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Peroxidase-Catalyzed Polymerization and Depolymerization of Coal in Organic Solvents

ALEXANDER M. BLINKOVSKY, JAMES P. McELDOON, JOHN M. ARNOLD, AND JONATHAN S. DORDICK*

Department of Chemical and Biochemical Engineering and Center for Biocatalysis and Bioprocessing, University of Iowa, Iowa City, IA 52242

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ABSTRACT

Peroxidases from horseradish roots (HRP) and soybean hulls (SBP) catalyze the efficient polymerization of a 4-kDa dimethylformamide (DMF)-soluble fraction of Mequininza (Spanish) lignite in 50% (v/v) DMF with an aqueous component consisting of acetate buffer, pH 5.0. Under these conditions, HRP and SBP catalyze the oxidation of free phenolic moieties in the coal matrix, thereby leading to oxidative polymerization of the low-molecular-weight coal polymers. The high fraction of nonphenolic aromatic moieties in coal inspired us to examine conditions whereby such coal components could also become oxidized. Oxidation of nonphenolic aromatic compounds was attempted using veratryl alcohol as a model substrate. SBP catalyzed the facile oxidation of veratryl alcohol at pH <3. HRP, however, was unable to elicit veratryl alcohol oxidation. The potential for SBP to catalyze interunit bond cleavage on complex polymeric substrates was examined using 1-(3,4-dimethoxyphenyl)-2-(phenoxy)propan-1,3-diol (**1**) as a substrate. SBP catalyzed the Cα-Cβ and β-ether bond cleavage of this compound, suggesting that similar reactions on coal, itself, could lead to depolymerization. Depolymerization of a >50 kDa coal fraction was achieved using SBP in 50% (v/v) DMF with an aqueous component adjusted to pH 2.2. Approximately 15% of the initial high-molecular-weight lignite fraction was depolymerized to

*Author to whom all correspondence and reprint requests should be addressed.

polymers <4 kDa in size. Hence, SBP is capable of catalyzing the depolymerization of coal in organic solvents, and this may have important ramifications in the generation of liquid fuels from coals.

Index Entries: Peroxidase from soybean hulls; coal depolymerization; enzymatic oxidation of veratryl alcohol.

INTRODUCTION

The liquefaction of coal represents a challenging approach to the development of nonpetroleum-derived fuel alternatives. Depolymerization of high-molecular-weight coal fractions to lower-molecular-weight polymers that have enhanced solubilities typically in organic solvents is the most attractive method of liquefaction. A number of chemical approaches to coal depolymerization rely on either reductive or oxidative reactions that break down the polydisperse, polycyclic aromatic coal matrix (*1,2*). Unfortunately, such chemical processes generally require high temperatures and pressures, and are, therefore, costly.

In addition to the chemical breakdown of coal, a wide variety of microorganisms can use coal as carbon and energy sources (*3–7*). The microbial depolymerization of coal has been found to be predominantly an extracellular process (*4,5*) in which oxidoreductases catalyze the cleavage of C—C, C—O, and C—S interunit linkages either within or between coal polymers. The structural similarity between low-rank coal (such as lignites) and lignin has prompted numerous groups to utilize lignin-degrading/altering microorganisms and their enzymes to depolymerize coal. These enzyme systems are almost exclusively comprised of oxidoreductases (e.g., peroxidases, oxygenases, hydrogenases, and dehydrogenases). Unfortunately, coal bioprocessing by these enzymes has been severely limited by the poor solubility of coal in aqueous solutions. It may be anticipated, therefore, that effective coal depolymerization must be performed in a nonaqueous environment. Fortunately, a wide variety of enzymes are catalytically active in organic solvents (*8,9*), including oxidative enzymes with demonstrated activity on coal (*10,11*).

Peroxidases have proven to be highly effective catalysts for the modification of coal and coal model compounds. For example, Dordick et al. used horseradish peroxidase (HRP), lactoperoxidase, and chloroperoxidase to catalyze the oxidation of dibenzothiophene (DBT) in the presence of 25% dimethylformamide (DMF) (*12*). Similarly, Klyachko and Klibanov used the peroxidatic activity of hemoglobin to oxidize DBT in a large number of nearly anhydrous solvents (*13*). More directly on coal itself, Scott et al. employed dioxane-soluble HRP to increase the solubilization of nitric oxide treated subbituminous coal in 85% dioxane solution (*14*). The enzymic reaction improved the solubilization of the coal by as much as 20% over that in the absence of enzyme in 85% dioxane. It is clear,

then, that peroxidases have some direct utility for the oxidation of coal. In the present work, we have focused our attention on identifying the conditions that favor the depolymerization of coal in the presence of organic solvents using two commercially available plant peroxidases: HRP and soybean hull peroxidase (SBP). The latter represents an additional source (over that from horseradish roots) for this popular enzyme.

