ENZYMATIC CONVERSION OF COAL IN NONAQUEOUS MEDIA

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ABSTRACT.
Two different peroxidases--from horseradish roots and soybean hulls--were studied as potential biocatalysts for modification of coal in aqueous-organic media. Both enzymes were found to cause polymerization of Mequininza lignite in a mixture (1:1 v/v) of DMF and acetate buffer (pH 5). At pH 2.2, incubation of the lignite with soybean peroxidase in 50% DMF resulted in both polymerization and depolymerization.

INTRODUCTION

It has been demonstrated earlier that both isolated enzymes[1,2] and intact microorganisms[3-5] can selectively modify coal. However, coal bioprocessing has not been highly successful. One of the most difficult problems that remains is the lack of sufficient enzyme-coal interactions that are necessary for substantial depolymerization and solubilization. The insolubility of coal in water severely limits the extents of either microbial or enzymatic degradation. Any truly effective coal depolymerization process, therefore, must take place in a non-aqueous medium that can solubilize at least some fractions of the coal. The use of enzymes in nonaqueous solvents[6,7] has now become common place and numerous enzymes and conditions have been studied in organic media. Specifically, peroxidases are known to be highly active in nonaqueous media, with turnover numbers often enhanced in organic solvents over that in water[8]. Several peroxidases (e.g., chloroperoxidase, lactoperoxidase, and horseradish peroxidase)[1,2] display activity on the oxidation of coal-related compounds including lignin[9]. In the present work, our efforts have focused on study of two different peroxidases as potential biocatalysts for modification of coal structure and for depolymerization of coal in aqueous-organic media. We have evaluated also multi-enzyme systems from whole cells of bacteria in respect of oxidation of a coal model compound.

MATERIALS AND METHODS

Mequininza lignite (from Spain) was used as a substrate. The lignite was not subjected to any treatment except for thorough grinding. Horseradish peroxidase (EC 1.11.1.7) type II was obtained from Sigma Chemical Co (St. Louis, MO). Peroxidase from soybean hulls (EC 1.11.1.7) was obtained from Enzymol, International (Columbus, OH). The enzymes were used without additional purification. Before incubation with the enzymes, the lignite was fractionated on lipophilic Sephadex LH-60. Gel permeation column chromatography on lipophilic Sephadex LH-60 is traditionally used as a tool to separate molecules of different molecular weight in a non-aqueous medium as well as to determine the molecular weight distribution. Intermolecular associations of coal polymers with the resin, and to each other, were eliminated by employing DMF supplemented with 0.1 M LiCl. The range of molecular weights which can be separated by this technique is 500 - 60000 D.

In a typical experiment, 7.5 mg of the soluble coal fraction was dissolved in 1 ml of pure DMF and 0.8 ml of the aqueous buffer (containing 1 mg of enzyme) was added. The reaction was initiated upon pumping 0.2 mL of hydrogen peroxide into the vial over a period of two minutes. The final concentration of hydrogen peroxide was 0.66-0.8 mM. A control reaction which included all reagents, except enzyme, was initiated concurrently. The incubation mixtures were shaken at 100 rpm at 30°C for 24 h during which time 0.1 mL aliquots were taken. Before analysis of the molecular weight distribution, the samples of the lignite after incubation with peroxidases were dried in vacuum at 45°C and redissolved in 0.1 mL of pure DMF. Supernatant was loaded onto a glass column (24 x 1 cm) packed with Sephadex and eluted at a flow rate 0.1 mL/min. The column was calibrated with polystyrenes with molecular weights of 2, 4, 15, and 50 kD. Fractions (1 mL) were measured via absorbance at 280 nm.

Dibenzothiophene and byphenyl were used as coal model substrates to study oxidation by whole cells of *Beijerinckia* and by the *Beijerinckia* enzyme system in aqueous-organic media. Cells were grown via the method of Laborde and Gibson[10], harvested and lyophilized. The freeze-dried cells were ground then to a fine powder. NADH was added to the incubation mixture as a cofactor of the oxygenase system for the disrupted cells. In a typical experiment, wet whole cells of *Beijerinckia* (0.3-0.4 g)were incubated with saturated concentrations of the substrates in 10 mL of phosphate buffer (0.05 M, pH 7.2) containing 0.1% of pyruvate and 1-20% (v/v) of organic solvents. In the case of the lyophilized cells (fine dry powder), incubation was carried out in acetate-phosphate buffer at pH 5.0-7.5 containing up to 50% of an organic solvent at a concentration of cells and NADH of 4 mg mL^{-1} and 1 mM, respectively. Periodically, aliquots of the reaction mixtures were withdrawn, dried in vacuum at 40°C, redissolved in pure acetonitrile, and analyzed with a Waters μBondapak C_{18} column (3.9 x 300 mm). The mobil phase consisted of an acetonitrile-water mixture; the flow rate was 1 mL min^{-1}. Absorbance peaks were monitored at 230 nm.

