**APPLICATIONS OF MICELLAR ENZYMOLOGY TO CLEAN COAL
TECHNOLOGY**

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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. 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 (Figure 1).

Dibenzothiophene (DBT) and ethylphenylsulfide (EPS) are serving as models of organic sulfur-containing components of coal in initial studies.

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 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

FIGURE 1. DBT, EPS and Metabolites in Sulfur Oxidation Pathway

been recognized. Other surfactants to be tested include Tritons, Tweens, CTAB and Brij 35.

1.2 Summary of Results

The aliphatic sulfur in EPS is oxidized by chloroperoxidase in aqueous solution, at a greater rate at pH 2.75 than at pH 5.0. The primary product was EPSx, although when EPSx was tested as starting material substantial conversion to EPSn occurred. Chloroperoxidase was active in reverse micelle solutions, as evidenced by chlorination of MC, and activity was greatest in solutions with the highest percent aqueous phase (5.4%). MC was found to partition into the aqueous phase from the organic solvent. In contrast, EPS was not significantly oxidized to EPSx by either chloroperoxidase or HRP in reverse micelle solutions. Several solution parameters were tested, including buffer composition, enzyme concentration and H₂O/AOT ratios. Neither acetate buffer, which favored the partition of EPS into the aqueous phase, nor phosphate buffer, shown to increase sulfoxidation in aqueous solution, resulted in significant EPSx production by chloroperoxidase in reverse micelle solutions. Increasing the aqueous phase content to 5.4% and the enzyme concentration was also without effect. HRP in reverse micelles was also not an effective catalyst for EPS sulfoxidation. Current studies are exploring a CTAB/1-hexanol system to determine if the use of a surfactant with a positive, rather than negative, charge has an influence on the oxidative activity of these enzymes.

Section 2

MATERIALS AND METHODS

2.1 Materials

Enzyme studies were performed with chloroperoxidase from Caldariomyces fumago and horseradish peroxidase (HRP). 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. HRP (Type II, Sigma) was maintained at -20 °C. EPS (Aldrich Chemical Co.), EPSx (K & K Laboratories, Inc.) and EPSn (Lancaster Synthesis Ltd.) were evaluated as enzyme substrates. Reverse micelle solutions were formed with the surfactant AOT (0.1 M), di (2-ethylhexyl) sodium sulfosuccinate from Sigma Chemical Co., the organic solvent isooctane (OmniSolv, EM Scientific) and a 1.8-5.4% (v/v) aqueous phase, either sodium acetate (0.1 M, pH 5.0) or potassium phosphate (0.015-0.1 M, pH 2.75). Hexane (Fisher Scientific), methanol (Fisher Scientific) and isopropanol (Fisher Scientific) were used in sample analysis. Solid phase extractions were performed on 20H (diol) Bond Elut (500 mg) and C-18 (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 by Standard Assay

The standard 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. The substrate (0.3 µmol/1 ml) is added to KCl (60 µmol/1.0 ml) and H₂O₂ (6 µmol/0.5 ml) in a total volume of 2.5 ml. The reaction

is initiated upon the addition of chloroperoxidase, 0.3 $\mu\text{g}/0.5\text{ ml}$ in potassium phosphate buffer (0.1 M, pH 2.75). Final concentrations of reagents at time zero were 0.1 mM monochlorodimedon, 0.02 M KCl, 2 mM H_2O_2 , 2.4 nM chloroperoxidase, and 0.1 M potassium phosphate. Absorbance was measured at 278 nm on an LKB Biochrom 4050 spectrophotometer using "Reaction Rate" software to collect absorbance data and calculate reaction velocity. Nonenzymatic blanks were also run using 0.5 ml of potassium phosphate buffer (0.1 M, pH 2.75) instead of the enzyme solution.

To test the activity of chloroperoxidase in reverse micelles, two solutions were made. The first contained 0.1 M AOT-isooctane with 0.1 mM monochlorodimedon, to which was added an aqueous phase (1.8-5.4%) 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 (1.8-5.4%) consisted of 20 mM H_2O_2 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 monitored, as described above, for 20-30 sec during which time the reaction rate was linear. Nonenzyme-containing solutions were also tested. All conditions were tested in triplicate. In order to examine initial reaction kinetics with solutions containing greater than a 2.3% aqueous phase, it was necessary to decrease the ionic strength of the aqueous solution so that turbidity problems were minimized. This change was achieved by using a 0.015 M phosphate buffer, since decreasing the KCl concentration adversely influenced the kinetics.

The activity of HRP was determined using phenol and 4-aminoantipyrine as substrates. The methodology is described in detail in the First Quarterly Report (p.5).

