Sixteenth Quarterly Report
Regulation of Coal Polymer Degradation by Fungi
(DE-FG22-94PC94209) -16

July 31, 1998

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Disclaimer

Abstract

Three phenomena which concern coal solubilization and depolymerization were studied during this reporting period. Previous investigations have shown that lignin peroxidases mediate the oxidation of soluble coal macromolecule. Because it appears to be a substrate, soluble coal macromolecule is also an inhibitor of veratryl alcohol oxidation, a reaction that is mediated by these enzymes. The mechanism of inhibition is complex in that oxidation (as assayed by decolorization) of soluble coal macromolecule requires the presence of veratryl alcohol and veratryl alcohol oxidation occurs only after a substantial lag period during which the soluble coal macromolecule is oxidized. In a previous quarterly report we proposed a reaction mechanism by which this may occur. During the present reporting period we showed that our proposed reaction mechanism is consistent with classical enzyme kinetic theory describing enzyme activity in the presence of a potent inhibitor (*i.e.,* an inhibitor with a very low K_I). The oxidative decolorization and depolymerization of soluble coal macromolecule was also studied. Because wood rotting fungi produce hydrogen peroxide via a variety of reactions, we studied the effect of hydrogen peroxide on soluble coal macromolecule decolorization and depolymerization. Results showed that substantial decolorization occurred only at hydrogen peroxide concentrations that are clearly non-physiological (*i.e*., 50 mM or greater). It was noted, however, that when grown on solid lignocellulosic substrates, wood rotting fungi, overtime, cumulatively could produce amounts of hydrogen peroxide that might cause significant oxidative degradation of soluble coal macromolecule. Thirdly, we have shown that during oxalate mediated solubilization of low rank coal, a pH increase is observed. During this reporting period we have shown that the pH of solutions containing only sodium oxalate also undergo an increase in pH, but to a lesser extent than that observed in mixtures containing sodium oxalate and low rank coal. It is our hypothesis that bicarbonate ion is formed during oxalate mediated solubilization of low rank coal and this is responsible for the increase in pH that is observed. A general reaction by which this occurs is proposed herein.

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Executive Summary

During this reporting period we continued our investigations concerning the ability of lignin peroxidases from the wood rotting fungus *Phanerochaete chrysosporium* to degrade soluble coal macromolecule. A kinetic analysis is provided that is consistent with and supports a reaction mechanism that we presented in our fifteenth quarterly report. We also studied the ability of hydrogen peroxide to oxidize soluble coal macromolecule. Hydrogen peroxide is produced by a variety of fungal processes. We found that the relatively high (50 mM or greater) concentrations necessary to mediate substantial oxidation of soluble coal macromolecule were clearly non-physiological. Lastly we continued to study the pH increase that accompanies oxalate mediated solubilization of low rank coal. It is our hypothesis that the pH increase observed is due to the formation of bicarbonate ion from oxalate ion.

Introduction

Wood rotting fungi and other fungi mediate solubilization and depolymerization of low rank coals (1). It is our goal to better understand the details by which these processes occur. It has been reported that lignin peroxidase (2) and Mn peroxidases (3,4) mediate the oxidative depolymerization of soluble low rank coal macromolecule. However, these systems need to be better characterized; especially in view of the fact that soluble coal macromolecule is a complex mixture and components of this mixture tend to self associate. This, of course, can make difficult the interpretation of depolymerization experiments. In this report, we describe some of our observations concerning the inhibition of lignin peroxidase by soluble coal macromolecule. During this reporting period, we also assessed the ability of hydrogen peroxide (a cosubstrate secreted by wood rotting fungi that is required for lignin peroxidase and Mn peroxidase activity) to mediate oxidative degradation of soluble coal macromolecule. Additionally, we reinvestigated the pH increase that occurs during the solubilization of low rank coal by sodium oxalate and we propose a reaction by which this pH increase may occur.

Methods and Materials

Lignin peroxidase activity was assessed using the veratryl alcohol oxidase assay at room temperature as described (5). Oxalate solubilized coal macromolecule was prepared as described by Bumpus *et al.* (6) and base solubilized coal macromolecule was prepared as described by Wondrack *et al.* (2) and

Results and Discussion

Oxidation of soluble coal macromolecule by lignin peroxidases.