RESULTS AND DISCUSSION

Proxidase-Catalyzed Polymerization of Lignite

It was previously shown[9] that horseradish peroxidase (HRP) is active in dioxane-water and DMF-water solutions in the one-electron oxidation of phenolic units of kraft lignin, a relatively inert polymeric compound. Horseradish peroxidase suspended in dioxane was also reported[1] to cause the limited solubilization and depolymerization of leonardite, a low rank coal.
In our prelimenary investigation we found that peroxidase from soybean hulls (SBP) possesses a much higher oxidative potential in comparison to horseradich peroxidase. Oxidative cleavage of *natural coal --Mequininza lignite-- has* been attempted using both peroxidases. This lignite represents a highly oxidized, non-bituminous coal.

The lignite powder is poorly soluble in tetrahydrofuran (THF) and dioxane (less than 1% w/w from total amount) and insoluble in water. Both THF and dioxane solubilize only low molecular weight fractions with molecular weight less than 2 kD (data not shown). DMF has been found to dissolve ca. 25% of coal. The solution in DMF contains a broad range of molecular weights as it is seen from Fig. 1, with some coal polymers soluble at molecular weights > 50 kD.
The DMF-soluble coal was fractionated on the GPC column and fraction number 11 (4 kD) was collected for further experimentation. At this molecular weight, both polymerization and depolymerization of the coal fraction can be monitored.

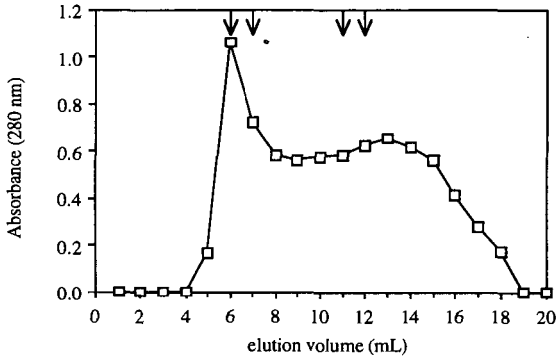

Fig. 1. Gel permeation chromatography of Mequininza lignite fractions soluble in DMF. Arrows indicate molecular weight markers: from left to right, 50, 15, 4, and 2 kD.

Lignite modification reactions were studied using HRP and SBP catalysis with this intermediate molecular weight fraction in 50% DMF at pH 5 (50 mM acetate buffer). In both cases, the incubations resulted in turbid solutions. Control reactions which included water insted of hydrogen peroxide did not lead to formation of turbudity. Sediments were not completely soluble in pure DMF. Figures 2 and 3 depict changes in the molecular weight profile of the lignite fraction for HRP and SBP as catalysts. In both cases, following 24 h, clear polymerization was observed. The highest soluble polymeric products had molecular weights > 50 kD. The DMF-insoluble fractions appear to consist of high molecular weight polymers produced during peroxidase catalyzed oxidative cleavage of coal structure. No evidence of depolymerization was detected. These findings are in accordance with previous literature obsrvations[1,10] that peroxidases are excellent catalysts to oxidize phenolic polymers such as lignins and coals, particularly in the presence of organic solvents.

Oxidative Power of Soybean Peroxidase -- Depolymerization of Coal in Aqueous DMF Solutions

In related research, we have studied the oxidative ability of SBP. The enzyme is highly active and stable under acidic conditions. At low values of pH, it has been observed that SBP is capable of oxidizing veratryl alcohol (personal communication of J. P. McEldoon). The catalytic activity of SBP on veratryl alcohol was strongly dependent on the pH and the presence of calcium ions. It was found that the pH optimum of veratryl alcohol oxidation is ca. 2.15 with very little oxidation above pH 3. Furthermore, the enzyme is strongly stabilized by $CaCl_2$ concentrations of up to 0.1 M. Evidently, the low pH results in a significant increase in the oxidation potential of SBP and enables a compound such as veratryl alcohol to be oxidized. Given the high oxidation potential of SBP at low pH, it occured to us that this enzyme is acting more as a lignin peroxidase than a typical plant peroxidase. In that regard, we proceeded to re-investigate the action of SBP on DMF-soluble lignite at pH 2.2. Figure 4 shows that both polymerization and depolymerization occured after 24 h incubation of fraction 11 of the lignite with SBP in 50% DMF, 0.1 M tartrate buffer, pH 2.2 containing 0.1 M $CaCl_2$. Approximately 10% of the initial lignin precipitated out of the enzyme reaction, indicating that some very high molecular weight material was produced. In the absence of enzyme, no coal modification took place.

