Characterization of Chemically Modified Enzymes for Bioremediation-Reactions

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Progress Report

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Research Objective

Many, if not most, biological transformation reactions of interest to U.S. Department of Energy (DOE) site remediation involve substrates that are only sparingly soluble in aqueous environments. Hence, destruction of these recalcitrant and toxic materials would benefit tremendously if their degradation could be performed in nonaqueous environments. Organic biocatalysis may be motivated by the nature of the substrate itself, augmented mass transport, ease of product recovery, or novel reaction pathways afforded by the organic solvent. For instance, polychlorinated biphenyls (PCBs) are sparingly soluble in water, but may be more effectively processed when solubilized by organic liquids.

However, naturally-occurring enzymes are not soluble in organic solvents. Indeed, most spontaneously denature and, depending on the solvent used, typically form' inactive and insoluble precipitates. The objective of the current work is to gain a fundamental understanding of the molecular and catalytic properties of enzymes that have been chemically-modified so that they are catalytically-active and chemically-/thermally-stable in organic solvents. The premise for this study is that highly stable enzymes which are catalytically active in both water and in a range of organic solvents are optimally suited for bioremediation where substrates of interest are more soluble and may be processed with greater specificity in nonaqueous solvents.

The proposed research program will enable the development of nonaqueous bioremediation technologies for the treatment of DOE sites contaminated with aqueous-insoluble organic compounds. Such compounds may include dense nonaqueous phase liquids, trichloroethylene (TCE), trichloroacetic acid, trans-dichloroethylene, diesel fuel, and PCBs). These compounds have been identified as targets for technology development in the "EM Technology Needs Database," and are contaminants at the following DOE sites: K-25 Site plumes; ORNL WAGs 1, 4, and 5; Paducah plumes; Portsmouth plumes; the X-701B Holding Pond; and the Y-12 Poplar Creek and Bear Creek Watersheds.

Research Statement

Experiments will be performed to screen enzymes for the degradation of target pollutants. Studies will also be carried out to explore the physical and catalytic properties of polyethylene glycol- (PEG)-modified hyperthermophilic hydrogenases and potential electron carriers as model systems for enzyme catalyzed reactions in organic solvents. In addition to the PEG-modification, other approaches, including the incorporation of enzymes into various polymer matrices and protein cross-linking, will be investigated to enhance the stability and activity of enzymes of interest in organic media. In a final step, we will explore how the modified hydrogenase and other enzymes may be used in remediation reactions via novel reaction pathways that may be afforded through organic biocatalysis. The following statements address these issues in further detail.

- **Screen Enzymes for Degradation of Organic Pollutants.** To identify enzymes with the potential to catalyze the degradation of organic pollutants of interest that are sparingly soluble in water. To understand the reaction pathway and characterize the products of degradation reactions. To examine the chemical and thermal stability of enzymes of interest. To optimize the reaction media in terms of substrate solubility and enzyme activity via the solvent engineering approach.

- **Characterization of PEG-Modified Hydrogenase and Modification of Electron Carriers.** To determine the extent of enzyme modification and how the modification affects enzyme stability, pH, and temperature optima as well as oxygen sensitivity. To correlate enzyme activity with the hydrophobic@ of the solvent, the degree of modification, and the nature of the electron carriers used. To study how lower molecular weight electron carriers may be modified to be soluble and active in organic solvents. To determine how modification affects redox potential, stability, electron transfer activity, and reactivity of the redox active metal center. To examine the modified hydrogenase for novel biocatalytic abilities in the area of bioremediation. This will include dehalogenation reactions, desulfurization, and hydrocarbon degradation.

- **Protein Engineering to Enhance Enzyme Activity and Stability.** To modify the microenvironment of the enzymes to improve their performance. Studies will include understanding the modifiers', surfactants', water's, and inhibitors' effects on the enzymes' physical configuration and chemical activities. To optimize the modification process to reduce enzyme denaturation and enhance enzyme stability and activity in organic media.

- **Bioremediation Reactions with Modified Proteins in** Organic **Media.** Continuous operation of modified enzymes in their optimized media at a gram scale to assess the engineering issue for larger scale practice. This will include the optimization of reactors, solvent/catalyst recycling, feeding control, and product separation.

