite

North Dakota lignite was ground, sieved (40 mesh), suspended in an equal weight of water, and concentrated nitric acid added to 20–40% con-

centration. The suspension was allowed to stand for 2–6 h while maintaining the temperature below 70°C. The material was then filtered, washed, and dried (*27,28*). This treatment was performed by JGS Research, Inc., Pittsburgh, PA.

One g of nitric acid-oxidized coal was suspended in 100 mL of 1 *N* NaOH (procedure 1) and stirred for 30 min. The suspension was then centrifuged at 4°C for 15 min at 10,000×G; the black insoluble material was discarded. The supernatant solution (Fraction I) containing about 50% of the original carbon was neutralized with 6 *N* HCl to pH 3.5 and centrifuged as above; the clear yellow supernatant (Fraction II) was discarded. The brown-black pellet, insoluble in the 1 *N* NaCl produced by neutralization, was suspended in 50 mL of water, allowed to stand for 20 min, and then centrifuged as above; the supernatant, Fraction IIIa, was discarded. The brown precipitate was again suspended in 50 mL of water, centrifuged after 20 min, and the supernatant solution, containing coal-derived material soluble at low ionic strength, was saved (Fraction IIIb). This fraction was used in gel filtration chromatographic characterization of the soluble polymer. Fraction IIIb was dialyzed against 50 m*M* EDTA in 20 m*M* sodium tartrate, pH 3.7, and against 20 m*M* sodium tartrate, pH 3.7, or deionized water. The dialysis membrane was BRL Prepared Dialysis Tubing (Bethesda Research Laboratories, Gaithersburg, MD), which has a molecular weight exclusion of 12,000–14,000 daltons. Dialyzed Fraction IIIB was used in all experiments with lignin peroxidase.

German Subbituminous Coal

A 50 g sample of ground German subbitminous coal from Ruhrkohle Oel and Gas GmbH, Bottrop, FRG, was suspended in water and treated with nitric acid (20% final concentration) at 50–70°C for 1 h by JGS Research Company, Inc. The insoluble material was washed and dried. The yield of water-insoluble, base-soluble material was 51.8 g with the consumption of 23.3 g of nitric acid. One carboxyl group was introduced per 352 g of base-soluble, acetone-insoluble material. The dried material was later processed to yield soluble polymer, essentially, as described above for North Dakota lignite, except for modifications designed to use less alkali.

One g of nitric acid-oxidized subbituminous coal was suspended in 100 mL of water, and NaOH was added to pH 10–11 (procedure 2). After centrifugation and recovery of the supernatant (Fraction I), as reported above, the pH of the supernatant was adjusted to 3.5 with HCl. Following centrifugation, polymeric material was salted out by adding NaCl to the supernatant to 1 *M* concentration, and the suspension centrifuged. The pellet was suspended in 100 mL of H_2O, and left at room temperature for 12 h. The mixture was then centrifuged, and the supernatant taken as the final pH 3.5 water soluble coal fraction (Fraction IIIb). This material was dialyzed against water before use.

Table 1
Carbon Content of Coal Fractions[a]

Coal fraction	Relative carbon content, percent		
	North Dakota lignite		German subbituminous coal
	Procedure 1	Procedure 2	Procedure 1
1% Powdered coal suspension	100	100	100
Alkaline extract, Fraction I	50	69	34
Soluble precipitate, pH 3.5 Fraction IIIb	35	61	28

[a]Aliquots of solutions or suspensions in 0.4 mL of H_2O were boiled for 20 min with 1 mL of dichromate reagent (2.5 g $Na_2Cr_2O_7 \cdot 2H_2O$ plus 10 mL H_2O plus 95% H_2SO_4 to 500 mL) (23). After cooling, 10 mL of water was added, and the absorbance of dichromate remaining was read at 370 nm; alternatively, solid sodium sulfite was added and the absorbance of chromous ion was read at 620 nm. Results are expressed as percent of color of an equivalent amount of the original coal suspension.

Characteristics of Soluble Coal Polymer

Table 1 summarizes the carbon content of fractions derived from North Dakota lignite by both procedures. Based on analysis for organic carbon, both German subbituminous coal and North Dakota lignite yielded a major fraction of the carbon as pH 3.5 water soluble material.

The ultraviolet absorption spectrum of polymer from North Dakota lignite showed no major features, only a smooth increase in absorbance to a maximum in the 200 nm region.

Fraction IIIb from North Dakota lignite was

1. Soluble in water, dimethyl formamide, dimethyl sulfoxide, and tetrahydrofuran;
2. Sparingly soluble in methanol; and
3. Insoluble in ethyl acetate, acetonitrile, isopropanol, hexane, and toluene.

Molecular Weight Measurements by Gel Permeation Chromatography

Coal polymer chromatographed normally in 0.02 *M* KPO_4–0.5% Tween 80, pH 7, with highly polar supports such as Sephadex. In contrast, the polymer adsorbed strongly to vinyl polymer (Fractogel) supports that are nonpolar in character; with Fractogel columns, elution was achieved with dimethylformamide.