MATERIALS AND METHODS

Ground Spanish Mequininza lignite was generously provided by Frank Roberto of the Idaho National Engineering Laboratory (Idaho Falls, ID). Horseradish peroxidase, Sephadex LH-60 resin, and H_2O_2 (30% solution in water) were obtained from Sigma Chemical Co. (St. Louis, MO). Peroxidase from soybean hulls was obtained from Enzymol International (Columbus, OH). All chemicals and solvents were of the highest quality commercially available. Prior to enzymic treatment, the lignite was dissolved in DMF and fractionated on the Sephadex LH-60 column (1 × 24 cm) eluted with DMF containing 0.1*M* LiCl at a flow rate of 0.1 mL/min. The salt prevented intermolecular association of individual coal polymers, thereby providing a truer molecular-weight profile (*15*). The column was calibrated with polystyrene standards (Polysciences, Warington, PA) of 2, 4, 15, and 50 kDa. Fractions corresponding to average molecular weights of 4 kDa and >50 kDa were obtained by pooling elutions within 1 mL of the peak representing the two standard molecular weights.

In a typical experiment, 5.0 mg of fractionated coal were dissolved in 1 mL DMF. The reaction was initiated by simultaneously pumping 0.6 mL of an aqueous buffer solution (50 m*M* Na acetate, pH 5.0 or 50 m*M* K tartrate buffer, pH 2.2 containing 0.1 M $CaCl_2$), containing 1 mg enzyme and 0.4 mL of a 2 m*M* H_2O_2 aqueous buffer solution into a 5 mL reaction vial over a period of 4 min. The reaction mixtures were shaken at 100 rpm and 30°C, during which time 0.1 mL aliquots were removed for molecular-weight determination. Before analysis of the aliquots, the samples were vacuum-dried at 45°C and dissolved in 0.1 mL anhydrous DMF. Two different control reactions were also initiated. The first control involved the exclusion of enzyme from the reaction mixture (to ascertain whether H_2O_2 alone could promote coal oxidation). The second control involved the exclusion of H_2O_2 (to ascertain whether the peroxide-independent oxidase reaction pathway of peroxidases [*16*] could promote coal oxidation). All reactions were performed in duplicate, and the results presented represent averages of each duplicate.

Peroxidase-catalyzed oxidation of veratryl alcohol was performed as follows: 33 μg of enzyme was added to 1 mL of a 2 m*M* veratryl alcohol solution in aqueous buffer (0.1 *M* K tartrate, glycine·HCl, or Na acetate depending on pH). The reaction was initiated by adding 0.2 m*M* H_2O_2, and the reaction followed spectrophotometrically for the appearance of

veratraldehyde (ϵ_{310} = 9500M^{-1}/cm). Oxidation of 1-(3,4-dimethoxyphenyl)-2-(phenoxy)propan-1, 3-diol (a lignin model compound) was performed under conditions similar to veratryl alcohol oxidation. The lignin model was generously provided by Kenneth Hammel at the USDA Forest Products Laboratory (Madison, WI).

RESULTS AND DISCUSSION

Low-rank coals, such as lignites, have many structural similarities to lignin, including the presence of free phenolic moieties and aromatic ether linkages (*17*) that are susceptible to enzymic oxidation and cleavage, respectively. The success of plant and fungal peroxidases in modifying lignin structure (*18,19*) provided the necessary precedence to utilize peroxidases to modify coal structure. Mequininza lignite was used in the present study because it is highly representative of the low-rank class of coals with a high phenolic content, thereby making it a reasonable target for peroxidase-catalyzed oxidation. The poor solubility of this lignite in water mandated the use of organic solvents for bioprocessing. To that end, a number of water-miscible solvents were screened for the solubilization of the lignite. The coal was very poorly soluble (<0.5 mg/mL) in both tetrahydrofuran and dioxane, solvents that are known to support peroxidase catalysis (*20*). Conversely, DMF can dissolve up to 10 mg/mL of the lignite at room temperature. Although HRP had no activity in anhydrous DMF, the enzyme was active in DMF/water mixtures (data not shown). Unfortunately, coal solubility drops precipitously in the presence of added water. To overcome the problem of maintaining soluble coal under a condition that supports enzyme activity, oxidation reactions were performed by slowly feeding aqueous solutions of enzyme and H_2O_2 to a homogeneous solution of coal in DMF with a final solution consisting of 50% (v/v) DMF. Thus, during most of the initial reaction period, the coal remains soluble, and the enzyme reacts with the soluble substrate.