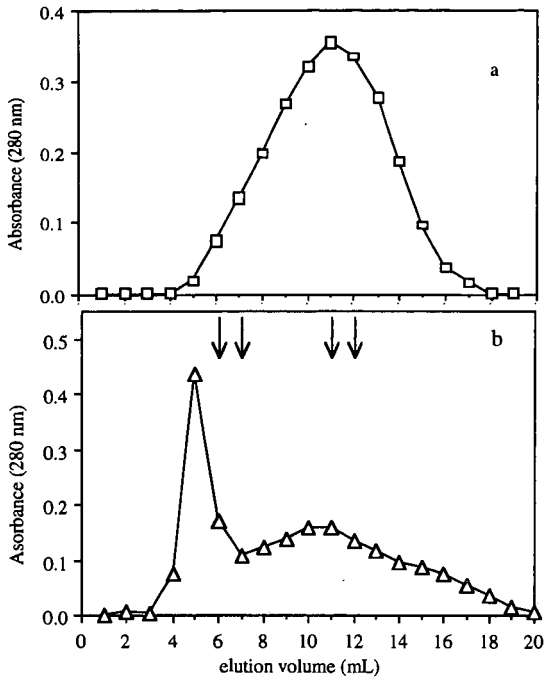

Fig. 2. Gel permeation chromatography of Mequininza lignite fraction No 11 (MW 4 kD) before incubation with horseradish peroxidase (a) and after 24 h incubation at pH 5 in 50% DMF (b).

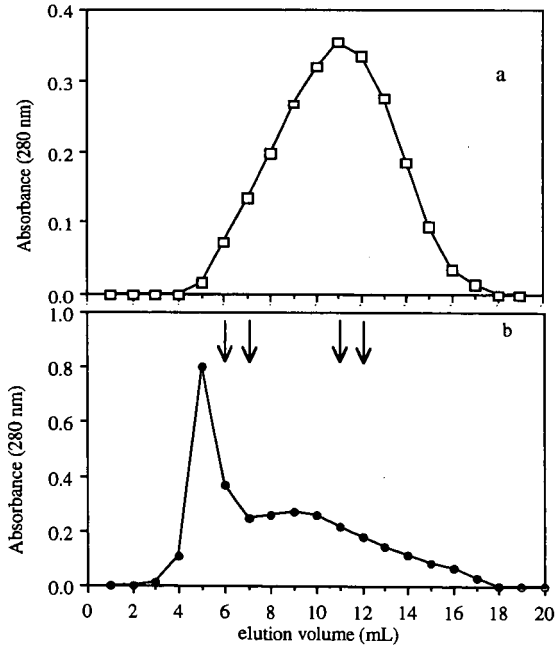

Fig. 2. Gel permeation chromatography of Mequininza lignite fraction No 11 (MW 4 kD) before incubation with soybean peroxidase (a) and after 24 h incubation at pH 5 in 50% DMF (b).

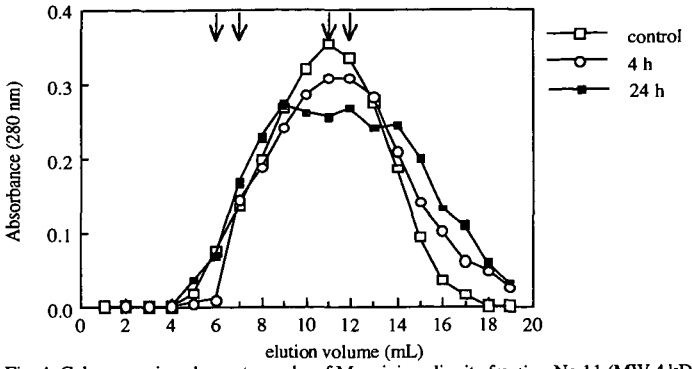

Fig. 4. Gel permeation chromatography of Mequininza lignite fraction No 11 (MW 4 kD) before incubation with soybean peroxidase and after incubation at pH 2.2 (0.1 M tartrate buffer, 0.1 M $CaCl_2$) in 50% DMF.

