Ninth Quarterly Report
Regulation of Coal Polymer Degradation by Fungi
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Disclaimer

Abstract

Several investigations have demonstrated that oxalate anion secreted by fungi is able to mediate solubilization of leonardite, a highly oxidized lignite. During this reporting period, we have used a biomimetic approach to study oxalate mediated solubilization of several Argonne Premium Coals. Results showed that, relative to leonardite, oxalate solubilized minimal amounts of these coals. Other studies showed that pH has a dramatic effect on solubilization of leonardite by several Lewis bases. In general, solubilization appeared to be a function of ionization of the Lewis base. Coal solubilization is estimated by an increase in the visible spectrum of aqueous solutions containing coal and a solubilizing agent. Because leonardite solubilization was studied over a broad pH range, it was necessary to determine if pH has a substantial effect on the absorbance of soluble coal macromolecule. Results showed that absorbance at 600 nm increased by ~56% between pH 4.5 and pH 12.0. Clearly, this increase must be considered when interpreting coal solubilization data. The decolorization of soluble coal macromolecule in nutrient nitrogen limited cultures of *Phanerochaete chrysosporium* was also studied. In stationary and agitated cultures, respectively, 83.8% $\pm$ 2.3% and 89.6% $\pm$ 1.0% of the coal macromolecule was decolorized during 8 days of incubation.

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Executive Summary

Research in our laboratory focuses on the metabolism of leonardite (a low rank coal) by lignin degrading fungi. A number of investigators have shown that oxalate ions that are secreted by such fungi chelate metal ions in leonardite; thereby breaking ionic bridges that link coal macromolecules. Once these ionic linkages are broken, the relatively polar coal macromolecules are water soluble. A major objective of our research is to determine if leonardite solubilization and its subsequent depolymerization are distinct events. Evidence to date suggests that this is, indeed, the case. While leonardite solubilization appears to be mediated by oxalate (and possibly other metal chelators), its subsequent metabolism appears to be mediated by the lignin degrading system. The details of subsequent metabolism by lignin degrading enzymes are, however, unclear and a topic of continued investigation.

Introduction

Our research efforts focus on the ability of lignin degrading fungi to solubilize and depolymerize a low rank coal. Previous investigations by others (1,2,3) have shown that fungi solubilize leonardite by secreting oxalate anions which break ionic linkages by chelating metals that link coal macromolecules. We have partially characterized oxalate mediated leonardite solubilization using a biomimetic approach in which sodium oxalate is used to solubilize leonardite. Briefly, we have shown that this process is relatively slow and continues at a measurable rate for weeks. We have also shown that, in addition to oxalate, phosphate and bicarbonate/carbonate ions are also able to mediate solubilization of leonardite. Furthermore, solubilization was found to be pH dependent. Much of this information has appeared in previous quarterly reports.

A major objective of this research is to test the hypothesis that leonardite solubilization and subsequent depolymerization of solubilized coal macromolecule are distinct events in lignin degrading fungi. Oxalate secreted by fungi has been shown to mediate solubilization of leonardite. Indeed, this compound likely has an important role in this process. It should be noted, however, that other common fungal metabolites may, in some species, be important in this process. For example, several Krebs cycle intermediates have been shown to mediate solubilization of leonardite *in vitro.* This could prove important in fungi that secrete high concentrations of such compounds. There have been reports (4,5) in the literature suggesting that lignin degrading enzymes, primarily laccases, may have a role in low rank coal solubilization. Subsequent investigations in our laboratory (unpublished) and by others (6) do not support a role for lignin degrading enzymes in leonardite solubilization. On the other hand, lignin peroxidase from *Phanerochaete chysosporium* has been reported to depolymerize coal macromolecule that was chemically solubilized from a North Dakota Lignite and a German Sub-bituminous coal (7). Furthermore, we have shown that decolorization of soluble coal macromolecule occurs in nutrient nitrogen limited (*i.e.,* ligninolytic) but not in nutrient nitrogen sufficient (*i.e.*, nonligninolytic) malt agar cultures of *P. chrysosporium.* It thus appears that leonardite solubilization and its depolymerization are, indeed, distinct events in lignin degrading fungi. Chelation of metal ions by oxalate (or, possibly other metal chelators) appears to be the dominant mechanism by which solubilization occurs while enzymes of the lignin degrading system appear to be responsible for further metabolism of coal macromolecule solubilized from leonardite.

