Oxidation of Dibenzothiophene Catalyzed by Surfactant-Hemoprotein Complexes in Anhydrous Nonpolar Organic Solvents

RYU, KEUN-GARP[1]*, YOUNG-RAE CHAE[1], AND O-YUL KWON[2]

[1]School of Chemical Engineering and Bioengineering, College of Engineering, The University of Ulsan, Ulsan 680-749, Korea
[2]Department of Environmental Engineering, Seoul National University of Technology, Seoul 139-743, Korea

Abstract In anhydrous organic solvents, the complexes formed between AOT (dodecylbenzene sulfuric acid sodium salt) and hemoproteins, such as hemoglobin, myoglobin, or cytochrome c, displayed remarkably higher activity than the hemoprotein powders to oxidize dibenzothiophene, a model compound of organic sulfurs contained in fossil fuels. In slightly hydrophobic organic solvents, such as ethyl acetate and butyl acetate, dibenzothiophene was completely oxidized catalytically by the cytochrome c-AOT complex with cumene hydroperoxide (α,α-dimethylbenzyl hydroperoxide) as an oxidant. In highly hydrophobic organic solvents, such as decane and hexadecane, however, the activity of the cytochrome c-AOT complex decreased, presumably due to the aggregation of the hemoprotein-AOT complex in these solvents.

Key words: Surfactant, hemoprotein, dibenzothiophene, anhydrous organic solvent

Development of enzymatic catalysis in organic solvents made many unconventional biocatalytic processes feasible [22]. The polyester synthesis by lipases [5], the polymerization of various phenols by peroxidases [17, 20], and peptide synthesis by proteases [13] are such examples. Desulfurization of organic sulfur compounds by enzymatic oxidation in organic solvents also draws much interest for environmental purposes, since fossil fuels contain various organic sulfur compounds which are considered as major air pollutants causing acid rain problems. Among various organic sulfur compounds contained in fossil fuels, sulfides and thiols are readily removed by the currently employed hydrodesulfurization processes [1]. However, aromatic sulfur compounds of thiophenes which constitute major sulfur fractions of heavy oils are not easily removed by hydrodesulfurization processes.

An alternative desulfurization process for the removal of thiophenes, especially dibenzothiophene, which is widely accepted as a model compound for thiophenes present in heavy oil, is microbial desulfurization using microbes which oxidize aromatic compounds [8, 14] or sulfonated aromatic compounds [6, 11, 18]. Microbial desulfurization methods, however, require the use of a large amount of water during the desulfurization process to support microbial activity. This aspect of microbial desulfurization becomes a potential drawback in the commercialization of the process.

Peroxidases and hemoproteins are known to oxidize various aromatic compounds [4], especially dibenzothiophene to dibenzothiophene-sulfone via dibenzothiophene-sulfoxide [8, 19, 21]. In order to increase the solubility of dibenzothiophene, water-miscible solvents were added into the aqueous solutions containing peroxidases or hemoproteins. Usually, aqueous buffers containing 25% (v/v) of a polar organic solvent were employed to almost completely oxidize dibenzothiophene [8, 19]. When the content of organic solvents was increased, the degree of the oxidation of dibenzothiophene was decreased significantly. Using hemoglobin as a biocatalyst in nearly anhydrous organic solvents containing 1% v/v of an aqueous buffer, less than 40% of dibenzothiophene was oxidized in polar organic solvents (1,2-propanediol, for example), while no oxidation occurred in nonpolar organic solvents (ethyl acetate, for example) [8]. Since fossil fuels resemble nonpolar organic solvents, such as hexadecane, in their physical properties, development of enzymatic method to oxidize dibenzothiophene in nonpolar organic solvents is crucial for the development of biocatalytic desulfurization of fossil fuels. One simple and efficient method to enhance the activity of enzymes in nonpolar organic solvents is to use enzyme-surfactant complexes [2, 6, 12, 14, 15]. In this work, we employed surfactant-hemoprotein complexes to oxidize dibenzothiophene dissolved in nearly anhydrous nonpolar organic solvents of different hydrophobicity.