We reasoned that a relatively low molecular weight fraction of lignite was more susceptible to polymerixation than depolymerization. To test this hypothesis, we examined the action of SBP in 50% DMF (pH 2.2) with a higher molecular weight fraction of DMF-soluble lignite. Figure 5 shows that the high molecular weight fraction (ca. 50 kD) was unaffected in the absence of the enzyme, yet was obviously depolymerized in the presence of SBP.

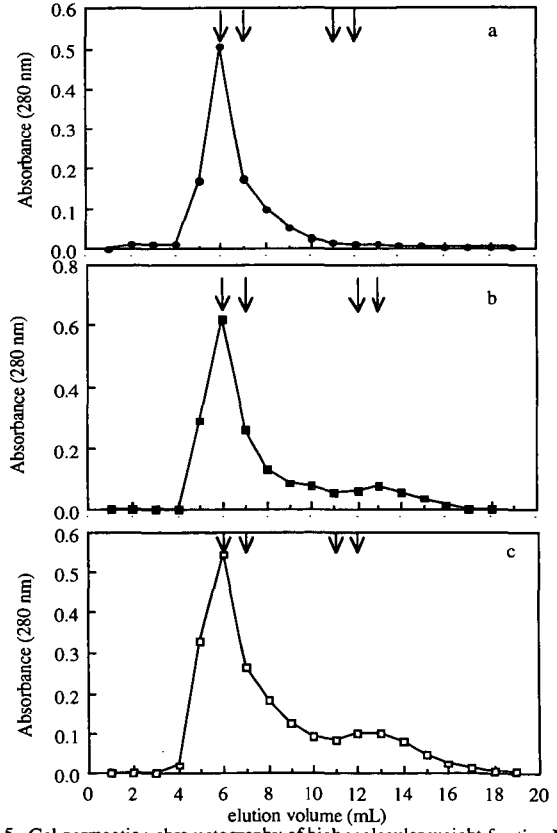

Fig. 5. Gel permeation chromatography of high molecular weight fraction No 6 (MW 50 kD) of Mequininza lignite before incubation (a) with soybean peroxidase and after 4 h (b) and 24 h (c) incubation at pH 2.2 (0.1 M tartrate buffer, 0.1 M $CaCl_2$) in 50% DMF.

Activity of Oxygenase Enzymes on Coal Model Compounds

The unique specificities and reactivitites of oxygenase enzymes in organic solvents can be exploited for biocatalytic transformation of coal. Mono- and dioxygenases from bacteria consist of a three-enzyme system comprised of an iron-sulfur hydroxylase/oxygenase, a ferridoxin electron transfer protein, and an NAD(P)H reductase. The reaction mechanism of this multienzyme system remains poorly characterized and the use of the isolated enzyme system has been poorly successful. Nevertherless, several promising biotransformations have been performed on coal-related compounds using whole cells. For example, Laborde and Gibson demonstrated[10] that fermentation of dibenzothiophene (DBT) with *Beijerinckia* B8/36 resulted in the accumulation of 1,2-dihydroxy-1,2-dihydrodibenzothiophene and dibenzothiophene-5-oxide in the cell culture. Further reatment of the first product by the *Beijerinckia* sp. led to the accumulation of 1,2-dihydroxydibenzothiophene. We have initially evaluated the catalytic activity of the *Beijerinckia* enzyme system in aqueous-organic media. The whole cells were unable to grow in the solvents, yet retained intrinsic enzyme activity. Conversions (ca. 25%) of both biphenyl and DBT were observed in the media containig up to 50% of DMF or dioxane. Moreover, several unidentified products that were not detected after fermentation of biphenyl and DBT with *Beijerinckia* sp.in aqueous media were found in aqueous-organic mixture. It was also found that the *Beijerinckia* enzyme system from the lyophilized cells is active in non-aqueous media in the presence of NADH. Cofactor regeneration system comprising horse liver alcohol dehydrogenase and ethanol was applied to provide long term activity of the oxygenase system from a fine powder of the freeze-dried cells of *Beijerinckia* sp.in non-aqueous media. Improvement of the ability of the *Beijerinckia* oxygenase enzyme system to catalyse coal model compounds degradation in aqueous-organic media is the subject of continuing investigations.

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