The DMF-soluble lignite has a broad molecular-weight profile with ca. one-third of the coal polymers having mol wt > 50 kDa (Fig. 1). Two fractions were collected corresponding to a low-molecular-weight coal polymer (4 kDa) and a high-molecular-weight coal polymer (>50 kDa). Our initial enzyme experiments were focused on the low-molecular-weight fraction, such that polymerizative and depolymerizative outcomes of coal oxidation could be observed.

Peroxidase-Catalyzed Oxidation of Lignite

Oxidation of DMF-soluble lignite was performed in a solution with a final composition of 50% (v/v) DMF in aqueous buffer, pH 5.0, using both HRP and SBP. Following 24-h incubations, the reaction mixtures were

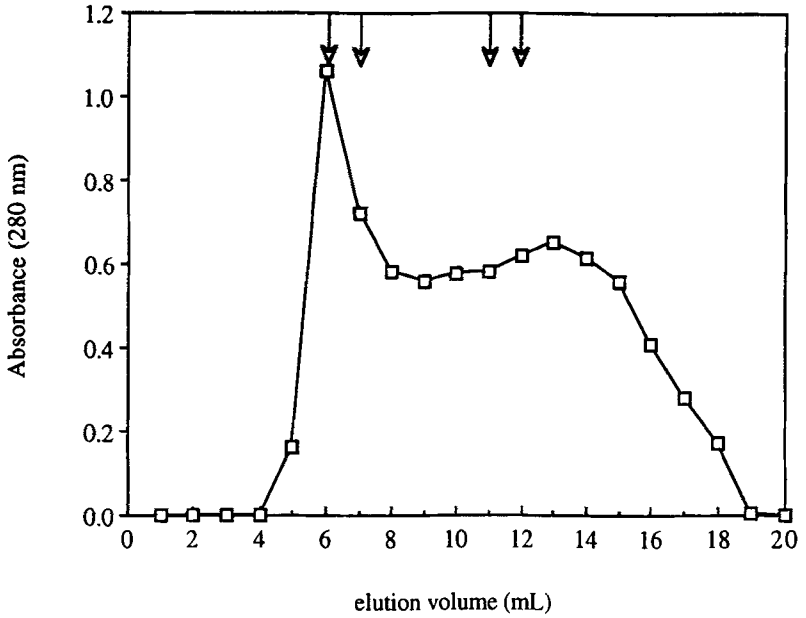


Fig. 1. Molecular-weight spectrum of Mequininza lignite soluble in DMF. Arrows indicate molecular-weight markers: from left to right, 50, 15, 4, and 2 kDa.

recovered, evaporated, and dissolved in anhydrous DMF. Not all of the lignite dissolved in anhydrous DMF, indicating that high-molecular-weight polymers were produced in the enzyme reactions. The DMF-insolubles were removed by centrifugation and found to contain 20 and 12% of the initial lignite in reactions catalyzed by HRP and SBP, respectively. The supernatants were then subjected to gel-permeation chromatography. Figure 2 depicts the molecular-weight changes that accompany the oxidation of lignite in 50% DMF with both HRP and SBP. Clear polymerization was observed with no evidence of depolymerization. Based on absorbance values, it is estimated that in each case, ca. 35% of the initial lignite was polymerized to species with mol wt > 50 kDa. Thus, HRP catalyzed the polymerization of 55% of the initial lignite, taking into account the polymerization to DMF-soluble and insoluble materials. In the absence of either enzyme or H_2O_2, no reaction was observed. Thus, coal oxidation by the plant peroxidases resulted in a similar outcome to those studies done on lignin oxidation by HRP (*18*).