2.2.2 Quantitation of EPS Sulfur Oxidation Products

2.2.2a Aqueous solution on C-18 column

Methanol (1 column volume, 2.8 ml) was used to solvate a C-18 Bond Elut column (500 mg). Excess methanol was removed with two column-volume washes of distilled water (15 ml/min). A 0.5 ml aliquot of standards or experimental samples in 0.1 M acetate (pH 5.0) or phosphate (pH 2.75) buffer was applied to the column and run to dryness at 4 ml/min. Hexane:isopropanol (85:15) was then used in seven 0.25-ml aliquots to elute the column. Eluates 3-7 were collected, the volume measured and aliquots analyzed by HPLC. Recovery of EPSx or EPSn (0.125 mM in acetate buffer) based on comparison of area values to those of 0.125 mM EPSx or EPSn in hexane:isopropanol (85:15) averaged $103 \pm 14\%$ and $94 \pm 6\%$ respectively.

2.2.2b Reverse micelle solution on 20H (diol) column

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 N_2 , 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%.

2.2.2c HPLC

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 μ l) were analyzed with a Shimadzu

HPLC system (LC-600 analytical pumps) using a Waters μ Porasil column (10 μ m, 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.

2.2.3 Enzyme Incubations with EPS, EPSx, and EPSn

2.2.3a Aqueous Solutions

EPS (0.5 mM) was incubated for 0.5 hours at 23°C with chloroperoxidase (2.2 μ M) and 1.0 mM H₂O₂ in 0.1 M acetate (pH 5.0) or 0.1 M phosphate buffer (pH 2.75). Controls included EPS, EPS with H₂O₂ but no enzyme, and 0.125 mM EPSx, each in the appropriate buffer. All conditions were tested in duplicate. Replicate aliquots (0.5 ml) were analyzed by C-18 elution and HPLC. EPSx concentrations in experimental samples were calculated on the basis of the area values in chromatographs relative to that of the 0.125 mM EPSx standard extracted from aqueous solution.

EPSx was purified by TLC (silica gel-60 F254, chloroform:acetone 80:20). Analysis of the isopropanol silica gel extracts by HPLC indicated that the EPSx peak area was increased from the original 94.8% to 99.7%. This purified material (0.5 mM in 0.1 M acetate buffer, pH 5.0) was incubated for 4.0 hours at 23°C with chloroperoxidase (2.2 μ M) and 1.0 mM H₂O₂. Controls included EPSx, EPSx with H₂O₂ but no enzyme, and 0.5 mM EPSn, each in 0.1 M acetate buffer (pH 5.0). Duplicate aliquots (0.5 ml) were analyzed by C-18 elution and HPLC. EPSx and EPSn concentrations in experimental samples were calculated on the basis of the area values in chromatograms relative to those of the standards extracted from aqueous solution.

EPSn was purified by TLC with the system described for EPSx. The EPSn peak area in the silica gel extracts increased from 62% to 94% of all peaks detected by HPLC. The purified material was then tested as a substrate, according to the protocol for EPSx described above.

2.2.3b Reverse Micelle Solutions

EPS (0.5 mM) was incubated with chloroperoxidase or HRP and H₂O₂ for 4.0 hour at 23°C in reverse micelle solutions. These solutions were made by combining one aqueous phase solution, containing both enzyme (22 or 44 µM) and peroxide (10 mM) in 0.1 M acetate or phosphate buffer, and the organic phase, 0.1 M AOT-isooctane, containing 0.5 mM EPS. The solutions were vortexed for 1 min and incubated for 4 hour. In each experiment controls included EPS without enzyme or peroxide and EPS with peroxide and no enzyme. All conditions were tested with duplicate incubations; replicate aliquots of each (0.5 ml) were analyzed by 20H (diol) extraction and HPLC.

2.2.4. Partition Coefficients of MC and EPS

The extraction of 0.1 M MC and 0.5 M EPS from isooctane into an equal volume of aqueous buffer solution was determined. Mixtures were vortexed for 1 min after combination, and then the absorbance of the isooctane phase measured and the residual concentration of MC or EPS determined. The absorbance of the aqueous phase was examined to confirm the results. Buffers included 0.1 M sodium acetate (pH 5.0), sodium citrate (pH 2.75) and potassium phosphate (pH 2.75).