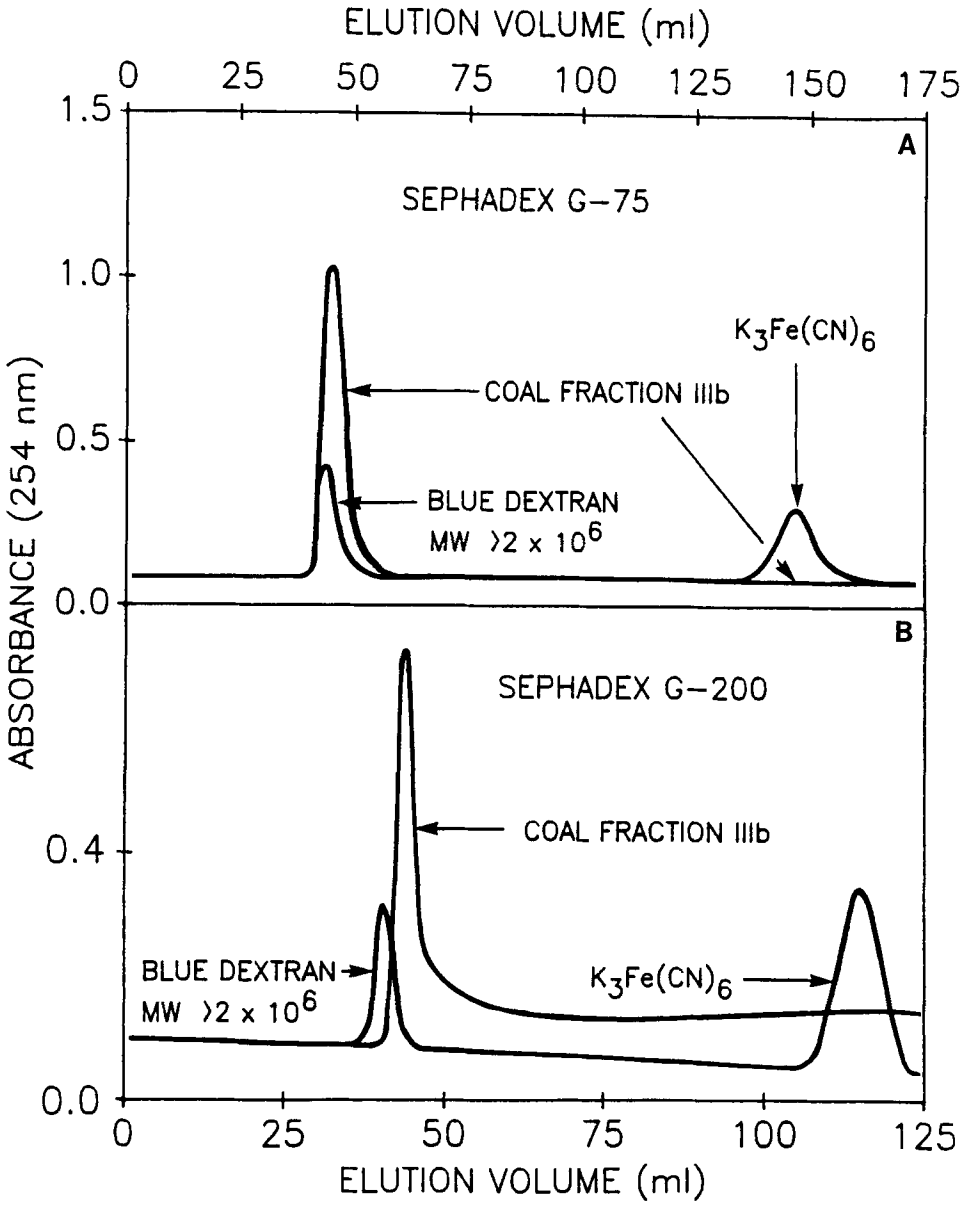

Fig. 1. Conventional gel filtration chromatography of soluble polymer from North Dakota lignite. Analyses were performed as described under Methods.

By visual observation of gel filtration behavior on conventional Sephadex columns, Fraction IIIb migrated as two brown bands of two widely different mobilities. One migrated with Blue Dextran on Sephadex G-75 and moved slightly slower than Blue Dextran on Sephadex G-200; the second component moved slightly slower than potassium ferricyanide on both G-75 and G-200. Figure 1 shows the absorption patterns at 254 nm for the same gel permeation separation on Sephadex G-75 (panel A), and G-200 (panel B) relative to Blue Dextran (MW $>2\times10^6$) and $K_3Fe(CN)_6$

(MW = 422). A sharp peak of relatively high molecular weight was observed. In contrast to the visual observation of a low molecular weight brown band, no low molecular weight material was observable at 254 nm. Similarly, analysis of water soluble material from German subbituminous coal by gel permeation HPLC revealed a major peak with a molecular weight of about 126,000 daltons (Fig. 2, panel A), based on the elution positions of thyroglobulin (MW = $>10^6$) and veratryl alcohol (MW = 168). The elution diagram showed some heterodispersity centered about 50,000 and 32,000 daltons; however, low molecular weight components in the range expected for monomeric products were absent.

