APPLICATIONS OF MICELLAR ENZYMEOLOGY TO CLEAN COAL TECHNOLOGY

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>INTRODUCTION</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Program Overview</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Summary of Results</td>
<td>4</td>
</tr>
<tr>
<td>1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>MATERIALS AND METHODS</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Materials</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Methods</td>
<td>5</td>
</tr>
<tr>
<td>2.1</td>
<td>Enzyme Activity in Reverse Micelle Solutions with MC as Substrate</td>
<td>5</td>
</tr>
<tr>
<td>2.2</td>
<td>Enzyme Activity in Reverse Micelle Solution with EPS as Substrate</td>
<td>6</td>
</tr>
<tr>
<td>2.2.1</td>
<td>Quantitation of EPS Sulfur Oxidation Products</td>
<td>6</td>
</tr>
<tr>
<td>2.2.2</td>
<td>Reverse Micelle Solutions</td>
<td>7</td>
</tr>
<tr>
<td>2.2.2a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.2.2b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>RESULTS</td>
<td>8</td>
</tr>
<tr>
<td>3.1</td>
<td>Comparison of Chloroperoxidase Activity in CTAB/1-Hexanol and AOT/Isooctane Reverse Micelle Solutions with MC as Substrate</td>
<td>8</td>
</tr>
<tr>
<td>3.2</td>
<td>Relationship of H₂O₂ to Chloroperoxidase Activity with MC as Substrate</td>
<td>11</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Dependence on H₂O₂ Concentration in Reverse Micelle Solution</td>
<td>11</td>
</tr>
<tr>
<td>3.2.2</td>
<td>Inhibition by Preincubation with H₂O₂</td>
<td>15</td>
</tr>
<tr>
<td>3.3</td>
<td>EPS Sulfoxidation by Chloroperoxidase in Reverse Micelle Solution</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>PLANS FOR THE TENTH QUARTER</td>
<td>24</td>
</tr>
<tr>
<td>5</td>
<td>BIBLIOGRAPHY</td>
<td>26</td>
</tr>
</tbody>
</table>

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LIST OF FIGURES

Figure 1: Enzymatic Sulfoxidation in Reverse Micelles
Figure 2: DBT, EPS and Metabolites in Sulfur Oxidation Pathway
Figure 3: Concentration vs Absorbance of MC in CTAB/1-Hexanol and AOT/Isooctane Reverse Micelle Solutions
Figure 4: Comparison of Chloroperoxidase Activity in CTAB/1-Hexanol and AOT/Isooctane Reverse Micelle Solutions
Figure 5: Dependence of Chloroperoxidase Activity in CTAB/1-Hexanol and AOT/Isooctane Reverse Micelle Solutions on MC Concentration
Figure 6: Effect of Preincubation Time in Reverse Micelle Solution on Chloroperoxidase Activity
Figure 7: Dependence of Chloroperoxidase Activity in Reverse Micelle Solution on H2O2 Concentration
Figure 8: Inhibition of Chloroperoxidase by Preincubation with H2O2 in Aqueous Solution
Figure 9: Effect of Chloroperoxidase Concentration (11-44 μM) on EPS Sulfoxidation in Reverse Micelle Solution
Figure 10: Effect of Chloroperoxidase Concentration (1-11 μM) on EPS Sulfoxidation in Reverse Micelle Solution
Figure 11: Time Dependence of EPS Sulfoxidation by Chloroperoxidase in Reverse Micelle Solution
Figure 12: Effect of H2O2 Concentration on EPS Sulfoxidation by Chloroperoxidase in Reverse Micelle Solution
Figure 13: Effect of Sequential H2O2 Addition on EPS Sulfoxidation in Reverse Micelle Solution

LIST OF TABLES

Table 1: Effect of Preincubation with H2O2 on Chloroperoxidase Activity in Reverse Micelle Solution
Section 1