Research Progress

Thus far in FY 1997, we have focused our research on screening enzymes for biodegradation reactions and optimization for the modification of hydrogenase to enhance its solubility and activity in organic solvents.

Screening Enzymes for Bioremediation Reactions

We have examined five redox enzymes with various model contaminants in both aqueous and organic solutions. The following describes this in more detail.

- Native and PEG-modified hydrogenase from a hyperthermophile, ***Pyrococcus furiosus,*** have been examined for the degradation of TCE. Experiments were carried out in aqueous buffer solution, and the reaction was monitored by gas chromatography (GC). No degradation reaction was observed. The addition of benzyl viologen, an electron carrier, did not stimulate the reaction. The enzyme was then examined with 2-chloropheno1, a compound that has a structure similar to that of PCBs but which is more soluble in aqueous solutions, and t-butyl mercaptan, a sulfur-containing hydrocarbon contaminant. Again, no degradation reaction was observed.

- A dehalogenase extracted from *E. coli* was tested with TCE in aqueous solution. No obvious reaction was observed.

- The catalytic behavior of ligninase (LiP) with 2-chlorophenol and pentachlorophenol has been characterized in both aqueous and organic solvents.

  Native LiP has been found to have very good activity toward the degradation of 2-chlorophenol. A typical reaction time course for this reaction at pH 4.8 and room temperature is shown in Fig. 1. Up to 80% of initially added chlorophenol was degraded. The products of this remediation reaction remain unknown in the literature. In the current work, the concentration of free chloride ions released during the degradation of chlorophenol was measured. As a result, 50% of total chlorine initially contained in the substrate was found in the final reaction mixture as free chloride ions. The difference between the chlorophenol concentration and chloride ion detection may imply that the degradation may also lead to products of chlorinated molecules other than chlorophenol or formation of chlorophenol polymer.

  The degradation reaction of 2-chlorophenol by LiP has been examined in toluene and acetonitrile. The native LiP shows poor activity in toluene, and no obvious reaction was observed in this solvent. In a test with a mixture of acetonitrile/buffer (10/90, v/v), a 5% degradation of chlorophenol has been achieved. The modification of the enzyme with PEG, however, can increase the enzyme activity. PEG-modified LiP can degrade -10% of 2-chlorophenol in toluene, and the modification can increase the enzyme activity by more then three times in the 10/90 acetonitrile/buffer mixture (a 17% degradation of 2-chlorophenol has been observed with the PEG-modified LiP). It is suspected that the modification with PEG may increase the stability of LiP against organic solvents and lead to higher apparent activities over the native enzyme.