Dialysis Experiments on German Subbituminous Coal

Additional tests based on dialysis behavior were performed to substantiate the high molecular weight composition of soluble coal polymer. Fraction IIIb was dialyzed for 24 h at 4°C against 0, 0.03 *M*, and 0.3 *M* KCl, each at pH 3.5 and pH 9.0. Membranes of about 12,000–14,000 dalton exclusion (Bethesda Research Laboratories, Gaithersburg, MD) were used. At pH 3.5, and all salt concentrations, no color, or UV absorbing material (254 nm) appeared in the dialysate, but a brown precipitate formed in the dialysis bag. At pH 9, and at all salt concentrations, a slight brown color appeared in the dialysate, but there was no UV absorbing material, and no precipitate formed in the bags.

It is unlikely that aggregation or micelle formation accounts for the high molecular weight behavior on gel permeation HPLC, as well as on dialysis at three salt concentrations, under both acidic acid and basic conditions.

Enzymatic Depolymerization of Coal

The procedure for obtaining a soluble polymeric coal material from North Dakota lignite is equally applicable to German subbituminous coal. Further, the soluble polymeric fractions from both coals appear to have a relatively uniform high molecular weight and to be largely free of low molecular weight components. Hence, these fractions are suitable for use as a substrate for lignin peroxidase in depolymerization studies. Reduction in molecular weight, caused by the action of lignin peroxidase, can be followed conveniently by changes in elution profile on gel permeation chromatography as utilized for initial characterization of soluble coal polymer fractions.

Soluble polymeric fractions (IIIb) from North Dakota lignite or German subbituminous coal were incubated with lignin peroxidase and H_2O_2 under a number of conditions. Reaction mixtures (1.0 mL) in capped tubes typically contained lignin peroxidase (0.2–1.0 U/mL) 20 m*M* sodium tartrate, pH 3.0, or 10 m*M* dimethylsuccinate, pH 4.5, 0.1 m*M* $MnSO_4$, 0.1% Tween 20, 0.45 – 1.0 m*M* H_2O_2 and 0.1 mL of coal Fraction IIIb (equivalent to 250–