Section 3

RESULTS

3.1 Activity of Chloroperoxidase in 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 reverse micelle solutions with H₂O/AOT ratios from 10:1 to 30:1, corresponding to an aqueous phase concentration of 1.8-5.4% . With the conditions tested, the absorbances at 278 nm of MC and non-MC containing solutions (without H₂O₂ or enzyme) were unaffected by the H₂O content. Peroxide-containing controls (without enzyme) exhibited insignificant levels of activity except in solutions with the highest water content (H₂O/AOT of 30:1). The net enzymatic reaction velocity corrected for enzyme concentration increased with the water content of the solutions (Figure 2). The rate was markedly less than that in aqueous solution (124.6 ± 10.8 abs units/min·uM enz.). This difference could not be accounted for by the MC extinction coefficients which were similar in aqueous and reverse micelle solutions (2.3% aqueous), 11.9 and 8.5 abs units/mM·cm, respectively.

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 Sulfoxidation by Chloroperoxidase-H₂O₂

3.2.1 Aqueous Solutions

3.2.1a. EPS Sulfoxidation in Phosphate (pH 2.75) and Acetate (pH 5.0) Buffers

The sulfoxidation of EPS (0.5 mM) by chloroperoxidase-H₂O₂ in 0.1 M acetate buffer, pH 5.0, was compared to that in 0.1 M phosphate buffer, pH 2.75 (Table 1). The magnitude of the enzymatically catalyzed sulfoxidation was about three-fold greater at the lower pH, with about 20% of the substrate converted to the sulfoxide in 0.5 hr. No other metabolites were observed.

FIGURE 2. Relationship between H₂O/AOT Ratio and Activity of Chloroperoxidase in Reverse Micelle Solution

**TABLE 1. Comparison of EPS Sulfoxidation by Chloroperoxidase-
H₂O₂ in Acetate (pH 5.0) and Phosphate (pH 2.75) Buffers**

BUFFER^b	CONCENTRATION OF EPSX (μM)^a	
	CONTROL	ENZYME
Acetate, pH 5.0	1.2 ± 0.2	32.0 ± 9.0
Phosphate, pH 2.75	2.1 ± 0.8	107.1 ± 12.0

^a Mean ± SD of replicate samples analyzed in duplicate.

^b EPS (0.5 mM) incubated in 0.1 M buffer solutions for 0.5 hr with 1.0 mM H₂O₂ alone or containing 2.2 μM chloroperoxidase

The recovery of EPSx from the two buffer solutions did not differ (109 and 116% from acetate and phosphate buffers respectively).

3.2.1b. EPSx and EPSn as Substrates

The sulfoxidation of EPSx in acetate buffer was reexamined with a more purified preparation of EPSx. As in the earlier study (Seventh Quaterly Report), EPSx was substantially oxidized by chloroperoxidase to EPSn and an additional derivative with a retention time similar to that of EPSn (Table 2). This latter material produced a peak area about three times that of EPSn, as was observed in the earlier experiment. In contrast, EPSn was not significantly metabolized by the enzyme under the conditions studied (Table 3). These results suggested therefore that the unknown derivative observed with EPSx as the starting material was not a product of EPSn oxidation but was directly formed from EPSx.

3.2.2 Reverse Micelle Solutions

EPS as a substrate for chloroperoxidase in reverse micelle solutions was tested under several conditions. These studies were carried out with 10 mM peroxide added in the aqueous phase, equivalent to ten-fold higher than the optimum for EPS oxidation in aqueous solution. As described above, chloroperoxidase was shown to be catalytically active in reverse micelle solutions containing this peroxide concentration, although the activity was substantially less than in aqueous solution. Concentrations of the enzyme (22 or 44 μ M) in the aqueous phase added to the organic solution exceeded the level of enzyme active: 1.) in monochlorodimedon chlorination in reverse micelle solution by ten or twenty fold and 2.) in EPS and EPSx sulfoxidation in aqueous solution by one or two-hundred fold.

In the first set of studies the buffer was changed to phosphate since this condition proved more favorable to EPS sulfoxidation under aqueous conditions. In addition, a solution with a higher percent water content (5.4%) was also studied since this procedure increased the total amount of enzyme in the solution and also

TABLE 2. Oxidation of EPSx by Chloroperoxidase-H₂O₂ in Aqueous Solution

Sample Conditions^a	Concentration of EPSx (mM)	Concentration of EPSn (mM)
Control	0.52 ± 0.03^b	0
Enzyme	0.20 ± 0.05.	0.22 ± 0.06

^a EPSx (0.5 mM) incubated for 4 hr in 0.1 M acetate buffer (pH 5.0) with 1.0 mM H₂O₂ alone or containing 2.2 μM chloroperoxidase

^b Mean ± SD of replicate samples analyzed in duplicate

TABLE 3. Incubation of EPSn with Chloroperoxidase-H₂O₂ in Aqueous Solution

SAMPLE CONDITIONS^a	CONCENTRATION OF EPSN ^b (mM)
EPSn Standard	0.38 ± 0.03
Control	0.41 ± 0.02
Enzyme	0.51 ± 0.06