Although subsequent metabolism of soluble coal macromolecule appears to be mediated by the lignin degrading system, the details by which this occurs remains to be elucidated. For example, although we have shown that ligninolytic cultures of *P. chrysosporium* decolorize coal macromolecule, we have also shown that relatively low concentrations of soluble coal macromolecule from leonardite inactivates lignin peroxidase *in vitro*. How this occurs is one of the topics of ongoing research in our laboratory. Current topics of research also focus on: 1) determining the extent to which other coals are solubilized by oxalate; 2) further assessing the effect of pH on leonardite solubilization by selected metal chelators; 3) developing a better method for

assessing the molecular weigh range of soluble coal macromolecule and 4) continued study of the decolorization of coal macromolecule by ligninolytic cultures of *P. chrysosporium.*

Results and Discussion

Solubilization of Oxidized and Nonoxidized Argonne Premium Coals by Sodium Oxalate.

Because leonardite is solubilized by oxalate ion, it was logical to ask if other coals are solubilized by this natural chelating agent. The effect of oxalate on the solubilization of several oxidized and nonoxidized Argonne Premium Coals is presented in figure 1. Results show that, relative to leonardite, only very small amounts of these coals are solubilized by oxalate ion.

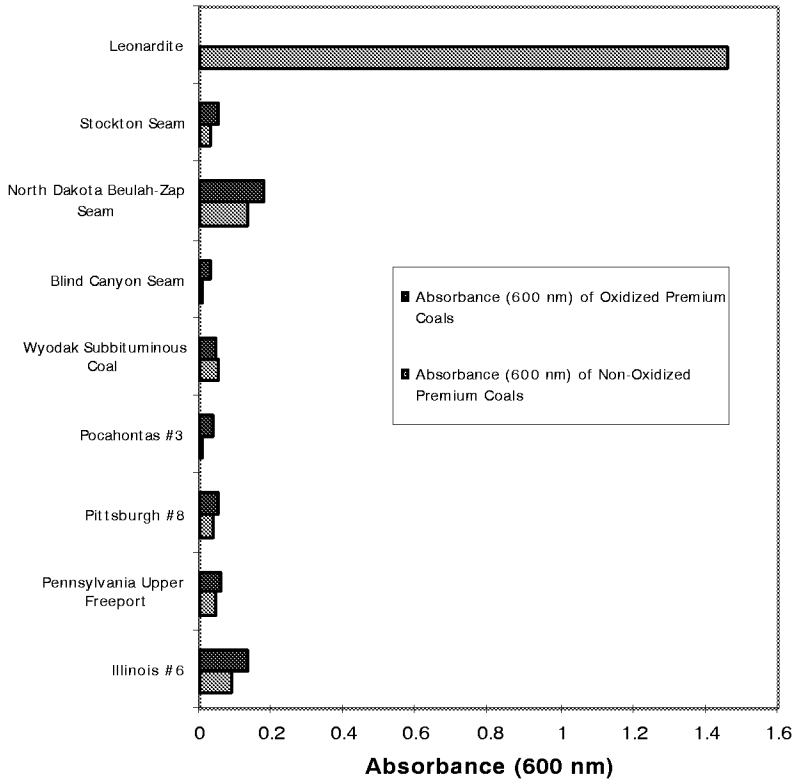

Figure 1. Comparison of oxalate mediated solubilization of leonardite with that of eight Argonne Premium Coals. For each coal, a 10 mL suspension containing twenty mg of each coal and 10 mL of 74.6 mM sodium oxalate was prepared and mixed for 24 h an aliquot was then centrifuged and the absorbance of the sample at 600 nm was determined.