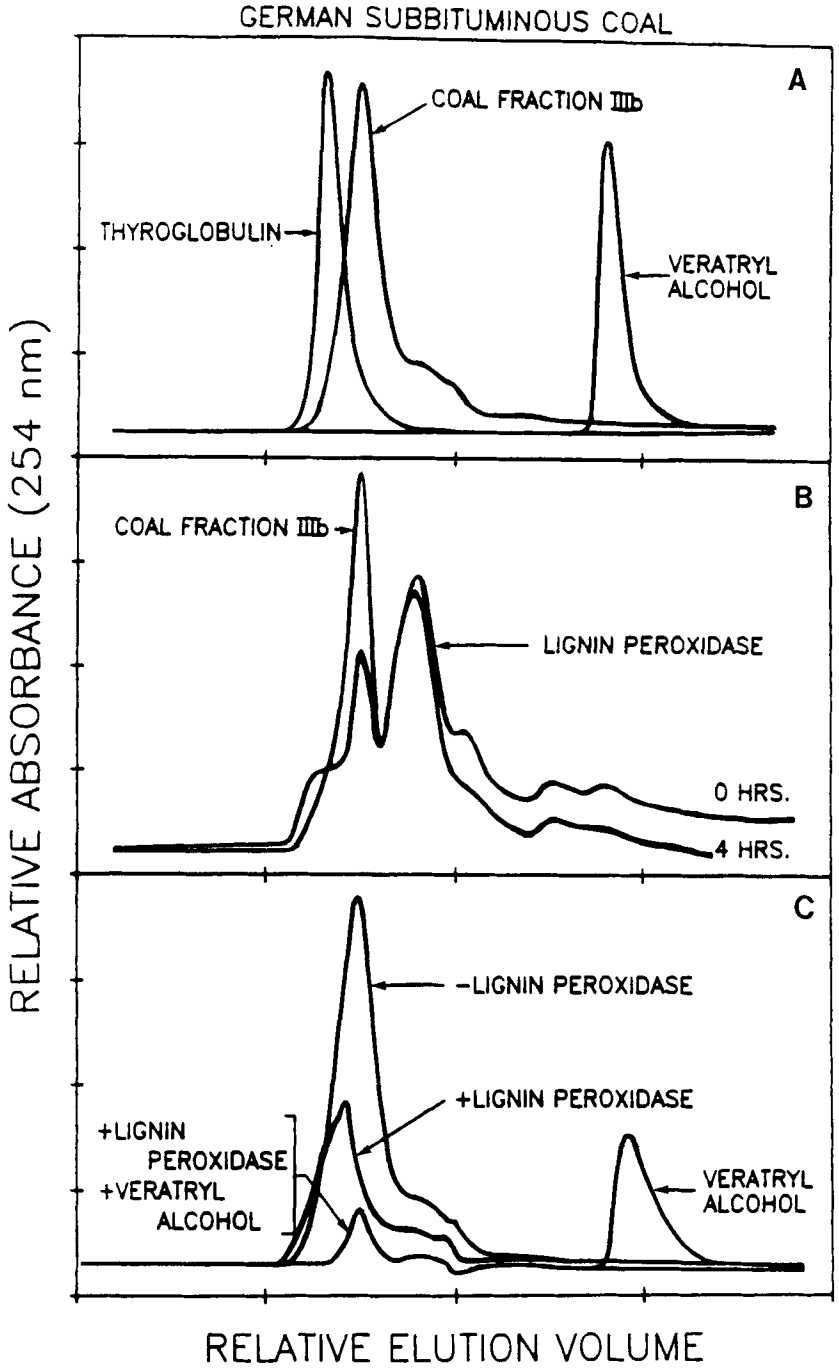

Fig. 2. Analyses of soluble polymer from German subbituminous coal by gel permeation high performance liquid chromatography. A. The elution behavior of coal polymer is compared to high and low molecular weight standards as described under Methods. B. Coal polymer (0.01 mL (25–30 μg/mL)) was incubated with 10 U lignin peroxidase for 1 h as described under Results. C. Coal polymer (0.1 mL (250–300 μg/mL)) was incubated with 10 U lignin peroxidase for 8 h. The reaction was initiated with 0.45 m*M* H_2O_2. Reaction mixtures were oxygenated every 15 min and incubated at 37°C with shaking. Veratryl alcohol (2 m*M*) was added as indicated. Samples were treated with dimethyl formamide/acetone described under Results.

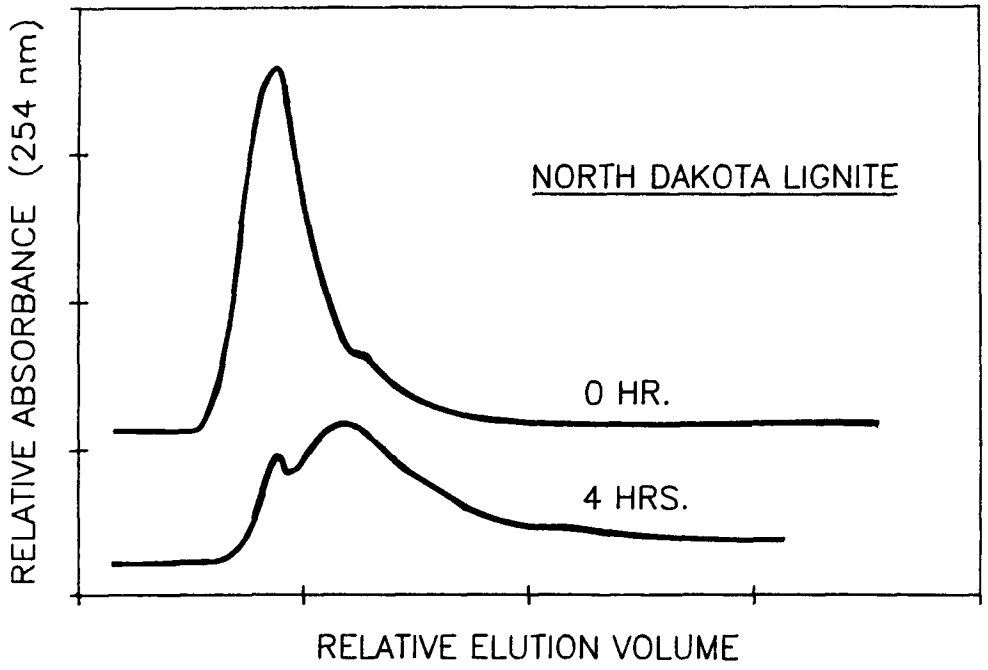

Fig. 3. Incubation of North Dakota lignite soluble polymer with lignin peroxidase. The reaction mixture contained 0.9 U of lignin peroxidase. Other conditions are as described under Results.

300 μg of coal organic material)/mL of reaction mixture. The substrate (soluble coal polymer) was added after the first addition of H_2O_2. The tubes were flushed with 100% O_2 and incubated at 37°C. Controls contained 2 m*M* veratryl alcohol as substrate instead of, or at times in addition to, Fraction IIIb. At indicated times samples (0.1 mL) were removed and analyzed directly (Fig. 3), or were first treated to dissociate any coal polymer–protein complex (Fig. 4) as follows: a sample of the reaction mixture (0.1 mL) was shaken with 0.2 mL acetone/dimethylformamide (1:1), the denatured protein was removed by centrifugation (2 min, ca. 14,000×g), and the supernatant air dried to remove the acetone. The residue was taken up in 0.02 *M* potassium phosphate –0.5% Tween 80, pH 7, for gel filtration HPLC analysis.