^a EPSn (0.5 mM) incubated for 4 hr in 0.1 M acetate buffer (pH 5.0) alone, with 1.0 mM H₂O₂ or with 2.2 μM chloroperoxidase-1.0 mM H₂O₂

^a Mean ± SD of replicate samples analyzed in duplicate.

enhanced its catalytic activity (based on MC results). These conditions did not, however, result in a significant increase in EPS sulfoxidation (Table 4). A fairly substantial peak was observed in enzyme-containing solutions at an R_f between EPS_n and EPS_x. However, this finding was not replicated in a subsequent experiment with the 2.3% aqueous phase conditions. When this study was repeated using acetate buffer at 3.2 or 5.4%, peak areas in enzyme-containing solutions did not differ significantly from controls (Table 5).

The partitioning of EPS between the aqueous and organic phases of the reverse micelle solutions probably differed depending on the aqueous buffer. EPS was substantially extracted from isooctane into an equal volume of acetate buffer but not at all by phosphate or citrate buffer (Table 6).

3.3 Sulfoxidation by Horseradish Peroxidase-H₂O₂

The activity of HRP tested by conventional methods in aqueous solution was within the expected range (0.12 abs. units/min) and did not differ in 0.0125 mM. potassium phosphate (pH 7.0) and sodium acetate (pH 5.0). The concentration of EPS_x after 4-hr incubation of EPS in a reverse micelle solution with HRP was only slightly greater than control. No other derivatives were increased in the HRP containing samples (Table 7).

Section 4

PLANS FOR THE SEVENTH 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. Comparison of enzyme activity in aqueous and reverse micelle solutions.
2. Examination of oxidative activity of enzymes in a reverse micelle solution with a non-sulfur containing surfactant, CTAB, and 1-hexanol as the organic solvent.

TABLE 4. Sulfoxidation of EPS by Chloroperoxidase-H₂O₂ in a Reverse Micelle Solution with Phosphate Aqueous Phase^a

AQUEOUS PHASE	CONCENTRATION OF EPSX (μ M) ^b	
	CONTROL	ENZYME
2.3%	1.3 $\pm$ 0.3 ^b	2.0 $\pm$ 0.3
5.4%	2.3 $\pm$ 0.6	2.2 $\pm$ 0.6

^a EPS (0.5 mM) incubated for 4 hr in 0.1 M AOT-isooctane with aqueous phase of 0.1 M phosphate (pH 2.75) buffer; buffer contained H₂O₂ (10 mM) alone or with chloroperoxidase (44 μ M).

^b Mean $\pm$ SD of replicate samples analyzed in duplicate.

TABLE 5. Sulfoxidation of EPS by Chloroperoxidase-H₂O₂ in a Reverse Micelle Solution with Acetate Aqueous Phase^a

AQUEOUS PHASE	CONCENTRATION OF EPSX (μM) ^a	
	CONTROL	ENZYME
3.2%, 22μM enz.	8.1 ± 1.4 ^b	3.4 ± 0.6
5.4%, 44μM enz	0.8 ± 0.2	0.6 ± 0.1

^a EPS (0.5 mM) in 0.1 M AOT-isooctane with 3.2 or 5.4% aqueous phase of 0.1 M acetate (pH 5.0) buffer; buffer contained H₂O₂ (10 mM) alone or with chloroperoxidase (22 or 44 μM)

^b Mean ± SD of replicate samples analyzed in duplicate.

TABLE 6 Percent Extraction of EPS from Isooctane into Aqueous Buffers^a

BUFFER	PERCENT EXTRACTION
Na Acetate, pH 5.0	37.8 ± 1.6
K Phosphate, pH 2.75	0
Na Citrate, pH 2.75	0

^a EPS (0.5 mM) in isooctane was shaken with an equal volume of 0.1 M buffer, and the residual concentration in the organic phase was determined from the absorbance at 254 nm.

TABLE 7. Sulfoxidation of EPS by Horseradish Peroxidase-H₂O₂ in a Reverse Micelle Solution

SAMPLE CONDITIONS^a	CONCENTRATION OF EPSX (μM)
Control	0.77$\pm$0.12^b
Enzyme	1.29$\pm$0.36

^a EPS (0.5 mM) incubated for 4 hr in 0.1 M AOT-isooctane with 2.3% aqueous phase of 0.1 M acetate (pH 5.0) buffer; buffer contained H₂O₂ (10 mM) alone or with horseradish peroxidase (22 μ M).

^b Mean $\pm$ SD of replicate samples analyzed in duplicate.

Section 5

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