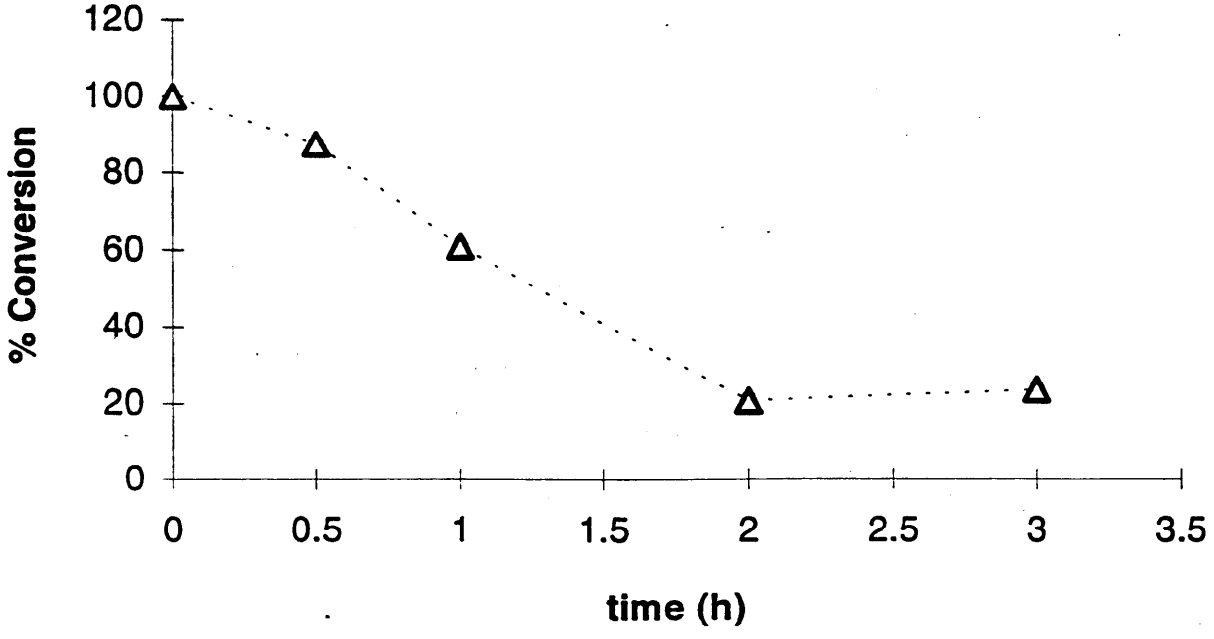

Figure 1. Time course of the degradation of 2-chlorophenol by ligninase.

The bioremediation potential of LiP was further examined with pentachlorophenol in both aqueous solution and organic solvents. Table 1 presents the experimental results with mixed solvents of aqueous buffer and acetonitrile. LiP showed very good activity in aqueous buffer and mixtures with up to 20% (v/v) acetonitrile, such that a 90% degradation can be achieved and more than 60% of total chlorine can be released as free chloride ions from pentachlorophenol. However, a higher content of acetonitrile tends to denature the enzyme, and no reaction was observed when the acentonitrile exceeded 40% (see Table 1).

Table 1. Degradation of pentachlorophenol by ligninase*

%-MeCN	0	10	20	40
%-degradation	53	94	72.5	0
%-release of Cl	28	66	9.9	0

'Note that solutions have been prepared with a 10 mM pH 4.8 acetate buffer. The enzyme and peroxide were added over a period of 3 hours. The reactant concentrations in the reactor were controlled to be: [Enzyme] = 0.5 Unit/ml, [pentachlorophenol] = 0.05 mg/ml, H_2O_2] = 0.5 mM.

Native peroxidases from horseradish (HRP) and soybean (SBP) have been examined for the degradation of 2-chlorophenol and pentachlorophenol. The primary results with HRP have been tabulated in Table 2.

Table 2. Percent of degradation of 2-chlorophenol and pentachlorophenol by HRP*

Solvent	Buffer	MeCN	MeCN/buffer (10/90, v/v)	Octane/THF (70/30, v/v)	Toluene/buffer (70/30, v/v)
2-Chlorophenol	~100	20	~100	20	85
Pentachlorophenol	55	–	58	0	—

Note that the solutions have been prepared with a 10mM pH 6 phosphate buffer. The enzyme and peroxide were added over a period of 3 hours. The reactant concentrations in the reactor were controlled to be: [Enzyme] = 0.5 mg/ml, [pentachlorophenol] = 0.05 mg/ml or [2-chlorophenol] = 1 mM, and [H_2O_2] = 1 mM.

HRP can remove nearly 100% of 2-chlorophenol from an aqueous solution. Precipitate was formed during this reaction, but a minimum of free chloride ions was detected in the reaction mixture. This reaction is suspected to be a polymerization reaction of the phenol-another important potential application of HRP in wastewater treatment. For pentachlorophenol, over 55% conversion has been achieved, with a detectable -10% of total chlorine released to aqueous solution as free chloride ions.

Unlike LiP, HRP seems to be active in nearly anhydrous MeCN such that -20% removal of 2-chlorophenol was achieved. Similar results were obtained with a mixed solvent of octane/tetrahydrofuran (70/30). Interestingly, the enzymes did not show my activity with pentachlorophenol in the same octane/tetrahydrofuran mixture. Pentachlorophenol is sparingly soluble in the pH 6 buffer solution (a solubility of <0.2mg/ml was detected). Addition of 10% of acetonitrile to the buffer can increase the solubility of pentachlorophenol by more than five times. HRP can still achieve a 58% conversion in this mixture.

The catalysis behavior of SBP with these degradation reactions was tested to be very close to that of HRP, basically the same results were obtained when HRP was replaced by SBP at the same reaction conditions.

In addition to the above enzyme screening tests, an analytical method for PCBs using gas chromatography (GC) with an electron capture detector has been established. Performance of this method is still under examination, but a good calibration has been obtained for a hexachlorobiphenyl, indicating it has a good potential for the characterization of the bioremediation reactions with PCBs.