*Corresponding author
Phone: 82-52-259-2822; Fax: 82-52-259-1689;
E-mail: kgryu@mail.ulsan.ac.kr

MATERIALS AND METHODS

Materials

Hemoglobin (bovine), myoglobin (horse heart), and cytochrome c (horse heart) were purchased from Sigma Chemical Co. Dibenzothiophene and dibenzothiophene-sulfone were from Aldrich Chemical Co. All organic solvents were of HPLC grade and dried over molecular sieves before use. H_2O_2 (30% solution), cumene hydroperoxide (80% solution), and t-butyl hydroperoxide (70% solution) were from Sigma.

HPLC Analysis

Oxidation of dibenzothiophene in n-octane was analyzed using a high performance liquid chromatograph (HPLC) equipped with a Waters μBondapak C_{18} column (3.9×300 mm) and a UV-visible detector. Dibenzothiophene and its oxidation products were detected at 232 nm. The mobile phase was an acetonitrile-water mixture (20:80, v/v) at a flow rate of 0.8 ml/min.

GC Analysis

Oxidation of dibenzothiophene in highly nonpolar organic solvents was analyzed, using a Hewlett-Packard gas chromatograph (HP 5890 model) equipped with a flame photometry detector. Reaction samples for analysis were centrifuged to remove any solids dispersed in organic solvents, and 1 μl of the supernatant was injected into a HP-5 capillary column (10 m×0.53 mm). Nitrogen was used as a carrier gas. The temperatures of the injector and the detector were 250°C and 200°C, respectively. The column temperature was 120°C for initial 2 min, then increased to 300°C at a rate of 10°C/min.

Preparation of Hemoprotein-Surfactant Complexes

Hemoprotein-surfactant complexes were prepared according to the method described by Fishman et al. [2]. In a 40 ml 0.05 M sodium acetate buffer, pH 6.0, 30 mg of each hemoprotein was dissolved, and the mixture was stirred for 20 min at 4°C. A solution containing 0.088 mmol of a surfactant in 1 ml warm ethanol was added dropwise to the enzyme solution. After mixing for 20 min, the dispersion was sonicated for 10 min. The enzyme-surfactant mixture was further stirred for 3 h, and then stored overnight at 4°C without stirring. The hemoprotein-surfactant precipitates formed were collected by centrifugation, and then lyophilized.

RESULTS AND DISCUSSION

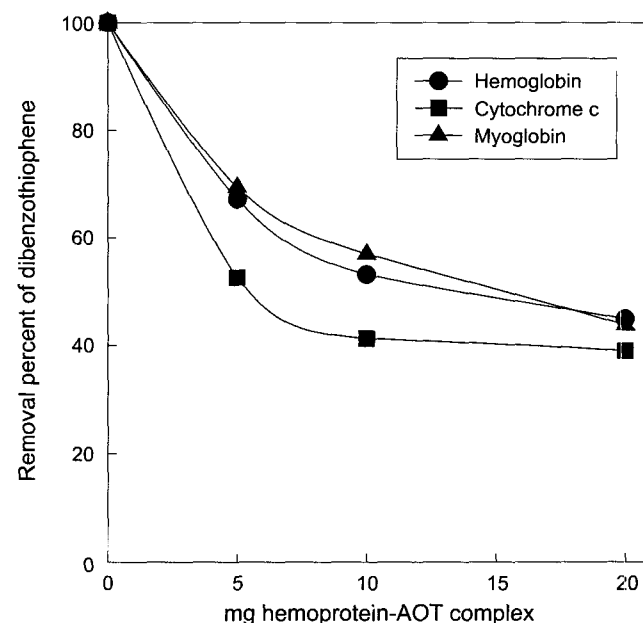### Oxidation of Dibenzothiophene in Anhydrous n-Octane by Hemoprotein-Surfactant Complexes

We chose three hemoproteins, such as hemoglobin, myoglobin, and cytochrome c, for this study, based on the previous results by Klyachko and Klibanov [8] that

Table 1. Percent oxidation of dibenzothiophene by different peroxides catalyzed by cytochrome c-AOT complex in anhydrous n-octane.