In previous quarterly reports we noted that soluble coal macromolecule appears to be a competitive inhibitor of lignin peroxidase mediated veratryl alcohol oxidation. We also noted that in some inhibition experiments a lag period occurred (Figure 1) while in other experiments no lag period was observed. This phenomenon has been reinvestigated. It appears that the lag period is only observed at relatively low concentrations of lignin peroxidase and soluble coal macromolecule. When the concentrations of both lignin and soluble coal macromolecule were increased at the same ratio, the lag period disappeared (Figure 2). The lag period and its

disappearance occurs when either oxalate solubilized coal or base solubilized coal is used.

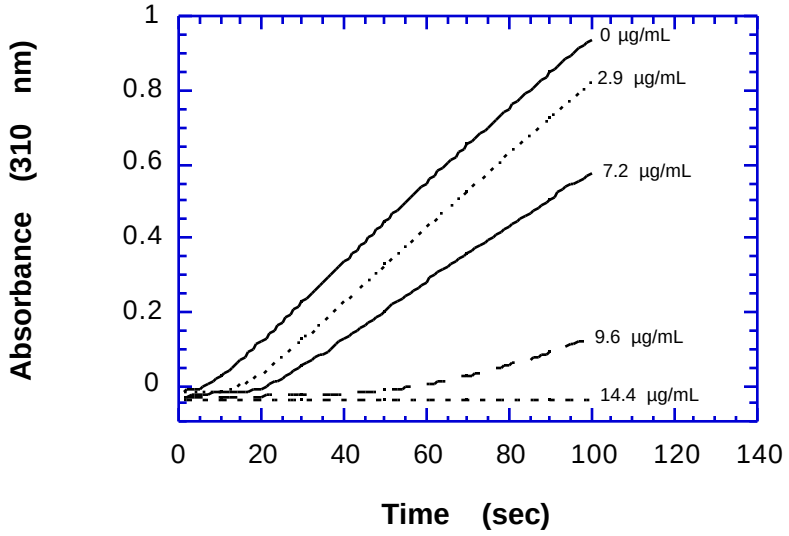


Figure 1. Effect of increasing concentration of soluble coal macromolecule on lignin peroxidase activity. The reaction took place in a volume of 1 ml in 220 mM sodium tartrate buffer, pH 4.5, containing 1.67 mM veratryl alcohol, 8.8 µg lignin peroxidase and 0.5 mM hydrogen peroxide. The concentration of oxalate solubilized coal macromolecule present varied from 0 to 14.4 µg/mL.

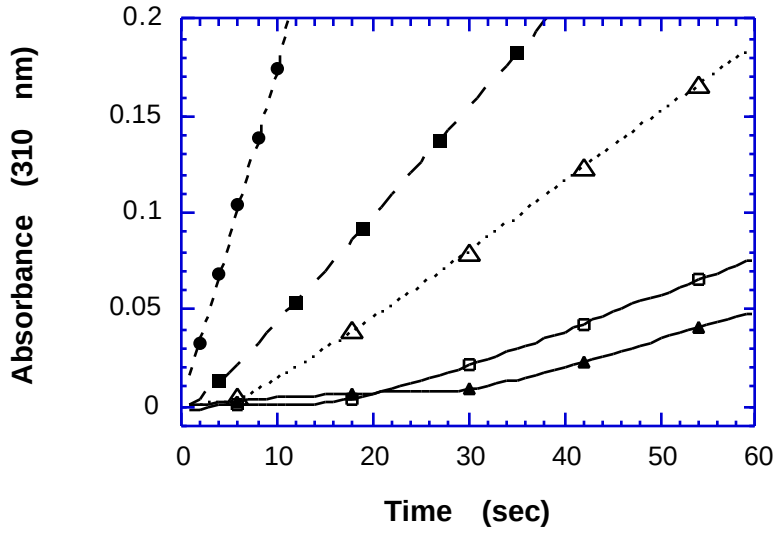


Figure 2. Effect on enzyme activity of increasing the amount of soluble coal macromolecule and lignin peroxidase at a constant ratio. Solid triangles-50 µg soluble coal macromolecule and 0.88 µg lignin peroxidase; Open squares-200 µg soluble coal macromolecules, 3.53 µg lignin peroxidase; open triangles-250 µg soluble coal macromolecule,4.4 µg lignin peroxidase; solid squares-350 µg soluble coal macromolecule, 6.16 µg, lignin peroxidase; Solid circles-0 ug soluble coal macromolecule, 0.88 µg lignin peroxidase. The reaction took place in a volume of 1 ml in 220 mM sodium tartrate buffer, pH 3.5, containing 1.67 mM veratryl alcohol and 0.5 mM hydrogen peroxide. For this experiment, base solubilized coal macromolecule was used.