INTRODUCTION

1.1 Program Overview

Full implementation of coal fuel sources will require more effective methods of providing "clean coal" as a fuel source. Methods must be developed to reduce the sulfur content of coal which significantly contributes to environmental pollution. This project is designed to develop methods for pre-combustion coal remediation by implementing recent advances in enzyme biochemistry. The novel approach of this study is incorporation of hydrophilic oxidative enzymes in reverse micelles in an organic solvent (Figure 1). Enzymes from commercial sources or microbial extracts are being investigated for their capacity to remove organic sulfur from coal by oxidation of the sulfur groups, splitting of C-S bonds and loss of sulfur as sulfuric acid. Dibenzothiophene (DBT) and ethylphenylsulfide (EPS) are serving as models of organic sulfur-containing components of coal in initial studies (Figure 2).

A goal of this project is to define a reverse micelle system that optimizes the catalytic activity of enzymes toward desulfurization of model compounds and ultimately coal samples. Among the variables which will be examined are the surfactant, the solvent, the water:surfactant ratio and the pH and ionic strength of the aqueous phase. Studies by several groups (Martinek et al., 1981; Kabanov et al., 1988; Martinek, 1989; Verhaert et al., 1990) have shown that the surfactant AOT over a broad concentration range in organic solvents produces micelles, comparatively uniform in
Figure 1: Enzymatic Sulfoxidation in Reverse Micelles

- E: enzyme in water pool
- $\rightarrow$: surfactant
- EPS: ethylphenylsulfide
- DBT: dibenzothiophene

Organic Solvent

ENZYMATIC SULFOXIDATION IN REVERSE MICELLES
Figure 2: DBT, EPS and Metabolites in Sulfur Oxidation Pathway

Ethylphenylsulfide [EPS] → Ethanol and Benzene Sulfonate

EPS Sulfoxide → Ethyl Sulfonate and Phenol

EPS Sulfone

Dibenzothiophene [DBT] → O-Hydroxybiphenyl

DBT Sulfoxide

DBT Sulfone → "DBT Sulfonate"

O,0'-Biphenol
diameter, which incorporate hydrophilic enzymes. The activity (kcat) of certain enzymes in this system is higher than in aqueous solution. This surfactant is therefore being examined first, although the potential disadvantages of an SO₃ containing molecule have been recognized. Other surfactants to be tested include Tritons, Tweens, CTAB and Brij 35.

1.2 Summary of Results

Chloroperoxidase was active in both AOT/iso octane and CTAB/1-hexanol reverse micelle solutions, as evidenced by chlorination of MC. The latter reverse micelle system produced more variable results with a lower reaction velocity than observed with the former solution. The activity of the enzyme decreased with incubation time at room temperature but the drop was not substantially different from that observed in aqueous solution. The enzyme was inhibited by brief preincubation with H₂O₂, which almost certainly explained the lack of EPS sulfoxidation in reverse micelle solutions, reported earlier. Definitive enzyme mediated sulfoxidation of EPS in an AOT/iso octane reverse micelle solution was demonstrated, when peroxide was added already diluted in AOT/iso octane as in MC studies. However, the sulfoxidation of EPS was incomplete, considerably less than that observed under aqueous conditions, and not enhanced by an increase in enzyme concentration. The sequential addition of peroxide during the incubation was found to markedly enhance sulfoxidation. This observation is currently being further explored. In addition, the CTAB/1-hexanol reverse micelle solution is being evaluated for its ability to support chloroperoxidase catalysed EPS sulfoxidation.
Section 2
MATERIALS AND METHODS