Peroxide	Reaction period	Percent removal
H_2O_2	4 h	8.5
t-Butyl hydroperoxide	4 h	14.9
Cumene hydroperoxide	4 h	26.3
H_2O_2	15 h	11.8
t-Butyl hydroperoxide	15 h	21.0
Cumene hydroperoxide	15 h	61.0

Experimental conditions: Reaction mixtures containing 5 mg cytochrome c-AOT complex, 0.15 mM dibenzothiophene, and 1 mM each peroxide in 1 ml anhydrous n-octane were incubated at 30°C with shaking at 150 rpm. Dibenzothiophene concentration was analyzed by HPLC.

these hemoproteins can catalyze complete oxidation of dibenzothiophene with H_2O_2 in an aqueous buffer containing 25% (v/v) water-miscible organic solvents. Therefore, we examined the formation of the complexes between hemoproteins and various surfactants. Among many surfactants tested, including tween 20, tween 80, steric acid, polypropylene glycol, Triton X-114, and AOT, only AOT formed light brownish complexes with hemoproteins. Therefore, we employed hemoprotein-AOT complexes for our further study. When measured by both the spectrophotometric method at 410 nm, the maximum wavelength of hemoproteins, and

Fig. 1. Effect of the amount of hemoprotein-AOT complexes on the oxidation of dibenzothiophene by cumene hydroperoxide in n-octane.

Hemoproteins used to form complexes with AOT were hemoglobin (●), myoblogin (▲), and cytochrome c (■). Reaction conditions: A solution containing 0.15 mM dibenzothiophene, 1 mM cumene hydroperoxide, and a different amount of each hemoprotein-AOT complex in 1 ml n-octane was incubated for 15 h at 30°C with shaking at 150 rpm.

by the micro-biuret method [3], nearly 100% of the initial amount of each hemoprotein was recovered in the hemoprotein-surfactant complexes.

n-Octane, which has a medium hydrophobicity among the nonpolar organic solvents considered in this study, was selected as a reaction medium for the initial study to compare the oxidation power of the three peroxides, H_2O_2, t-butyl hydroperoxide and cumene hydroperoxide, in the oxidation of dibenzothiophene with cytochrome c-AOT complex as a catalyst. Results summarized in Table 1 show that cumene hydroperoxide was the most efficient oxidant to oxidize dibenzothiophene, followed by t-butyl hydroperoxide. H_2O_2, which is a representative oxidant used with peroxidases in aqueous media, poorly oxidized dibenzothiophene in n-octane. The oxidizing activity of cumene hydroperoxide and t-butyl hydroperoxide being higher than H_2O_2 is presumably due to the enhanced solubility of these two hydroperoxides in n-octane. In fact, cumene hydroperoxide possesses the most nonpolar substituents among the three peroxides. At maximum, almost 61% of the initial amount of dibenzothiophene was oxidized by cumene hydroperoxide catalyzed by the cytochrome c-AOT complex in 15 h.

Figure 1 shows the effect of the amount of each hemoprotein-AOT complex on the oxidation of dibenzothiophene by cumene hydroperoxide in n-octane. Cytochrome c-AOT complex was the most efficient among the three hemoproteins employed. The oxidation of dibenzothiophene was increased as the amount of hemoprotein-AOT increased up to 10 mg/
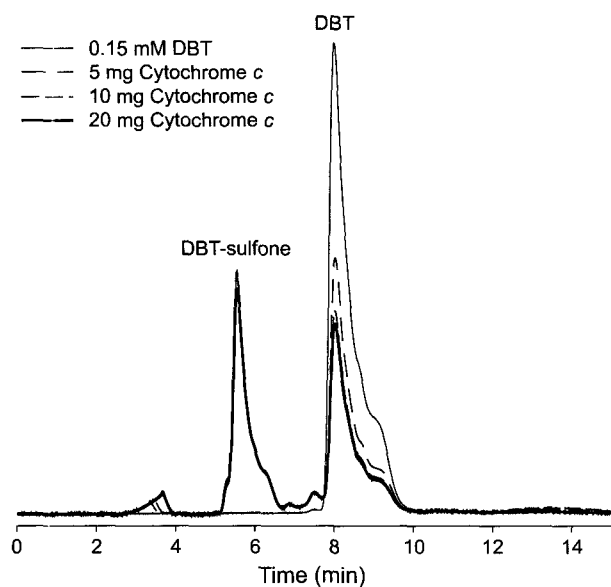
ml and was not further increased, although the amount of hemoprotein-AOT complex increased further to 20 mg/ml. Figure 2 shows the examples of HPLC chromatographs of the reaction mixtures after the oxidation of dibenzothiophene with different amounts of cytochrome c-AOT complex for 15 h in n-octane. Dibenzothiophene-sulfone was detected as the major oxidation product of dibenzothiophene, while no significant production of the oxidation intermediate, dibenzothiophene-sulfoxide, was detected. From these results from the initial study, we selected cumene hydroperoxide and cytochrome c-AOT complex for further study as the most efficient catalytic system.