The relationship describing the initial velocity of an enzyme reaction in the presence of an inhibitor is as follows:

$$v_o = \frac{[V_{max}]\,[S]}{K_m[1 + I/K_I] + [S]}$$

Equation 1

Where:

Vo = initial velocity.

Vmax = maximum velocity.

Km = the Michaelis-Menton Constant for the enzyme.

I= the concentration of the enzyme inhibitor.

K_I = the Inhibitor constant.

This equation predicts that in the presence of a very potent competitive inhibitor (*i.e.,* one with a very small K_I) that is also a substrate, the enzyme's affinity for the inhibitor is such that the inhibitor is preferentially attacked and substantial catalysis of the substrate occurs only when the inhibitor concentration reaches very low levels. This phenomenon is illustrated in figure 3 for a hypothetical enzyme reaction and inhibitors having K_I values of 1×10^{-3} mM, 1×10^{-5} mM and 1×10^{-7} mM. As expected, as the enzyme mediates a decrease in inhibitor concentration, the initial velocity of the enzyme reaction increases. However, in the presence of the most potent inhibitors (those having K_I values of 1×10^{-5} mM and 1×10^{-7} mM) it appears that the substrate is converted to product only after a substantial lag period during which time the inhibitor was reduced to very low concentrations·

This is consistent with the reaction mechanism (Scheme 1) we have proposed for oxidation of soluble coal macromolecule by lignin peroxidase. In this mechanism, hydrogen peroxide first oxidizes the enzyme to the reactive peroxidase intermediate known as Compound I which mediates the one electron oxidation of veratryl alcohol to veratryl