2.1 Materials

Enzyme studies were performed with chloroperoxidase from Caldariomyces fumago. Chloroperoxidase, 2.7 mg/ml in 0.1 M sodium phosphate buffer (pH 4.0), was obtained from Sigma Chemical Co. and refrigerated at 0 °C. EPS (Aldrich Chemical Co.) and MC were evaluated as enzyme substrates. Two reverse micelle solutions were studied: 1) surfactant AOT (0.1 M, di (2-ethylhexyl) sodium sulfosuccinate from Sigma Chemical Co.), organic solvent isooctane (OmniSolv, EM Scientific), and 2.3% (v/v) aqueous phase (potassium phosphate, 0.1 M, pH 2.75); 2) surfactant CTAB (0.1 M, hexadecyltrimethylammonium bromide, Sigma Chemical Co.), solvent 1-hexanol (Fisher Scientific) and 2.3% (v/v) aqueous phase (potassium phosphate, 0.1 M, pH 2.75). Hexane, chloroform, acetone and isopropanol, all from Fisher Scientific, were used in sample analysis. Solid phase extractions were performed on 20H (diol) Bond Elut (500 mg) Bond Elut columns from AnalytiChem Inc. (Van Horne, 1990). A Scientific Industries rotator was used for agitation of incubation samples.

2.2 Methods

2.2.1 Enzyme Activity in Reverse Micelle Solutions with MC as Substrate

The assay method for measuring chloroperoxidase activity is based on procedures developed by Morris and Hager (1966) and Hager et al. (1966) and involves monitoring the enzymatic chlorination of
monochlorodimedon to form the dichloro derivative, 1,1-dimethyl-4,4-dichloro-3.5'-cyclohexanedione. To test the activity of chloroperoxidase in reverse micelles, two solutions were made. The first contained 0.1 M AOT/isooctane or CTAB/1-hexanol with 0.1 mM monochlorodimedon, to which was added an aqueous phase (2.3%) containing enzyme (4.4 μM) and KCl (0.04 M) in phosphate buffer (pH 2.75). The second solution contained the same organic phase but the aqueous phase (2.3%) consisted of 20 mM H₂O₂ in phosphate buffer. These solutions were combined in a 1:1 ratio and vortexed for 10 sec. After an additional 10-sec delay, the absorbance of the solution was measured for 20-30 sec at 278 nm on an LKB Biochrom 4050 spectrophotometer using "Reaction Rate" software to collect absorbance data and calculate reaction velocity. Nonenzyme-containing solutions were also tested. All conditions were tested in triplicate.

2.2.2 Enzyme Activity in Reverse Micelle Solution with EPS as Substrate

2.2.2a Quantitation of EPS Sulfur Oxidation Products

Isopropanol (1 column volume, 2.8 ml) was used to solvate a 20H (diol) Bond Elut column (500 mg). Excess isopropanol was removed with two column-volume washes of isooctane at a flow rate of 15 ml/min. A 0.5 ml aliquot of standards or experimental samples in reverse micelle solutions was applied and run to dryness at a flow rate of 4.8 ml/min. The sample was eluted five times with 0.25 ml aliquots of 80:20 chloroform:acetone. Eluates 2-5 were collected, solvent evaporated under N₂, and the residue redissolved in 85:15 hexane:isopropanol (0.1 ml) for HPLC analysis. Recovery of 0.009 mM
or 0.125 mM EPSx from reverse micelle solution, based on comparison of area values to that of EPSx in hexane:isopropanol (85:15), consistently averaged about 100%.

EPS (0.5 mM), EPSx (0.009 or 0.125 mM), and EPSn (0.5 mM) were added alone to hexane:isopropanol (85:15, v/v). These standards and aliquots of eluates from extraction columns (20 μl) were analyzed with a Shimadzu HPLC system (LC-600 analytical pumps) using a Waters μPorasil column (10 μm, 3.9x300 mm). This normal phase chromatographic system was based on the method used by Light et al. (1982) and Waxman et al. (1982) to separate sulfones and sulfoxides. The mobile phase consisted of 85:15 hexane:isopropanol at a flow rate of 1.2 ml/min for 15.0 min. The detector was a SPD-6AV UV-VIS spectrophotometer set at 254 nm. Chromatograms were produced on a Chromatopac CR 501 data processor.