The polymerization of the low-molecular-weight lignite fraction was not surprising. Peroxidases are expected to catalyze the oxidation of free phenolics in the lignite matrix to phenoxy radicals. These radicals undergo nonenzymatic radical transfer/termination reactions with other coal-based phenoxy radicals or phenolics, respectively, which results in polymerization. Clearly, the simple oxidation of phenolic groups only

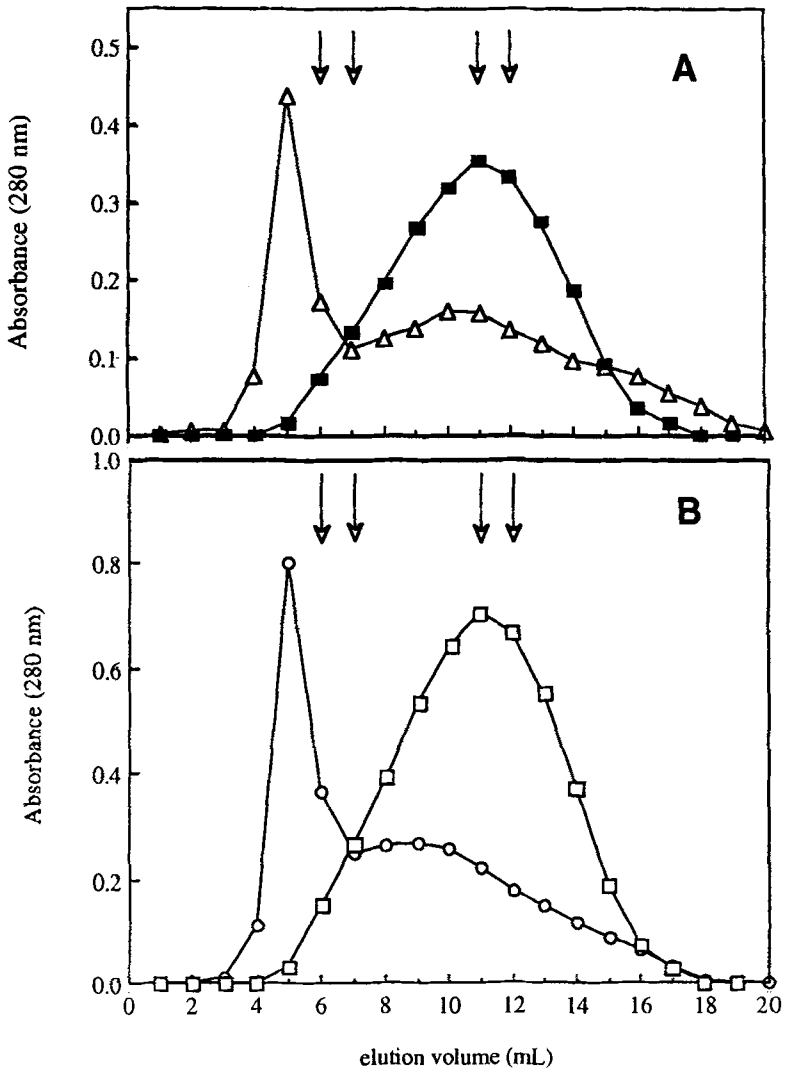


Fig. 2. Gel-permeation chromatography of Mequininza lignite fraction with average mol wt 4 kDa. (**A**) Incubation with horseradish peroxidase (HRP) in the absence of enzyme (■), in the presence of enzyme (△); (**B**) incubation with soybean peroxidase (SBP) in the absence of enzyme (□), in the presence of enzyme (○). Incubation of the lignite fraction with H_2O_2 in the absence of either enzyme did not alter the molecular-weight profile of the lignite. Fractionated lignite's molecular-weight profile was identical to the nonenzymatic controls depicted. Arrows indicate molecular-weight markers: from left to right, 50, 15, 4, and 2 kDa.

cannot lead to direct depolymerization of coal polymers. Under the conditions employed, both HRP and SBP efficiently oxidize phenolic moieties in the coal polymers, yet cleavage of interunit linkages is not favored. Altering the reaction conditions to favor interunit bond cleavage was deemed necessary to elicit coal depolymerization.