Incubation of soluble polymer from North Dakota lignite with lignin peroxidase for 4 h greatly decreased the amount of original polymer as evidenced by the decrease in area of the peak representing the original polymer and the appearance of a broad and trailing peak of somewhat lower molecular weight (Fig. 3). However, there is no evidence for formation of low molecular weight fragments. In another incubation, fivefold more enzyme was used, and the incubation was run at pH 4.5 instead of 3.0 (Fig. 4). In addition, the acetone–dimethylformamide procedure was used to dissociate any bound products from the enzyme. In this protocol

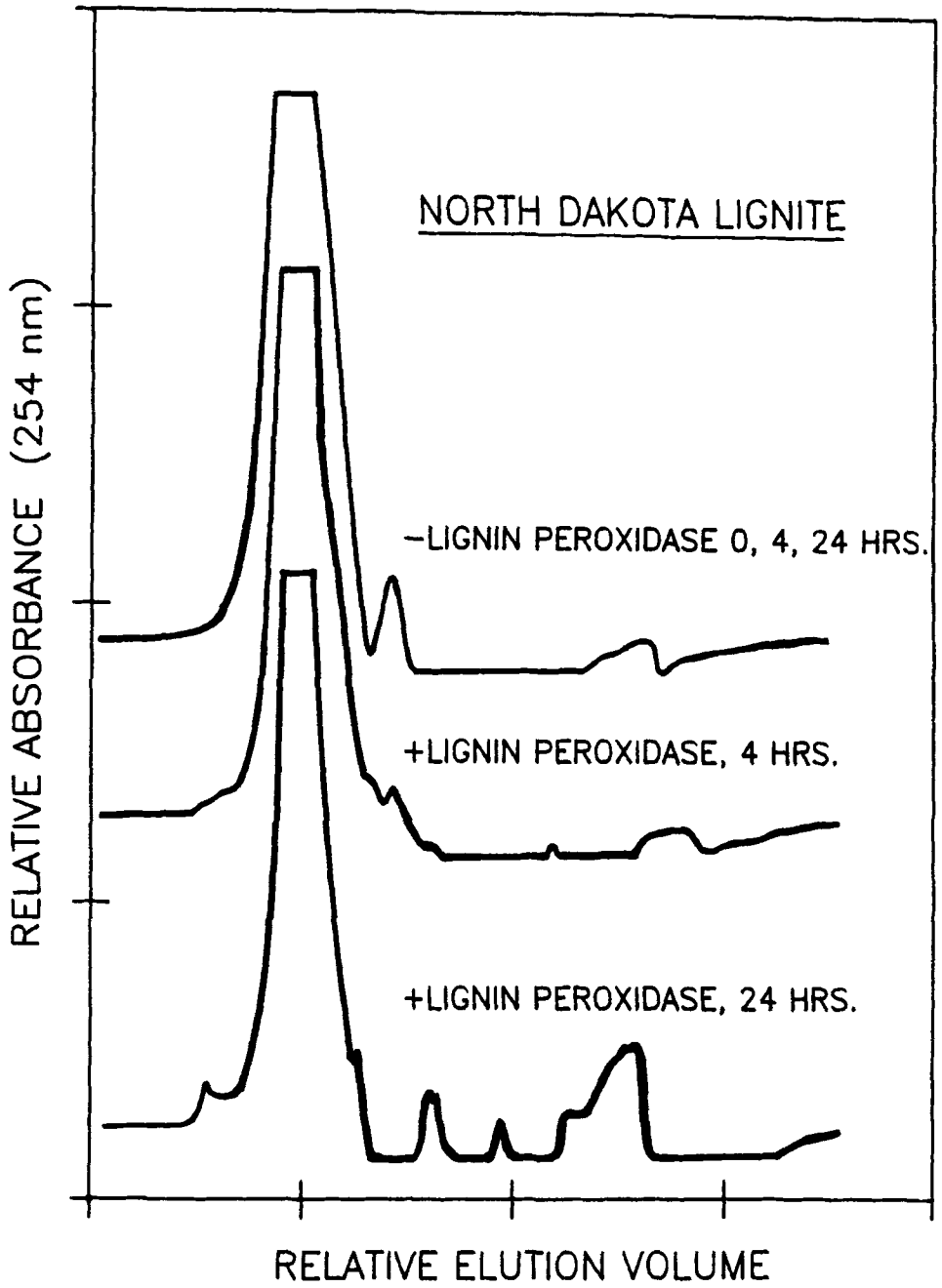

Fig. 4. Enhanced formation of fragments from North Dakota lignite soluble polymer by lignin peroxidase. The reaction mixture contained 4.5 U/mL of lignin peroxidase, 250–300 μg coal fraction, and 10 m*M* dimethylsuccinate buffer, pH 4.5. H_2O_2 was added in 0.15 μmol/mL portions at 0, 2, 4, and 6 h. Samples were removed at the times indicated and shaken with dimethylformamide/acetone as described under Results.

there was much less total depolymerization, but many new components of both higher and lower molecular weight than the starting material were detected. Figure 4 also shows that in the absence of lignin peroxidase, the elution profile of coal polymer is stable over a 24-h period.