Oxidation of Dibenzothiophene by Cytochrome c-AOT Complex in Various Nonpolar Organic Solvents

Solvent hydrophobicity can be represented by log P values, which are the logarithm of the partition coefficient of a solvent in an octanol-water two-phase system as defined by Laane et al. [10]. More hydrophobic solvents have larger log P values. We investigated the catalytic activity of cytochrome c-AOT complex in various anhydrous organic solvents of different hydrophobicity. The log P values of the solvents used in this study varied from 0.68 (ethyl acetate) to 8.8 (hexadecane). As summarized in Table 2, the oxidation of dibenzothiophene by cumene hydroperoxide catalyzed by cytochrome c-AOT complex decreased as solvent hydrophobicity increased. In ethyl acetate (log P= 0.68) and butyl acetate (log P=1.7), which are the least hydrophobic organic solvents examined in this work, the initial amount of dibenzothiophene was completely oxidized with cumene hydroperoxide. This result is quite in contrast with the result by Klyachko and Klibanov [8] in which no oxidation of dibenzothiophene was found with hemoglobin powder in anhydrous ethyl acetate. Therefore, we could conclude that the surfactant activated hemoproteins in organic solvents. In hexadecane (log P=8.8), the most

Fig. 2. HPLC chromatographs of the reaction mixtures after the oxidation of dibenzothiophene for 15 h with different amount of cytochrome c-AOT complex in n-octane.

Reaction conditions: A solution containing 0.15 mM dibenzothiophene, mM cumene hydroperoxide, and a different amount of cytochrome c-AOT complex in 1 ml n-octane was incubated for 15 h at 30°C with shaking at 150 rpm.

Table 2. Percent oxidation of dibenzothiophene by cumene hydroperoxide catalyzed by cytochrome c-AOT complex and the native cytochrome c powder, respectively, in various anhydrous organic solvents.

Solvent	Log P	Cyochrome c-AOT complex	Cytochrome c powder
Ethyl acetate	0.68	100.0	1.7
Butyl acetate	1.7	100.0	0.5
Octanol	2.9	84.8	2.9
n-Octane	4.5	55.5	4.0
Decane	5.6	57.2	3.1
Hexadecane	8.8	21.3	3.6

Reaction mixtures containing 20 mg cytochrome c-AOT complex, 0.15 mM dibenzothiophene, and 1 mM cumene hydroperoxide in 1 ml each anhydrous organic solvent were incubated at 30°C with shaking at 150 rpm. Dibenzothiophene concentration was analyzed by GC. Solvent log P values were obtained from Laane et al. [10].

nonpolar organic solvent used in the present study, however, only 21% of dibenzothiophene was oxidized in 15 h. The decreased oxidation of dibenzothiophene in solvents of higher log P values seems to be due to the aggregation of the hemoprotein-AOT complex in those solvents. In ethyl acetate, the hemoprotein-AOT complex was observed to be finely dispersed. Since hexadecane is recognized as a model solvent to represent diesel oil, the decreased oxidation of dibenzothiophene by cytochrome c-AOT complex in hexadecane indicates that further study is necessary to enhance the activity of hemoprotein-surfactant complexes in highly hydrophobic organic solvents to make enzymatic desulfurization feasible.

Acknowledgment

REFERENCES

1. Chang, J. H., S. K. Rhee, Y. K. Chang, and H. N. Chang. 1998. Desulfurization of diesel oils by a newly isolated dibenzothiophene-degrading *Norcadia* sp. strain CYKS2. *Biotechnol. Prog.* **14:** 851- 855.

2. Fishman, A., S. Basheer, S. Shatzmiller, and U. Cogan. 1998. Fatty-acid-modified enzymes as enantioselective catalysts in microaqueous organic media. *Biotechnol. Lett.* **20:** 535- 538.

3. Itzhaki, R. F. and D. M. Gill. 1964. A micro-biuret method for estimating proteins. *Anal. Biochem.* **9:** 401- 410.

4. Jeon, J. H., Y. J. Han, T. G. Kang, E. S. Kim, S. K. Hong, and B. C. Jeong. 2002. Purification and characterization of 2,4-dichlorophenol oxidizing peroxidase from *Streptomyces* sp. AD001. *J. Microbiol. Biotechnol.* **12:** 972- 978.