Table 1
Soybean Peroxidase-Catalyzed Oxidation of Veratryl Alcohol Under Acidic Conditions[a]

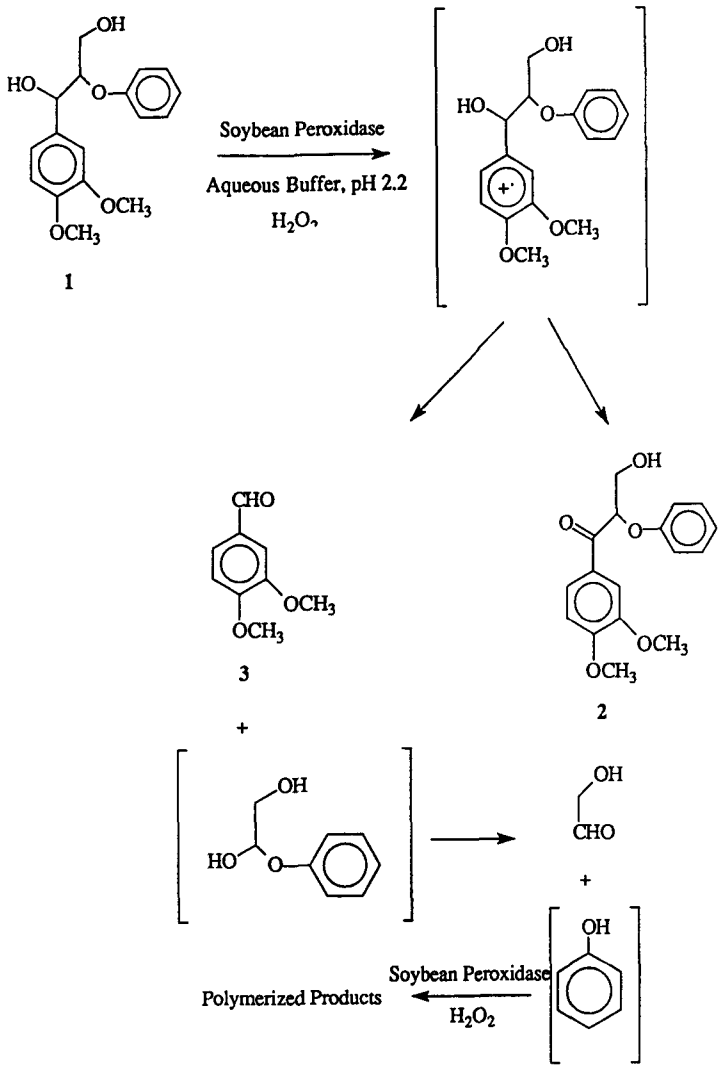
pH	Initial rate[b]
2.0	0.18
2.2	0.29
2.4	0.27
2.8	0.17
3.0	0.11
4.0	0
5.0	0

[a]Reaction conditions as described in Materials and Methods. Tartrate buffer used for pH range from 2.0 to 2.4; glycine · HCl buffer used for pH range from 2.8 to 3.0; and acetate from pH ≥ 4.0.
[b]Initial rate expressed in μmol/mg enzyme/min. The enzyme concentration was 33 μg/mL.

Activity of Peroxidases Under Acidic Conditions

As with lignin, aromatic ether bonds constitute a major fraction of the interunit linkages present in coal polymers. Lignin peroxidase (LiP, from *Phanerochaete chrysosporium*), with its high oxidation potential as compared to more common peroxidases, is capable of oxidizing nonphenolic aromatic compounds and can break α- and β-ether linkages in lignins and lignin model compounds (*19,21*). The oxidation of veratryl alcohol (3, 4-dimethoxybenzyl alcohol) is the most commonly used measure of LiP activity on nonphenolic aromatic compounds (*19,21*). LiP's activity on veratryl alcohol is optimal at low-pH values (e.g., pH <4), most probably because of the higher oxidation potential of the heme iron under acidic conditions (*22*). It occurred to us that common peroxidases may also have the ability to oxidize veratryl alcohol under similarly acidic conditions. To that end, both HRP and SBP were employed as potential catalysts in veratryl alcohol oxidation at pHs ranging from 2 to 5 with tartrate buffer (pH 2.0–2.4), glycine·HCl buffer (pH 2.8–3.0), and acetate buffer (pH ≥ 4.0) (Table 1). $CaCl_2$ was added to maintain peroxidase stability under acidic conditions (*23*). Not to our surprise, HRP was unable to oxidize veratryl alcohol at any pH, a finding in agreement with previous attempts to obtain LiP-type activity from HRP (*24*). Conversely, SBP was highly active in oxidizing veratryl alcohol at pH <3 with an optimum at pH 2.2.