These results also suggest that only the most highly oxidized coals are susceptible to substantial oxalate mediated solubilization.

Studies of oxalate solubilization of coal can be used as an investigative tool to partially

assess the mechanism by which coals are solubilized by fungi. As a case in point, Stewart and associates (8) showed that substantial solubilization of Pittsburgh #8 bituminous coal by *Penicillium sp.* and *Cunninghamella sp.* occurred only after preoxidation of the coal by exposure to heat (150^{o}C) for seven days. Of interest is the fact that neither *P. chrysosporium* nor *T. versicolor* solubilized this material. Of further interest is the fact that our studies show that oxalate solubilization of preoxidized Pittsburgh #8 bituminous coal is, at best, minimal. Taken together these studies indicate that certain fungi must possess a mechanism for coal solubilization that is not based on the action of oxalate ion.

Effect of pH on the biomimetic solubilization of leonardite.

In our last quarterly report we showed that leonardite solubilization by several chelators is pH dependent. In this experiment, leonardite was incubated for 24 hr in selected buffers. Samples were then centrifuged, diluted with water and absorbance at 600 nm was determined. This was an error in experiment design as samples should have been diluted with the buffer in which they were incubated. We have, therefore, repeated this experiment using appropriate buffers for sample dilution. Results were very similar to those in which water was used as the diluent.

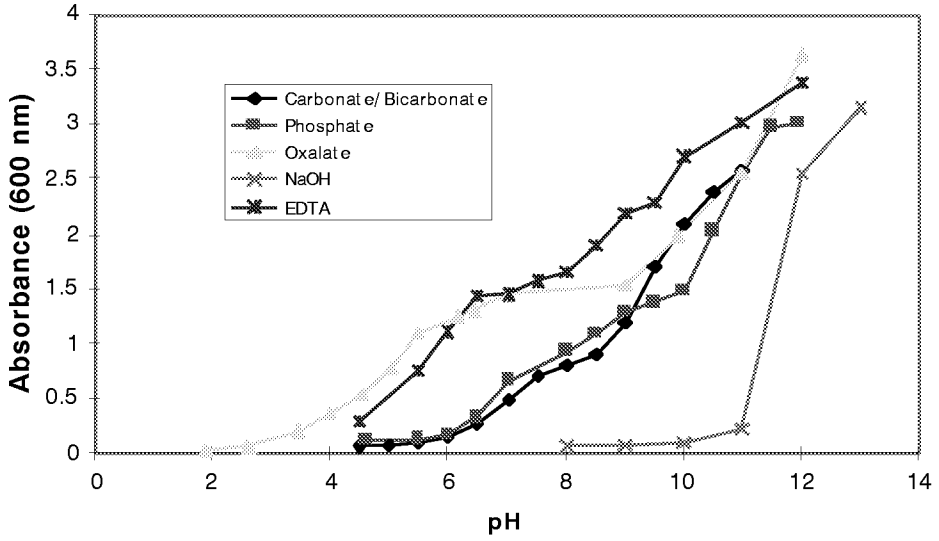

Figure 2. Effect of pH on solubilization of leonardite in the presence of EDTA, sodium oxalate, potassium phosphate, sodium bicarbonate/carbonate or sodium hydroxide. Twenty mg of leonardite was incubated on a rotatory shaker for 24 h in 10 mL of 74.6 mM sodium oxalate, 75 mM potassium phosphate, sodium bicarbonate/carbonate or sodium hydroxide at the indicated pH. After incubation, the absorbance at 600 nm was acquired.

Effect of pH on absorbance

The extinction of a chromophore often varies with pH. Because coal solubilization experiments have been carried out over a wide pH range, we found it necessary to determine what effect pH may have on the absorbance of soluble coal macromolecule. Results demonstrated that the absorbance at 600 nm of soluble coal macromolecule increases with increasing pH between pH 4.5 and pH 10.0. A more pronounced increase in absorbance occurred between pH 10.0 and 12.0. These pH dependent changes need to be considered when interpreting coal solubilization experiments.

