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Oxidation of Polycyclic Aromatic Hydrocarbons Catalyzed by Soybean Peroxidase

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Abstract

Soybean peroxidase (SBP) catalyzes the oxidation of a variety of polycyclic aromatic hydrocarbons (PAHs) in the presence of water-miscible organic cosolvents, including acetonitrile, tetrahydrofuran, and dimethylformamide (DMF). Oxidation was optimal at pH 2.0–2.5, with substantially lower reactivity at pH 1.5 as well as at pH > 3.0. Despite the low pH activity optimum, SBP had an observed half-life of 120 h at pH 2.5. Conversions of greater than 90% were observed with anthracene and 9-methylanthracene in the presence of 50% (v/v) DMF. Anthracene oxidation yielded exclusively anthraquinone, thereby demonstrating that SBP catalyzes a formal six-electron oxidation of the unactivated aromatic substrate to the quinone. A mechanism is proposed to account for this reaction that includes the initial one-electron oxidation of the PAH followed by addition of water to the oxidized PAH. 9-Methylanthracene was more reactive than anthracene, and its enzymatic oxidation yielded two products: anthraquinone and 9-methanol-9,10-dihydroanthracene. The former product indicates that loss of the methyl group occurs during enzymatic oxidation. These results suggest that SBP could be useful in the conversion of PAHs into more environmentally benign materials.

Index Entries: Peroxidase; oxidation of polycyclic aromatic hydrocarbons; water-miscible organic cosolvents; anthracene.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are produced ubiquitously as a result of industrial pyrolysis and organic combustion, posing a serious risk to the environment and human health *(1–3)*. Moreover, serious aquatic pollution results from nautical vessel effluents and spills *(1)*. Various methods have been employed for the removal of PAHs from the environment or the conversion of PAHs into more benign chemical species in polluted sites and waste streams. These techniques include solvent extraction, activated-carbon filtration, photooxidation, or the use of lipophilic adsorbents *(4)*. These methods are often too costly, however, particularly for dilute concentrations of PAHs present in water, soil, and organic liquids (e.g., diesel fuels *[5]*).

As a class, PAHs are relatively unreactive chemically. However, PAHs are particularly good substrates of the cytochromes P-450 found in mammalian livers, where they are converted into epoxides that may bind to DNA *(6)*. These epoxides, particularly from PAHs with exposed "bay regions" (e.g., chrysene), are highly potent xenobiotics and suspected carcinogens *(7,8)*. Thus, enzymes are capable of oxidizing PAHs. Unfortunately, the cytochromes P-450 are unstable membrane-bound enzymes and are not commercially available in large quantities. Their use in large-scale remediation is therefore impractical.

Haemmerli et al. *(9)* showed that a nonmammalian, noncytochrome P-450, lignin peroxidase ([LiP], from the white rot fungus, *Phanerochaete chrysosporium*) was capable of oxidizing benzo(*a*)pyrene in aqueous solutions. More recently, Vazquez-Duhalt et al. *(10)* found that LiP could catalyze the oxidation of a number of PAHs in aqueous solutions and aqueous-organic mixtures to oxidized products including quinones and hydroxylated derivatives. LiP, however, like the cytochromes P-450, is a poorly stable enzyme and is available only in limited supply.

An alternative to LiP is the abundant peroxidase from soybean hulls (soybean peroxidase [SBP]). McEldoon et al. *(11,12)* observed that SBP displayed the potent oxidative activity of LiP while exhibiting high stability toward acidic pHs and high temperature. In the present study, we demonstrate that SBP, a commercially available and inexpensive enzyme, is capable of catalyzing the efficient oxidation of a wide variety of PAHs. This finding may prove useful in large-scale bioremediation efforts.

Materials and Methods

Materials

SBP was obtained from Enzymol (Columbus, OH) as a solid powder (140 purpurogalin units/mg solid). PAHs were obtained from Aldrich (Milwaukee, WI). Hydrogen peroxide (30% solution in water) was obtained from EM Science (Gibbstown, NJ). Silica gel (flash chromatography grade)

was purchased from J. T. Baker (Phillipsburg, NJ). All other chemicals employed were of the highest purity commercially available.

Measurement of PAH Oxidation Rates and Identification of Oxidation Products

A typical reaction mixture contained from 5 to 30 µ*M* PAH dissolved in 0.1 *M* glycine-HCl buffer (pH 2.5) containing 10–50% (v/v) of a water-miscible organic solvent (tetrahydrofuran [THF], acetonitrile, or dimethylformamide [DMF]), 0.1 *M* $CaCl_2$, and 20 µg/mL of enzyme. Reactions were run in triplicate and were initiated on addition of 1.0 m*M* H_2O_2. PAH oxidation was monitored spectrophotometrically at appropriate wavelengths (anthracene, 252 nm; pyrene, 335 nm; acenaphthene, 290 nm; 9-methylanthracene, 259 nm; fluorene, 305 nm; chrysene, 247 nm; and phenanthrene, 293 nm in 1.0-mL quartz cuvets.

Product identification was performed via gas chromatography-mass spectrometry (GC-MS) (Shimadzu QP-5000 GC-MS equipped with an HP-5 column [Hewlett-Packard, Palo Alto, CA]) with an initial oven temperature of 150°C, a temperature ramp of 5°C per minute to 285°C, and maintaining this temperature for 5 min). Reactions analyzed by GC-MS consisted of 1 m*M* PAH dissolved in aqueous buffer containing 50% (v/v) DMF, 2.0 m*M* H_2O_2, and an enzyme concentration of 1 mg/mL.