2.2.2b Reverse Micelle Solutions

EPS (0.5 mM) was incubated with chloroperoxidase and H₂O₂ for 0.5-4.0 hour at 23°C in reverse micelle solutions. These solutions were made by: 1) combining one aqueous phase solution, containing both enzyme and peroxide in 0.1 M phosphate buffer, and the organic phase, 0.1 M AOT-isooctane, containing 0.5 mM EPS; 2) making separate enzyme-containing and peroxide-containing reverse micelles solutions and then combining them. The solutions were vortexed for 1 min and then incubated. In each experiment controls included EPS without enzyme or peroxide and EPS with peroxide and no enzyme. All conditions were tested with at least duplicate incubations; replicate aliquots of each (0.5 ml) were analyzed by 20H (diol) extraction and HPLC.
Section 3
RESULTS

3.1 Comparison of Chloroperoxidase Activity in CTAB/1-Hexanol and AOT/Isooctane Reverse Micelle Solutions with MC as Substrate

The activity of chloroperoxidase, measured as the chlorination rate of MC, was tested in 0.1 M AOT-Isooctane and 0.1 M CTAB/1-hexanol reverse micelle solutions with 2.3 % aqueous phase. The concentration-absorbance relationship of MC at 278 nm in these two solutions was similar (Figure 3). Enzyme activity data are therefore presented as absorbance units per min since correction for differences in extinction coefficient appeared unnecessary. Peroxide-containing controls (without enzyme) exhibited insignificant levels of activity.

The enzymatic reaction velocity was significantly higher in AOT/Isooctane as compared to CTAB/1-hexanol reverse micelle solutions (Figure 4). The rate was significantly increased in AOT/Isooctane solutions when the enzyme concentration was increased (p<0.0001, Student's t-test). In contrast, the rate was not significantly greater when the same increase in enzyme level was tested in CTAB/1-hexanol solutions; greater variability was also noted. Subsequently, a summary of five experiments indicated that for identical experimental conditions the coefficient of variation for the reaction rate in AOT/Isooctane was 15.6% whereas that for CTAB/1-hexanol was 62.2%. The mean rate in CTAB/1-hexanol averaged 57.8% of that in AOT/Isooctane. In both reverse micelle solutions the 0.1 M concentration of MC appeared to generate
Figure 3: Concentration vs Absorbance of MC in CTAB/1-Hexanol and AOT/Isooctane Reverse Micelle Solutions

\[ y = 0.34003 + 8.8444x \quad R^2 = 0.999 \quad (\text{CTAB/1HEXANOL}) \]

\[ y = 0.64703 + 7.3568x \quad R^2 = 0.987 \quad (\text{AOT/ISOOCTANE}) \]
Figure 4: Comparison of Chloroperoxidase Activity in
CTAB/1-Hexanol and AOT/Isooctane Reverse
Micelle Solutions

![Graph showing comparison of Chloroperoxidase Activity in CTAB/1-Hexanol and AOT/Isooctane Reverse Micelle Solutions]
maximal velocities; 50 and 100% increases in MC concentrations did not significantly increase reaction rates (Figures 5).

The stability of the enzyme in these two reverse micelle solutions was also compared. In this study the enzyme-containing reverse micelle solution was formed and the rate of MC chlorination then tested immediately or after a 2 or 4 hour incubation at room temperature. New peroxide-containing reverse micelle solutions were generated just prior to enzyme testing to eliminate a potential time-dependent change in this component of the assay mixture. The results, depicted in Figure 6, indicated that the rate of loss of enzyme activity was similar in the two types of reverse micelle solutions. After 4 hours the per cent of activity in the AOT/isoctane solution compared to 0 time, 45.5%, was similar to that observed when the enzyme stability was tested in aqueous solution (p. 10, Seventh Quarterly Report).

A partitioning experiment determined that 0.1 mM MC in isooctane was entirely extracted into an equal volume of 0.1 M phosphate buffer (pH 2.75).