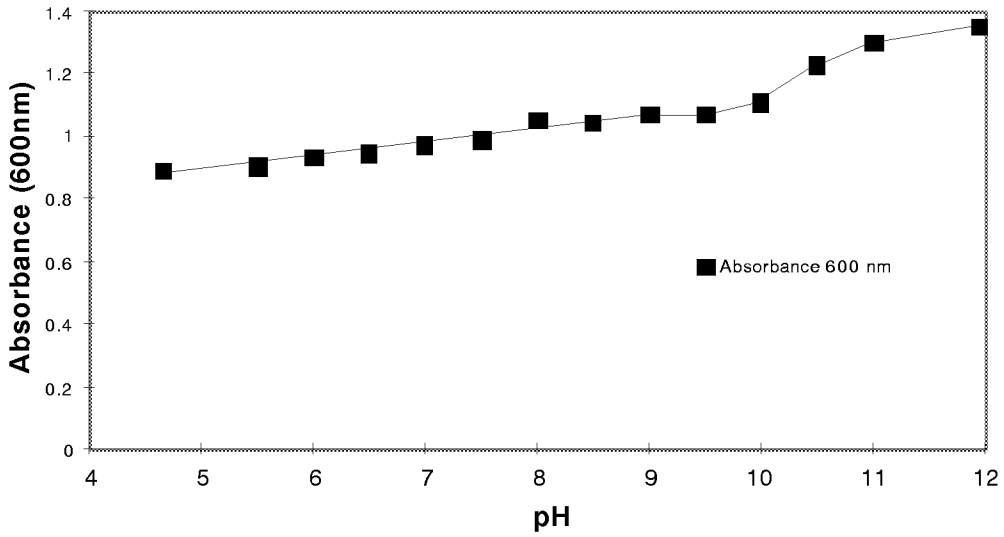

Figure 3. Effect of pH on the absorbance of solubilized coal macromolecule at 600 nm. Oxalate solubilized coal macromolecule was dissolved in 10 mL of 75 mM potassium phosphate buffer at the indicated pH values.

Molecular Weight Determination

The molecular weight of coal macromolecule is estimated by GPC-HPLC using proteins of known molecular weight as standards. In these assays, coal macromolecules elute as a relatively symmetrical peak. However, unlike the protein standards, it is known coal macromolecule is a mixture rather than a homogeneous entity and the retention time of the peak is only a rough estimate of the mean

molecular weight of the coal macromolecules that comprise this mixture. In order to estimate the molecular weight range of coal macromolecules that are present in this mixture, a 20 μl aliquot of concentrated coal macromolecule was assayed by GPC-HPLC and fractions were collected at one-half minute intervals. Aliquots (20 μl) of these fractions were then individually assayed by GPC-HPLC.