The results for polymer from German subbituminous coal are similar to those for North Dakota lignite. There was a substantial disappearance of the original coal polymer, and the formation of lower molecular weight fragments (Fig. 2, panels B and C). In panel B, where coal polymer concentration was 25-30 μg/mL, the profile at zero time showed the major peak at 1×10^5 daltons with a shoulder at 2.2×10^4 daltons, and small peaks at 7,000 and 1,000 daltons. The other large peak at 11 min is lignin peroxidase, which in this incubation is in excess and appears as a separate peak. After incubation for 1 h at 37°C, there is a marked disappearance of the 10^5 MW peak and detectable loss of the 2.2×10^4 dalton component. However, no new low molecular weight peaks were seen. As in Fig. 4, the presence of new components of greater than the original molecular weight can be seen (Fig. 2, panel B). In panel C, where the coal polymer concentration was tenfold higher (250–300 μg/mL), a major disappearance of coal polymer takes place in 8 h. Lignin peroxidase is not present in the elution diagram because of dimethyl formamide/acetone treatment of the sample. The addition of veratryl alcohol, a substrate for lignin peroxidase, enhanced the depolymerization of coal polymer (Fig. 2, panel C) as evidenced by the near total disappearance of the coal polymer peak. It is noteworthy that no fragments appeared in the incubation containing veratryl alcohol even though the absorbance representing high molecular weight material virtually disappeared.

Inhibition of Lignin Peroxidase by Soluble Coal Polymer

In most experiments where the coal polymer concentration exceeded that of lignin peroxidase, no peak was seen for lignin peroxidase at its elution position on gel permeation HPLC. Instead, lignin peroxidase eluted with the major coal polymer peak. This indicates possible formation of a coal–peroxidase complex. In other experiments, addition of soluble coal polymer inhibited veratryl alcohol oxidation as measured in the spectrophotometric assay for lignin peroxidase. Incubation of coal polymer with H_2O_2 alone for 5–10 min had no adverse effect on the ability of H_2O_2 to activate the peroxidase. Hence, a possible loss of H_2O_2 in a reaction with coal polymer is not the cause of the inhibition. Addition of bovine serum albumin (30 μg/mL) to the assay before coal polymer prevented inactivation, whereas addition after coal polymer did not.

Figure 5 shows a double reciprocal plot of velocity of veratryl alcohol oxidation versus veratryl alcohol concentration at various soluble coal substrate concentrations. The unweighted linear regression lines tend to