The ability of SBP to catalyze oxidation of veratryl alcohol suggests that the enzyme is capable of generating an aromatic cation radical. Such a radical has been implicated in the ability of LiP to catalyze the oxidation of nonphenolic aromatics, including veratryl alcohol (*18,21*). The ability of



Scheme 1

SBP-catalyzed oxidation reactions to proceed through an aromatic cation radical intermediate was demonstrated using 1-(3,4-dimethoxyphenyl)-2-(phenoxy)propan-1,3-diol (**1**) as substrate. In the presence of 33 μg/mL SBP, ca. 10% of a 1 m*M* solution of the ether substrate in aqueous buffer, pH 2.2, was converted to both an oxidized intermediate (ketone product, **2**) and veratraldehyde (**3**) (Scheme I) in 30 min. The formation of veratraldehyde implies that the C_α-C_β bond is cleaved, which results in the generation of a hemiacetal on the C_β carbon, which further breaks down to give free phenol (which would undergo further polymerization catalyzed by SBP in the presence of H_2O_2) and a hydroxyaldehyde. Thus, β-ether bond cleavage occurs, although via a C_α—C_β bond cleavage reaction. These results clearly demonstrate that SBP is capable of oxidizing

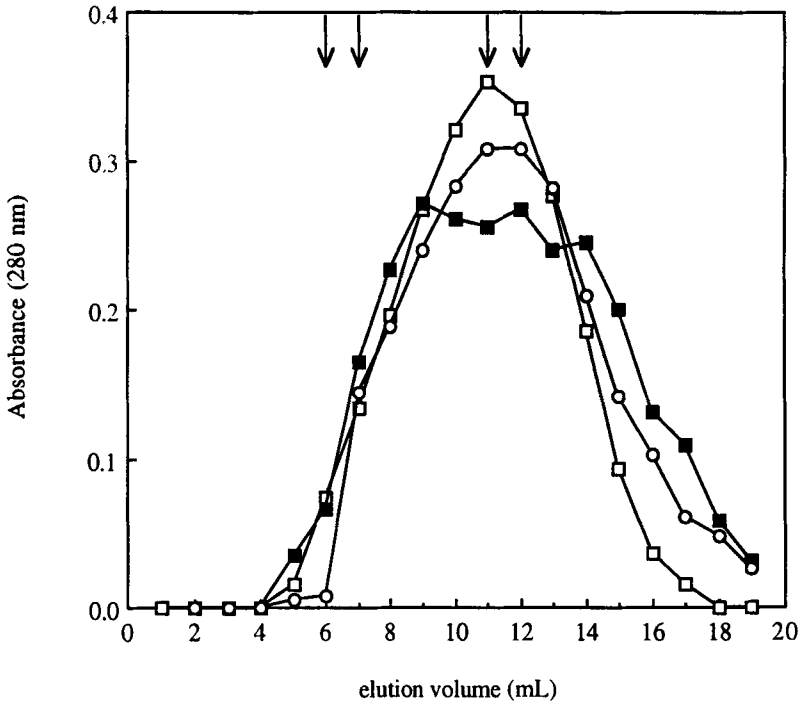


Fig. 3. Gel-permeation chromatography of Mequininza lignite fraction with average mol wt 4 kDa before incubation with soybean peroxidase (□), and after incubation at pH 2.2 (0.1*M* tartrate buffer, 0.1*M* $CaCl_2$) in 50% DMF for 4 h (○) and 24 h (■). Arrows indicate molecular-weight markers: from left to right, 50, 15, 4, and 2 kDa.

nonphenolic aromatic compounds and can catalyze β-ether bond cleavage. Thus, under acidic conditions, SBP appears to have the necessary catalytic machinery to depolymerize coal polymers.

Depolymerization of Coal Polymers Catalyzed by Soybean Peroxidase

The SBP-catalyzed oxidation of the 4-kDa coal fraction was performed in 50% (v/v) DMF with an aqueous phase containing 0.1*M* $CaCl_2$ at pH 2.2 (tartrate buffer). In contrast to the pH 5 reaction, SBP was capable of both polymerizing and depolymerizing the low-molecular-weight coal polymer (Fig. 3) even after 4 h. Following 24 h of incubation, ca. 10% of the initial coal fraction was found to be DMF-insoluble, indicating that some very high-molecular-weight polymers were formed. Inspection of Fig. 3, however, shows that depolymerization was evident as indicated by the increased absorbance in fractions 14–19. Both control reactions appeared to leave the lignin fraction unaltered.