5. Kim, D. and J. S. Dordick. 2001. Combinatorial array-based enzymatic polyester synthesis. *Biotechnol. Bioeng.* **76:** 200- 206.

6. Kim, E., I. Ahn, L. W. Lion, and M. L. Shuler. 2001. Enhanced *in-situ* mobilization and biodegradation of phenanthrene from soil by a solvent/surfactant system. *J. Microbiol. Biotechnol.* **11:** 716- 719.

7. Kim, H. Y., T. S. Kim, and B. H. Kim. 1991. Isolation and characterization of a dibenzothiophene-degrading sulfate-reducing soil bacteria. *J. Microbiol. Biotechnol.* **1:** 1- 5.

8. Klyachko, N. L. and A. M. Klibanov. 1992. Oxidation of dibenzothiophene catalyzed by hemoglobin and other

9. Kwon, H. H., E. Y. Lee, K. S. Cho, and H. W. Ryu. 2003. Benzene biodegration using the polyurethane biofilter immobilized with *Stenotrophomonas maltophilia* T3-c. *J. Microbiol. Biotechnol.* **13:** 70- 76.

10. Laane, C., S. Boeren, and K. Vos. 1985. On optimizing organic solvents in multi-liquid phase biocatalysis. *Trends Biotechnol.* **3:** 251- 252.

11. Monticello, D. J. 2000. Biodesulfurization and the upgrading of petroleum distillates. *Curr. Opin. Biotechnol.* **11:** 540- 546.

12. Moon, H.-J., Y.-K. Lim, H.-S. Kim, D.-Y. Kwon, and W.-J. Chung. 2002. Glycolipid biosurfactants produced by *Pseudomonas aeruginosa* D2D2 from diesel-contaminated soil. *J. Microbiol. Biotechnol.* **12:** 371- 376.

13. Nagashima, T., A. Watanabe, and H. Kise. 1992. Peptide synthesis by proteases in organic solvents: Medium effect on substrate specificity. *Enzyme Microb. Technol.* **14:** 842- 847.

14. Noda, S., N. Kamiya, M. Goto, and F. Nakashio. 1997. Enzymatic polymerization catalyzed by surfactant-coated lipases in organic media. *Biotechnol. Lett.* **19:** 307- 309.

15. Okahata, Y. and T. Mori. 1997. Lipid-coated enzymes as efficient catalysts in organic media. *TIBTECH* **15:** 50- 54.

16. Park, I. H. and J. O. Ka. 2003. Isolation and characterization of 4-(2,4-dichlorophenoxy)butyric acid-degrading bacteria from agricultural soils. *J. Microbiol. Biotechnol.* **13:** 243- 250.

17. Samuelson, L. 2001. Mechanistic study of the peroxidase-catalyzed polymerization of sulfonated phenol. *Macromolecules* **34:** 3522- 3526.

18. Setti, L., P. Farinelli, D. D. Martino, S. Frassinetti, G. Lanzarini, and P. G. Pifferi. 1999. Developments in destructive and non-destructive pathways for selective desulfurizations in oil-biorefining processes. *Appl. Microbiol. Biotechnol.* **52:** 111- 117.

19. Stachyra, T., D. Guillochon, S. Pulvin, and D. Thomas. 1996. Hemoglobin, horseradish peroxidase and heme-bovine serum albumin as biocatalyst for the oxidation of dibenzothiophene. *Appl. Biochem. Biotechnol.* **59:** 231- 244.

20. Uyama, H., N. Maruichi, H. Tonami, and S. Kobayashi. 2002. Peroxidase-catalyzed oxidative polymerization of bisphenols. *Biomacromolecules* **3:** 187- 193.

21. Vazquez-Duhalt, R., D. W. S. Westlake, and P. M. Fedorak. 1993. Cytochrome c as a biocatalyst for the oxidation of thiophenes and organosulfides. *Enzyme Microb. Technol.* **15:** 494- 499.

22. Zaks, A. and A. M. Klibanov. 1988. Enzymatic catalysis in nonaqueous solvents. *J. Biol. Chem.* **263:** 3194- 3201.