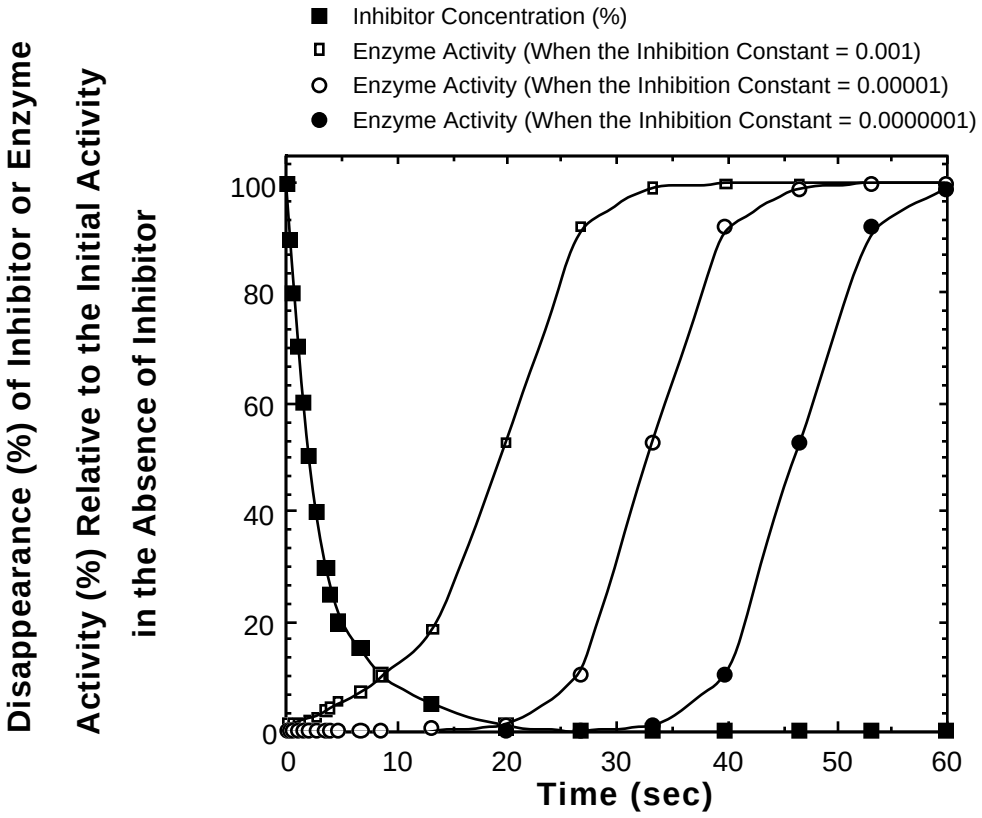


Figure 3. Effect of potent enzyme inhibitors on the initial activity of an enzyme as a function of time. Theoretical enzyme activity data was calculated using equation 1, assuming that the Km of the enzyme for the substrate was 0.089 mM and that the initial concentration of the the substrate was 2.0 mM. The rate equation for a first order reaction was used to calculate enzyme mediated disappearance of the inhibitor. A $t_{1/2}$ of 2 sec and a first order rate constant (k) of 0.3465 sec^{-1} was used in these calculations.

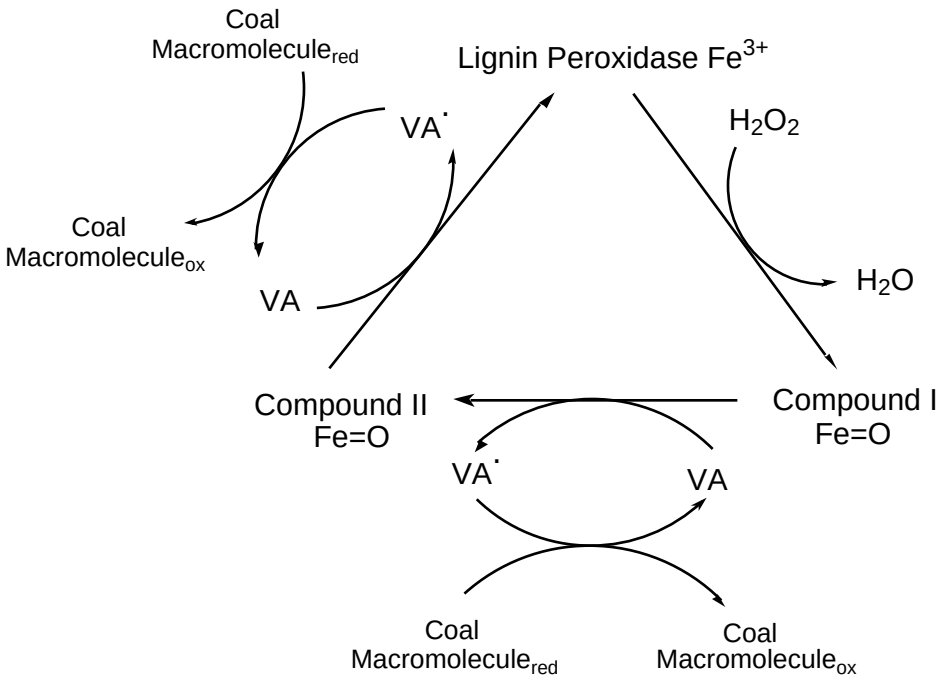
alcohol cation radical. Normally, this radical undergoes another one electron oxidation by lignin peroxidase to form veratryl aldehyde, the product whose absorbance at 310 nm is measured in this assay. However, in the presence of soluble coal macromolecule, the veratryl alcohol cation radical functions as a secondary oxidant by oxidizing the soluble coal macromolecule. In this process, the coal macromolecule is decolorized and the veratryl alcohol radical is converted back to veratryl alcohol.

It should be noted that equation 1 predicts that catalysis of substrate does occur, but at very low levels. For example, although the velocities of the reactions occurring in the presence of the inhibitors having K_I values of 1×10^{-5} mM and 1×10^{-7} mM appear to be zero in figure 3, they actually increase from 0 to 1.1% and from 0 to 0.11%, respectively, during the first 20 seconds of the apparent lag periods.