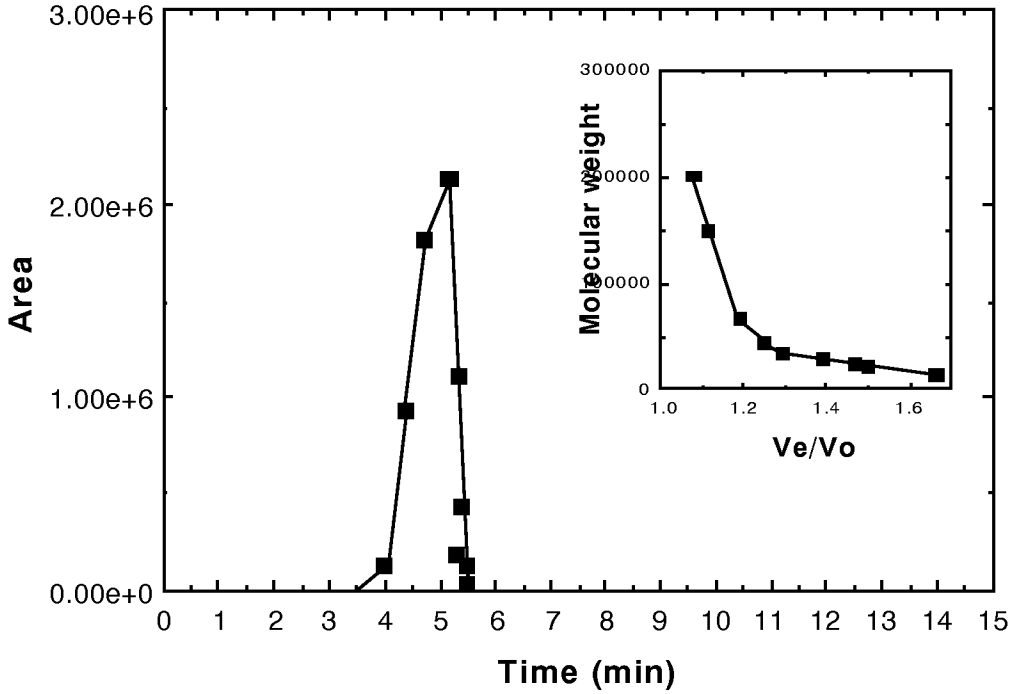

Figure 4. Estimation of the molecular weight range of soluble coal macromolecule. During GPC-HPLC of a 20 μL aliquot of solubilized coal macromolecule (2.1 mg/mL), fractions were collected every 0.5 min. Aliquots (20 μL) of fractions containing material have UV absorbance at 280 nm were then reinjected into the HPLC and average molecular weight of material in each fraction was determined. Inset graph. standard curve used to estimate mean average molecule weight of soluble coal macromolecule. Molecular weight standards used to construct this standard curve included sweet potato ß-amylase (MW 200,000), yeast alcohol dehydrogenase (MW 150,000), bovine serum albumin (MW 66,000), chicken egg ovalbumin (MW 45,000), carbonic anhydrase (MW 29,000), bovine pancreas trypsinogen (MW 24,000), soybean trypsin inhibitor (MW 20,100), bovine ß-lactoglobulin (MW 18,400 (elutes as a dimer)) and chicken egg white lysozyme (MW 14,300).

Results (Figure 4) demonstrated that the molecular weight of solubilized coal macromolecule ranged from about 14,000 (the molecular weight cut-off of the dialysis tubing used to prepare the sample) to approximately 66,000.

Decolorization of Coal Macromolecule in Liquid Cultures.

Previous investigations in our laboratory have documented the decolorization of coal macromolecule by *P. chrysosporium* in malt agar cultures. Furthermore, in initial investigations we have shown that coal macromolecule is decolorized in liquid cultures. Decolorization might be useful as a surrogate assay for depolymerization. However, these initial studies have been inconclusive as we have not yet been able to conclusively correlate decolorization, measured at 600 nm, with a decrease in GPC-HPLC peak area, measured at 280 nm. Thus, it may be that the absorbance of the chromophore in coal macromolecule is reduced without a substantial accompanying decrease in molecular weight. This possibility is still under investigation. Our most recent experiments show that substantial decolorization occurred in stationary and agitated nutrient nitrogen limited cultures of *P. chrysosporium* during an eight day incubation period. In stationary and agitated cultures, respectively, 83.8% $\pm$ 2.3% and 89.6% $\pm$ 1.0% decolorization was observed after 8 days of incubation at 39^{o}C. GPC-HPLC analysis of these samples is in progress.

Conclusions

Studies to date indicate that coal solubilization and depolymerization by lignin degrading fungi are distinct events. It also appears that, in the case of *P. chrysosporium*, the nutritional state of the fungus is important for subsequent metabolism as decolorization occurs in nutrient nitrogen limited (ligninolytic) cultures, but not in nutrient nitrogen sufficient (nonligninolytic) cultures. The details and mechanism by which further metabolism of coal macromolecule occurs is unclear and requires further study.

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