PEG-Modification of Hydrogenase

PEG (Mw 5000) was attached to hydrogenase by tailoring it to the primary **amino** residues of the protein. The PEG-modified hydrogenase has a solubility in anhydrous toluene of ~5 mg/ml. The modified and solubilized enzyme was then investigated to examine its potential as a biocatalyst for sulfur conversion reactions in organic solvents-in which great interest has been found for desulfurization of fossil fuels. However, the enzyme showed a minimum activity for the sulfur reduction reaction in nearly anhydrous toluene. Addition of bulk water to form a two-phase reaction mixture led to an obvious reduction in sulfur dissolved in toluene such that a large amount of H_2S has been detected from the headspace gas in the reactor. Based on this observation, a reaction mechanism has been proposed.

$$2\ H_2O + H_2 + S - H\ 2\ S + 2\ O\ H - + 2\ H +$$

It was also observed that the enzyme lost its activity after it had been in contact with toluene for ~ 1 h. Efforts have been carried out to improve the stability of PEG-hydrogenase in toluene by optimizing the modification procedure: altering ionic strength and temperature. It was expected that such an environmental change could lead to a corresponding change in the configuration of the protein molecules and, thus, reduce enzyme denaturation during the modification process and increase the extent of modification. No significant positive results were obtained.

Tolerance of the PEG-hydrogenase against various inhibitors, including CO, NO, $NaNO_2$, NaCN, NaN_3, and H_2S, has been examined. The modified enzyme did not show any obvious improved tolerance over the native enzyme. However, these substances show higher inhibiting effect on the sulfur reduction reaction than the H_2 evolution reaction. For example, the H_2 evolution reaction was only significantly inhibited by NO (-70% activity **loss)** whereas sulfur reduction was also inhibited by $NaNO_2$ (~70% activity loss), and **NaN_3** (~60% activity loss) in addition to NO (100% activity loss).

Other Modification Approaches

HRP, LiP, and SBP have been solubilized into organic solvents by formation of reversed micelles with AOT, a cation surfactant. However, no activity in isooctane or an isooctane-tetrahydrofuran (70/30), v/v) mixture was observed for the degradation of 2-chlorophenol and pentachlorophenol.

Other methods, including incorporation of the enzyme into polymer matrices and protein cross-linking, are being investigated to increase the stability of the enzymes of interest in organic solvents.

Summary of Accomplishments

Studies with five redox enzymes have been carried out to screen their potential as biocatalysts for remediation reactions. TCE, mercaptans, 2-chloropheno1, and pentachlorophenols have been used as model pollutants.

LiP has been found to be active in catalyzing the degradation of 2-chlorophenol and pentachlorophenol in aqueous solution. PEG modification may increase the solvent tolerance of LiP, and a three-fold enhancement of activity has been achieved in an aqueous solution containing 10% acetnonitrile.

Peroxidases from horseradish and soybean showed good activity for the degradation of pentachlorophenol. These enzymes tend to polymerize 2-chlorophenol and remove the pollutant from its aqueous solution in a form of precipitate, which may serve as an alternative approach for chlorophenol-containing wastewater treatment.

The PEG-modified hydrogenase can be dissolved into organic solvents including toluene and acetonitrile. Sulfur reduction reaction by this enzyme in organic solvents requires the presence of water, which may participate in the reaction pathway.

Publications and Abstracts

Kaufman, E. N., C. A Woodward, C. H. Kim, and M. W. W. Adams. 1997. “Enzyme-Catalysis in Organic Solvents: Characterization and Use of Polyethylene Glycol-Modified Enzymes for Bioremediation Reactions.” Presented at the Biotechnology for Fuels and Chemicals Symposium, Colorado Springs, Col., May 1997.

Kim, C. H., and M. W. W. Adams. 1997. “Enzymatic Catalysis in Organic Solvent.” Presented at the 4th Annual Hyperthermophile Symposium, Del Mar, Calif., May 1997.

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First International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Columbus, Oh., May 1998.

Woodward, C. A. and E. N. Kaufman. 1996. “Enzymatic Catalysis in Organic Solvents: Polyethylene Glycol-Modified Hydrogenase Retains-Sulfhydrogenase Activity in Toluene.” Presented at the AIChE Annual Meeting, Chicago, November 1996.