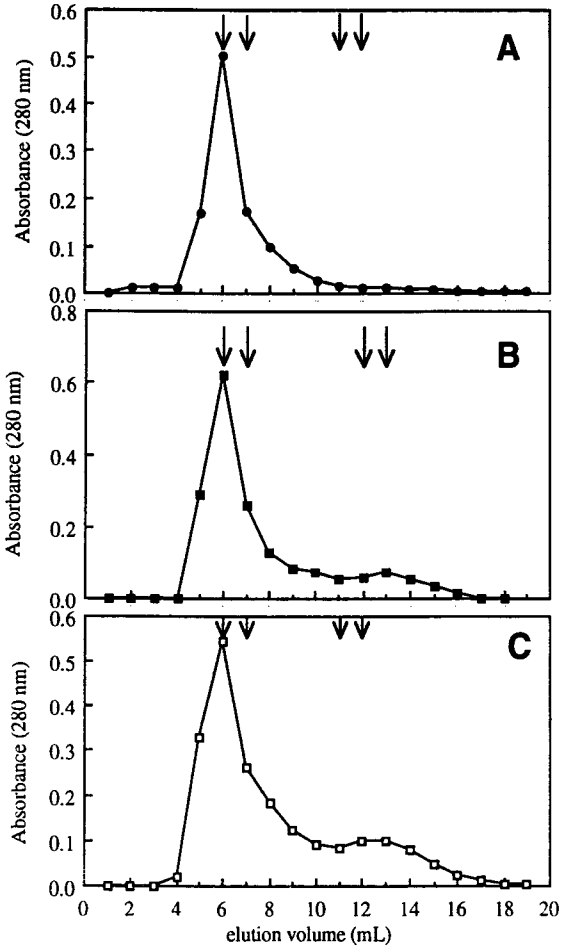


Fig. 4. Gel-permeation chromatography of Mequininza lignite fraction with average mol wt >50 kDa. (**A**) incubation in the absence of soybean peroxidase for 24 h; (**B**) incubation with soybean peroxidase after 4 h; (**C**) incubation with soybean peroxidase for 24 h. Conditions are as described in the legend to Fig. 3 and in the text. Arrows indicate molecular-weight markers: from left to right, 50, 15, 4, and 2 kDa.

Although depolymerization of the coal polymer was observed, a large fraction of the coal polymer polymerized. The preponderance of low-molecular-weight coal polymers in the 4-kDa fraction would favor polymerization as a result of radical transfer and termination reactions for the simple reason that such radical transfer reactions are more likely to result between two low-molecular-weight coal polymers leading to higher-molecular-weight polymers. Such a situation can be at least partly avoided by employing a higher-molecular-weight coal fraction. To that end, we employed a coal polymer fraction with an average molecular weight >50 kDa. SBP-catalyzed oxidation of the 50-kDa lignite fraction resulted in clear evidence of depolymerization (Fig. 4). After 24 h, ca. 15%

of the initial high-molecular-weight coal fraction was depolymerized to <4 kDa, and <5% of the initial coal was found to be DMF-insoluble. Finally, both control reactions left the coal polymer unaltered.

In conclusion, SBP is an effective catalyst for lignite depolymerization. The oxidation of the coal polymer by SBP under acidic conditions must involve both phenoxy radical oxidation (and subsequent polymerization of distinct coal polymers) and ether bond cleavage between nonphenolic aromatic linkages that comprise a large portion of the coal matrix, thereby leading to depolymerization. It may be expected that the ratio of depolymerization/polymerization would become even more favored as the rank of the coal increases owing to a lower fraction of phenolic moieties present in higher-rank coals, although the recalcitrant nature of high-rank coals may reduce the overall reactivity toward SBP. We are in the process of examining SBP catalysis on several different coal samples.

Although the exact mechanism of coal depolymerization catalyzed by SBP remains unknown, the ability of SBP to oxidize veratryl alcohol and depolymerize coal has important ramifications in fields distinct from coal bioprocessing. For example, the oxidative power of SBP may prove effective as an alternative to LiP in the oxidative depolymerization of lignin and decolorization of bleaching effluents (*25*). Such reactivities are presently under investigation.

ACKNOWLEDGMENTS

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