3.2 Relationship of H₂O₂ to Chloroperoxidase Activity with MC as Substrate

3.2.1 Dependence on H₂O₂ Concentration in Reverse Micelle Solution

The effect of varying the H₂O₂ concentration on chloroperoxidase activity in the AOT/isoctane reverse micelle solution was determined. As indicated in Figure 7, maximal activity was observed with peroxide concentrations of 10-40 mM in the
Figure 5: Dependence of Chloroperoxidase Activity in CTAB/1-Hexanol and AOT/Isooctane Reverse Micelle Solutions on MC Concentration

**CTAB/1-HEXANOL**

- EXP1
- EXP2

**AOT/ISOOCTANE**

- EXP1
- EXP2
Figure 6: Effect of Preincubation Time in Reverse Micelle Solution on Chloroperoxidase Activity

[Graph showing the effect of preincubation time on chloroperoxidase activity for CTAB/Hexanol and AOT/Isooctane solutions.]

REACTION VELOCITY (ABS UNITS/MIN)

TIME (HOURS)
Figure 7: Dependence of Chloroperoxidase Activity in Reverse Micelle Solution on H$_2$O$_2$ Concentration
final aqueous phase of the reverse micelle solutions.

3.2.2 Inhibition by Preincubation with \textbf{H}_2\textbf{O}_2

Addition of peroxide to chloroperoxidase (4.0 nM) in aqueous solution without substrate present was found to markedly inhibit the chlorination of MC on subsequent addition of the peroxide-enzyme combination (within 5-15 min) to MC in phosphate buffer (Figure 8). This study was designed such that the final peroxide concentration in all cases was 3.3 mM. In one experiment where enzyme was preincubated with 10 mM peroxide, after combination with the substrate solution, additional peroxide was added equivalent to the amount used in control studies. However, activity was even further decreased. An inhibitory effect was also observed when the peroxide-enzyme solution was tested by addition to MC in AOT/isooctane (Table 1). These observations indicated that the most recent protocol for testing EPS sulfoxidation by this enzyme in reverse micelle solutions was inappropriate. The procedure of combining peroxide and enzyme in aqueous solution prior to addition to surfactant/solvent had been adopted in June of 1991 (p. 8-9, Seventh Report), when initial studies with peroxide and enzyme separately added to EPS-containing reverse micelle solutions had proved negative. Subsequent work described below modified the procedure to attempt to avoid the pronounced inhibitory effect of peroxide.
Figure 8: Inhibition of Chloroperoxidase by Preincubation with H₂O₂ in Aqueous Solution
Table 1: Effect of Preincubation with $\text{H}_2\text{O}_2$ on Chloroperoxidase Activity in Reverse Micelle Solution

<table>
<thead>
<tr>
<th>CONDITION</th>
<th>ENZYME\textsuperscript{b} (abs units/min)</th>
<th>BLANK (abs units/min)</th>
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</thead>
<tbody>
<tr>
<td>Control\textsuperscript{c}</td>
<td>0.508 ± 0.075 (3)</td>
<td>0.032 ± 0.006 (2)</td>
</tr>
<tr>
<td>Preincubation: 10 mM $\text{H}_2\text{O}_2$\textsuperscript{d}</td>
<td>0.039 ± 0.025 (3)</td>
<td>0.026 ± 0.006 (2)</td>
</tr>
<tr>
<td>Control: 1 hr later</td>
<td>0.478 ± 0.083 (2)</td>
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\textsuperscript{a} Change in absorbance units of 0.1 mM MC contained in all 0.1M AOT:isooctane solutions, measured at 278 nm for 1 minute.

\textsuperscript{b} Chloroperoxidase concentration in aqueous phase: 2.2 uM; concentration based on total volume: 50 nM.

\textsuperscript{c} Enzyme in 0.04 M KCl-phosphate (pH 2.75) added to 0.1 M AOT:isooctane, combined with an equal volume of 20 mM $\text{H}_2\text{O}_2$ in phosphate added to 0.1 M AOT:isooctane.