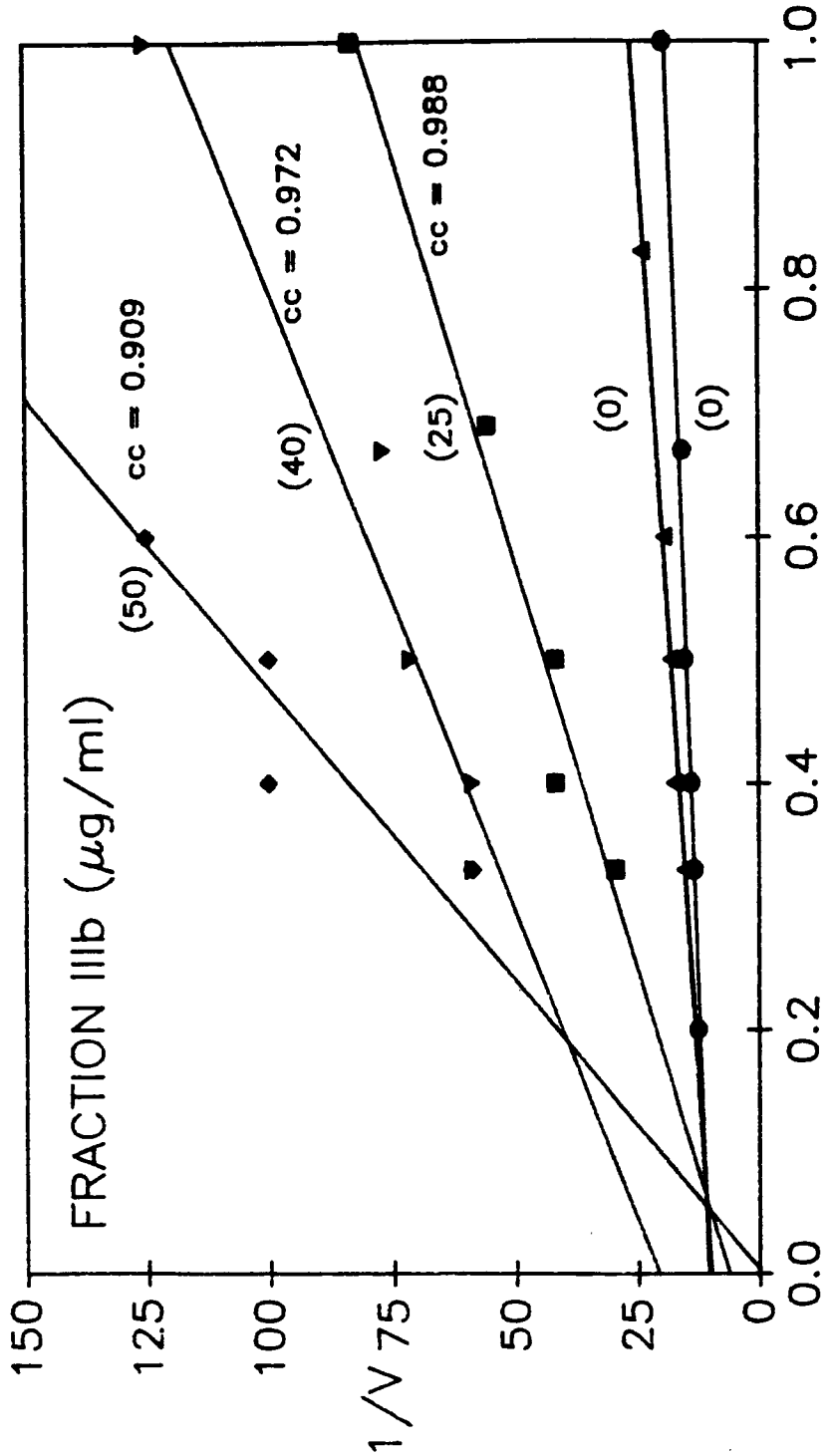

Fig. 5. Double reciprocal plots of velocity of veratryl alcohol oxidation by lignin peroxidase versus substrate concentration at several coal polymer concentrations. Velocity measurements were made with the spectrophotometric enzyme assay procedure described in Methods. The 1 mL reaction mixtures contained 20 m*M* tartrate, pH 3.0, 0.1 m*M* $MnSO_4$, 0.001 U of lignin peroxidase and 0.45 m*M* $H2O_2$. Coal fraction in amounts indicated was added 2 min after addition of H_2O_2.

converge at the y axis, as is characteristic of competitive inhibition. There is no convergence at the negative x axis as in noncompetitive inhibition, and the lines are not parallel as in uncompetitive inhibition.

DISCUSSION

The oxidation of lignite and low-ranked coals with nitric acid is known to yield a major proportion of the organic carbon as an alkali-soluble, acid-insoluble material often referred to as "humic acids" (*29–31*). Recent reports suggest that fungi carry out superficially similar oxidations. The molecular weight of the biologically produced material is high (>10,000 daltons). Therefore, the extent to which depolymerization has occurred in the biosolubilization process by microbial cultures is not known. However, it must not be extensive in view of the predominance of high molecular weight fragments in the biosolubilized material (*2,6*).

The addition of powdered lignite to liquid cultures of *P. chrysosporium* did not induce production of lignin peroxidase (*32*). Although it is likely that many of the fungi known to solubilize low-ranked coals can secrete lignin peroxidase under some conditions, there is no evidence that a lignin peroxidase-type depolymerization, as reported here, is a prominent part of the biosolubilization reported for fungal cultures. Hence, it is suggested that biosolubilization is a first-phase and that biodepolymerization could be a second-phase in the fungal mineralization of coal.

Lignin peroxidase is considered to be a powerful nonspecific, single electron oxidant. The cation radicals generated can rearrange nonenzymatically in a number of ways, including C–C bond breaking, and condensation in noneyzymatic reactions (*16–18*). This accounts for the large number of different types of reactions that lignin peroxidase has been observed to catalyze. Therefore, it is to be expected that many points of attack are available in the coal polymer and that many rearrangement, cleavage, and condensation products will be formed from subsequent reactions of carbon-centered radicals in the polymer. This has been partly confirmed by the observations of formation of both higher and lower molecular weight polymers at the expense of starting polymer (Fig. 2B and 4).

It has been observed that veratryl alcohol stimulates depolymerization of coal polymer and that veratryl alcohol oxidation by the peroxidase is inhibited by the polymer. A common mechanistic basis for these observations may exist (*15,33,34*). It has been postulated by Harvey et al. (*33*), that the action of lignin peroxidase can be indirect. For instance, veratryl alcohol can act as a mediator via formation of its cation radical in reduction of the Fe(IV)-oxo form of the peroxidase (*34*). The cation radical is the oxidant that then attacks the substrate, in this case the coal polymer; the electron removed regenerates veratryl alcohol to complete the cycle. Since the coal polymer effectively removes the veratryl alcohol cation radical by

reduction, the formation of veratraldehyde is suppressed, and this is interpreted as inhibition of the peroxidase by coal polymer in the spectrophotometric assay. In addition, veratryl alcohol is known to protect the peroxidase from inactivation by H_2O_2 (*15*).

It is also possible that the binding of coal polymer to the peroxidase inhibits veratryl alcohol oxidation in a kinetic sense by one or more of the following

(a) After binding of the polymer in the active site and direct C–C bond cleavage, polymer fragments to not dissociate;
(b) Parts of the coal polymer structure act as unreactive substrate-analogs that bind at the active site; or
(c) Hydrophobic parts of lignin peroxidase bind to the coal polymer in nonactive site regions so as to block the active site.

The first two possibilities better fit the competitive kinetic behavior.

Although there is a substantial loss of coal polymer during incubation with lignin peroxidase, only part of the polymer disappearance can be accounted for in lower and higher molecular weight components based on absorbance at 254 nm. In view of the many reactions catalyzed by the peroxidase, it is possible that ring opening and loss of UV absorbance accounts for some of the apparent loss in polymer.