It was, therefore, of interest for us to determine if very low rates of reaction were occurring in incubations containing coal macromolecule, veratryl alcohol and lignin peroxidase. In most experiments in which coal macromolecule was present, measurable rates during the first part (*i.e.,* the first 10 seconds) of the lag periods were not found (figure 1). However, this too is consistent with and, indeed, predicted, by our proposed reaction mechanism (Scheme 1) in which the veratryl alcohol cation radical is reduced back to veratryl alcohol thus preventing accumulation (and measurement) of veratryl aldehyde, the product of this reaction.

Veratryl aldehyde would be expected to accumulate only when the concentration of oxidizable coal macromolecule becomes limiting. Figure 2 shows that when the concentrations of lignin peroxidase and coal macromolecule are increased at the same ratio, the lag period disappears. This too supports our proposed reaction mechanism because increasing enzyme concentration would be expected to increase the rate of veratryl alcohol cation radical formation which, in turn, would cause an increase in the rate of coal macromolecule oxidation resulting in the disappearance of the lag when higher enzyme concentrations are used.

In addition to oxidizing the veratryl alcohol cation radical back to veratryl alcohol, it appears that at higher concentrations soluble coal macromolecule some functional groups on soluble coal may cause it to function as a reversible competitive inhibitor (*i.e.,* as an inhibitor that reversibly binds the active site but is not oxidized by the enzyme) (2) . Further complicating the kinetics of inhibition is the observation that sufficiently high concentrations of coal macromolecule causes formation of precipitable inactive enzyme-coal macromolecule complexes. Thus, in addition to the two types of inhibition described above, coal macromolecule also functions an a non-mechanism based inactivator of the enzyme.



Scheme 1. Proposed reaction mechanism for oxidation of soluble coal macromolecule by lignin peroxidase from *Phanerochaete chrysosporium*.

On the role of extracellular oxidases in the biodegradation of soluble low rank coal macromolecules.

In previous quarterly reports we noted that in nutrient nitrogen limited culture, *P. chrysosporium* is able to decolorize soluble coal macromolecule. The fact that decolorization occurred in nutrient nitrogen limited cultures and not in nutrient nitrogen sufficient cultures suggests involvement of the lignin degrading system of this fungus. Previous reports by others (2) have shown that soluble coal macromolecule is a competitive inhibitor of lignin peroxidase mediated veratryl alcohol oxidations. It was also reported that lignin peroxidases mediate depolymerization of soluble coal macromolecule. We have confirmed that coal macromolecule is a lignin peroxidase inhibitor and we have shown that the mechanism of this inhibition is complex. We also showed that lignin peroxidase can mediate partial decolorization of soluble coal macromolecule. However, we have found no compelling evidence that unequivocally, demonstrates that lignin peroxidases mediate substantial depolymerization of soluble coal macromolecule. Indeed, our observations (noted in previous quarterly reports) that soluble coal macromolecule undergoes self associations suggests that reports of such depolymerization should be interpreted with caution. Further investigations

using Mn peroxidase and a laccase are planned.

Additionally, we are studying the effect of hydrogen peroxide on soluble coal macromolecule. A variety of fungal enzymes result in hydrogen peroxide formation. Interestingly, hydrogen peroxide producing enzymes are among the enzymes produced in ligninolytic cultures of *P. chrysosporium.* Because hydrogen