\textsuperscript{d} Enzyme and 10 mM $\text{H}_2\text{O}_2$ in phosphate added after 5 min to 0.1 M AOT:isooctane, combined with 0.04 M KCl-10 mM $\text{H}_2\text{O}_2$ added to 0.1 M AOT:isooctane.
3.3 EPS Sulfoxidation by Chloroperoxidase in Reverse Micelle Solution

EPS as a substrate for chloroperoxidase in reverse micelle solutions was tested under several conditions. These studies were carried out with 20 mM peroxide in phosphate buffer added to AOT/isooctane, equivalent to the optimum for MC chlorination in the comparable experimental system. Concentrations of the enzyme in the aqueous phase added to the organic solution were initially tested at 44 μM which exceeded the level of enzyme active in MC chlorination in reverse micelle solution by twenty fold and in EPS sulfoxidation in aqueous solution by two-hundred fold. In this initial study the production of EPSx after 2 hours was five-fold greater in enzyme-containing reverse micelle solutions than in controls. This encouraging observation led to further studies with a range of enzyme concentrations. The results of the two studies, shown in Figures 9 and 10, demonstrated significant enzyme-dependent sulfoxidation of EPS in the AOT/isooctane reverse micelle solution. The magnitude of EPSx production in two hours did not show a positive correlation with enzyme concentration. Furthermore, only about 10% of the substrate was oxidized to EPSx with no other metabolites detectable.

Additional studies were carried out to explore the factors limiting the extent of EPS sulfoxidation, since increasing the amount of enzyme in the reverse micelle solution did not enhance the conversion. The next experiment, summarized in Figure 11, revealed little difference in the extent of product formation after 2.0 hours as compared to a much shorter incubation time, 0.5 hours. This
Figure 9: Effect of Chloroperoxidase Concentration (11-44 µM) on EPS Sulfoxidation in Reverse Micelle Solution
Figure 10: Effect of Chloroperoxidase Concentration (1-11 μM) on EPS Sulfoxidation in Reverse Micelle Solution
Figure 11: Time Dependence of EPS Sulfoxidation by Chloroperoxidase in Reverse Micelle Solution
finding was inconsistent with earlier studies of enzyme activity as a function of incubation time, based on MC as substrate, and suggested either product inhibition or depletion of peroxide might be limiting further EPS sulfoxidation. EPSx had previously been shown to be a substrate for chloroperoxidase in aqueous solution so that the former hypothesis seemed less likely. Therefore, in the next experiment, a four-fold higher peroxide concentration was tested. The magnitude of EPSx formation after a 0.5 hr incubation was increased by the higher peroxide level but only by 34% (Figure 12). The effect of sequential peroxide addition to the incubation mixture was examined next. In this experiment aliquots of the reverse micelle solutions were removed for EPSx determination after 0.5 hr incubations, and at that time additional phosphate buffer (2.3% of the total volume), either alone or containing 10 mM peroxide, was added. Marked enhancement of EPS sulfoxidation was produced by the second addition of peroxide (Figure 13, "samples" 1=just before and 0.5 hr after second peroxide addition, 2=just before and 0.5 hr after addition of phosphate control, dark bars are "before" condition). The extent to which this effect was dependent on enzyme catalysis cannot be determined from this experiment, since a nonenzyme-containing control was not included. Subsequent work will explore this question.
Figure 12: Effect of H$_2$O$_2$ Concentration on EPS Sulfoxidation by Chloroperoxidase in Reverse Micelle Solution

H$_2$O$_2$ CONCENTRATION (mM) IN AQUEOUS PHASE

EPS CONCENTRATION (µM)
Figure 13: Effect of Sequential \( \text{H}_2\text{O}_2 \) Addition on EPS Sulfoxidation in Reverse Micelle Solution

![Graph showing the effect of sequential \( \text{H}_2\text{O}_2 \) addition on EPS sulfoxidation.](image)
Section 4

PLANS FOR THE TENTH QUARTER

1. Further study of chloroperoxidase and bacterial and mammalian enzymes (Holland, 1988, Kobayashi et al., 1986, and Rettie et al., 1990) for sulfur oxidizing activity.

2. Methodology for peroxide addition to maximize EPS sulfoxidation by chloroperoxidase in reverse micelle solution.

3. Development of analytical assay for EPSx in a CTAB/1-hexanol reverse micelle solution and determination of EPS sulfoxidation by chloroperoxidase in this solution.
Section 5

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