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REFERENCES

1. Cohen, M. S., and Gabriele, P. D. (1982), *Appl. Environ. Microbiol.* **44,** 23–27.
2. Scott, C. D., Strandberg, G. W., and Lewis, S. N. (1986), *Biotechnol. Prog.* **2,** 131–139.
3. Strandberg, G. W. and Lewis, S. N. (1987), *J. Ind. Microbiol.* **1,** 371–275.
4. Wilson, B. W., Lewis, E. J., Stewart, D. L., and Li, S. M. (1985), pp. 31.1–31.8 in Proceedings of The Tenth Annual EPRI Contractors' Conference on Clean Liquid and Solid Fuels, April 23–25, 1985, Palo Alto, CA, Electric Power Research Institute, Palo Alto, CA.
5. Ward, H. B. (1985), *Sys. Appl. Microbiol.* **6,** 236–238.
6. Wilson, B. W., Bean, R. M., Franz, J. A., Thomas, B. L., Cohen, M. S., Aronson, H., and Gray, Jr., E. T. (1987), *Energy & Fuels* **1,** 80–84.
7. Pyne, J. W., Jr., Stewart, D. L., Fredrickson, J., and Wilson, B. W. (1987), *Appl. Envion. Microbiol.* **53,** 2844–2848.

8. Wilson, B. W., Lewis, E. J., Stewart, D. L., Li, S. M., Bean, R. M., Chess, E. K., Pyne, J., Cohen, M., and Aronson, H. (1986), pp. IV88-IV98, in Proceedings of Department of Energy Coal Liquification Contractors' Meeting, Pittsburgh, PA, November 19-21, 1985, NTIS, Springfield, VA.
9. Tien, M. and Kirk, T. K. (1983), *Science* **221,** 661-663.
10. Glenn, J. K., Morgan, M. A., Mayfield, M. B., Kuwahara, M., and Gold, M. H. (1983), *Biochem. Biophys. Res. Commun.* **114,** 1077-1083.
11. Tien, M. and Kirk, T. K. (1984), *Proc. Natl. Acad. Sci. USA* **81,** 2280-2284.
12. Gold, M. H., Kuwahara, M., Chiu, A. A., and Glenn, J. K. (1984), *Arch. Biochem. Biophys.* **234,** 353-362.
13. Umezawa, T., Shimada, M., Higuchi, T., and Kusai, K. (1986), *FEBS Lett.* **205,** 287-292.
14. Umezawa, T. and Higuchi, T. (1987), *Agric. Biol. Chem.* **51,** 2281-2284.
15. Haemmerli, S. D. Leisola, M. S. A., Sanglard, D., and Fiechter, A. (1986), *J. Biol. Chem.* **251,** 6900-6903.
16. Hammel, K. W., Kalyanaraman, B., and Kirk, T. K. (1986), *J. Biol. Chem.* **261,** 16948-16952.
17. Kersten, P. J., Tien, M., Kalyanaraman, B., and Kirk, T. K. (1985), *J. Biol. Chem.* **260,** 2609-2612.
18. Hammel, K. E., Tien, M., Kalyanaraman, B., and Kirk, T. K. (1985), *J. Biol. Chem.* **260,** 8348-8353.
19. Hammel, K. E., Kalyanaraman, B., and Kirk, T. K. (1986), *Proc. Natl. Acad. Sci. USA* **83,** 3708-3712.
20. Kirk, T. K., S. Croan, and Tien, M., Murtagh, K. E., and Farrell, R. L. (1986), *Enzyme Microb. Technol.* **8,** 27-32.
21. Archibald, F. S. and Fridovich, I. (1982), *Arch. Biochem. Biophys.* **214,** 452-463.
22. Glenn, J. K. and Gold, M. H. (1985), *Arch. Biochem. Biophys.* **242,** 329-341.
23. Johnson, M. J. (1949), *J. Biol. Chem.* **181,** 707-711.
24. Beyer, M. (1987), *Biotechnol. Lett.* **9,** 19-24.
25. van Krevelen, D. W. (1950), *Fuel* **29,** 269.
26. Jüntgen, H., in Biotechnologie im Steinkohlenbergbau, Steinkohlenbergbauverein Workshop Coal Recovery, Second Generation, Jan 1983, Essen, FRG, p. 28.
27. Schutz, J. G. and Sabourin, T., US Patent 4,052,488 (1977).
28. Schultz, J. G. D., and Zajac, J., US Patent 4,235,728 (1980).
29. Juettner, B., Smith, R. C. and Howard, H. C. (1935), *J. Am. Chem. Soc.* **57,** 2322-2326.
30. Chowdhury, J. K., and Biswas, A. B. (1942), *J. Indian Chem. Soc.* **19,** 289-298.
31. Charmbury, H. B., Eckerd, J. W., La Torre, J. S., and Kinney, C. R. (1945), *J. Am. Chem. Soc., 625#628.*
32. Wondrack, L., Szanto, M., and Wood, W. A. (1987) (unpublished observations).
33. Harvey, P. J., Shoemaker, H. E., and Palmer, J. M. (1986), *FEBS Lett.* **195,** 242-246.
34. Renganathan, V., Miki, K., and Gold, M. H. (1985), *Arch. Biochem. Biophys.* **241,** 304-314.