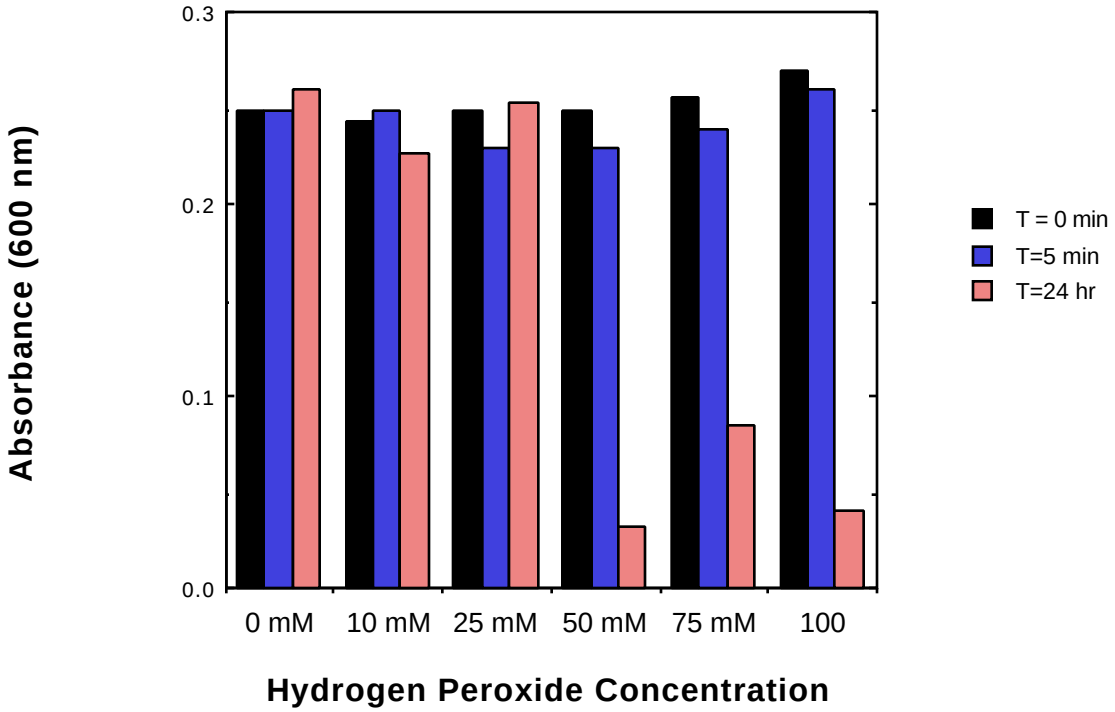


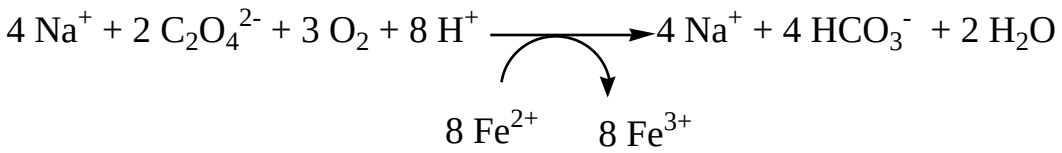
Table 1. Ability of hydrogen peroxide to decolorize soluble coal macromolecule.

peroxide itself is a good oxidant, we studied the ability of this compound to decolorize and depolymerize soluble coal macromolecule. Table 1 shows that hydrogen peroxide does, indeed, decolorize soluble coal macromolecule. However, the concentrations of hydrogen peroxide required to do this are clearly too high to be considered physiological and would, indeed, be toxic to the fungus. Furthermore *P. chrysosporium*, like all aerobic organisms, produces enzymes (catalases and peroxidases) that destroy hydrogen peroxide and thereby protect the organism from

this toxic metabolite. Thus, it is very unlikely that such concentrations would be produced *in vivo*. However, if one considers the fact that, on a suitably large amount of a good growth substrate, the amount of hydrogen peroxide produced by a microorganism over time might be sufficient to cause substantial oxidative degradation of susceptible compounds. This would assume, of course, that all of the hydrogen peroxide produced was not destroyed enzymatically. For this reason, we have initiated studies to determine if hydrogen peroxide that is slowly generated enzymatically is capable of degrading soluble coal macromolecule.

Further studies on the solubilization of low rank coal by sodium oxalate.

We have characterized sodium oxalate mediated solubilization of leonardite (Bumpus *et al.* 1998). An unexpected observation was that during the solubilization process, an increase in the pH of the system was observed and this was accompanied by an increase in the visible absorbance of the solubilized coal. We have further studied this phenomenon. In a recent experiment we showed that controls containing only sodium oxalate in water also exhibited a time dependent increase in pH. However, the observed increase was less dramatic than that observed during the solubilization of low rank coal. A major difference between the control and the solubilization procedure is that the coal contains substantial amounts of metal ions that would be available to participate in redox reactions. Given this fact and the observation that the pH values obtained would be consistent with production of bicarbonate ions we suggest that the pH increase observed is due to the oxidation of oxalate ion to form bicarbonate ion along with the concomitant reduction of molecular oxygen to form water. An empirical equation for this reaction is as follows:



The detailed reaction mechanism by which this occurs is likely to be quite complex.

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