^{13}C-nuclear magnetic resonance (NMR) analysis was performed on the products of 9-methylanthracene oxidation to help elucidate the mechanism of SBP-catalyzed oxidation. To that end, 9-methylanthracene was oxidized in a 1-L solution consisting of 30% (v/v) CH_3CN in aqueous buffer containing 30 mg of SBP, 0.1 m*M* 9-methylanthracene, and 1.0 m*M* H_2O_2 (added continuously and dropwise throughout the reaction). The reaction was terminated after 24 h by extraction of the substrate and PAH-derived products with methylene chloride, followed by evaporation of the methylene chloride and purification of the PAH-derived products by silica gel flash chromatography (4:1, hexane:ethyl acetate). Products of 9-methylanthracene oxidation were analyzed by ^{13}C-NMR (Brüker WM 360 MHz, Billerica, MA) in DMSO-d_6 with tetramethylsilane (TMS) as internal reference: Major product: δ68.8, 43.1, 134.4, 122.8, 123.1, 126.0, 125.8, 135.4, 32.8; Minor product δ168.1, 134.7, 131.5, 129.3.

Results and Discussion

The ability of SBP to catalyze the oxidation of the nonphenolic aromatic compound veratryl alcohol *(11)* strongly suggested that other nonphenolic aromatic compounds may also serve as substrates of the enzyme. Because of the growing importance of removing PAHs from contaminated sites and waste streams, we examined the feasibility of using SBP as a catalyst to oxidize and increase the water solubility (and hence reduce the toxicity) of PAHs. Anthracene, a common PAH, was chosen as a model substrate for this study.

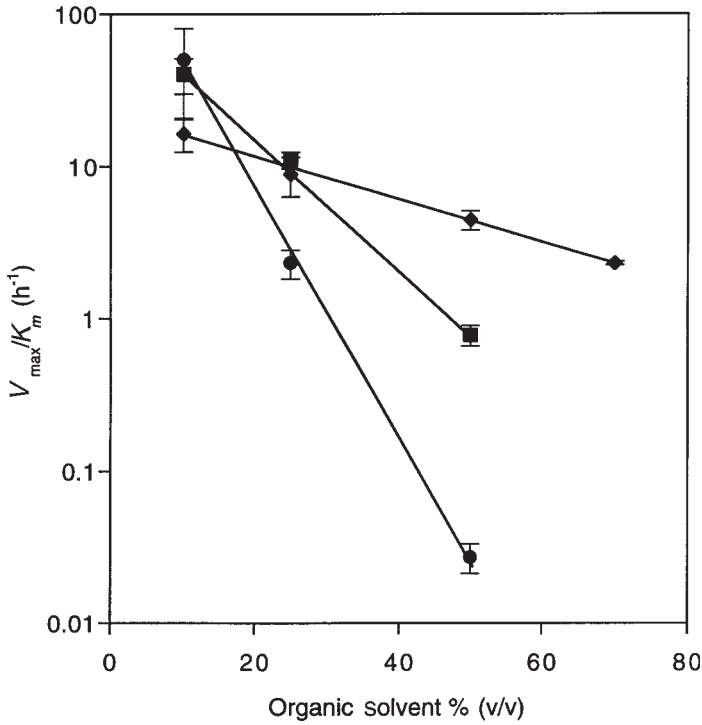
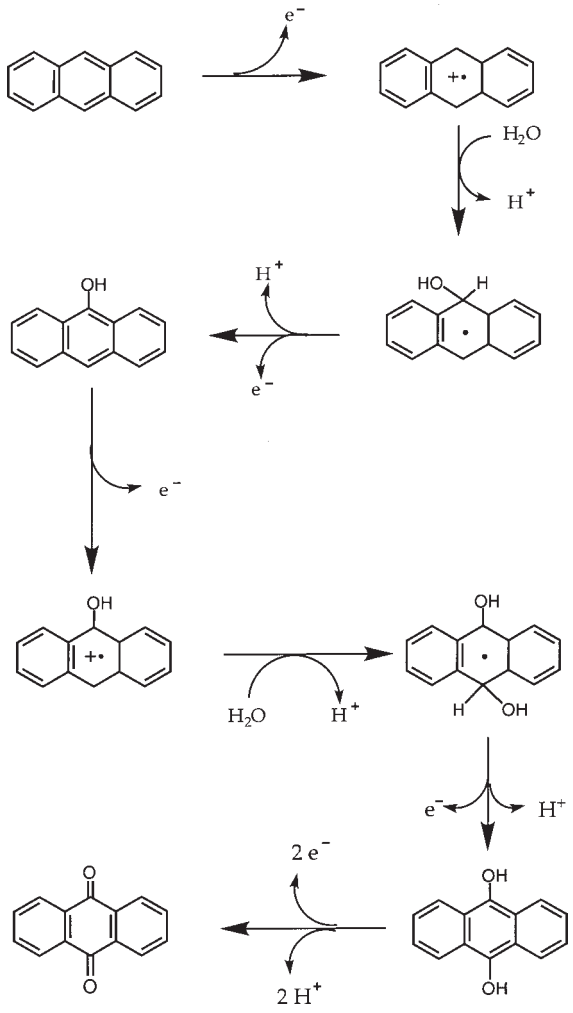


Fig. 1. The cosolvent effect on anthracene oxidation catalyzed by SBP in glycine HCl buffer, pH 2.5: (◆) DMF; (■) acetonitrile; (●) THF. Error bars represent ± 2 SDs for triplicate experiments.

Anthracene Oxidation

The poor aqueous solubility of anthracene (which is representative of nearly all PAHs) necessitated the addition of organic cosolvents such as THF, DMF, or acetonitrile. In aqueous buffer (pH 2.5, containing 10% [v/v] THF to improve PAH solubility), the value of V_{max}/K_m for SBP-catalyzed oxidation of anthracene was 46.7 h^{-1}. Increasing the THF concentration to 50% (v/v) decreased V_{max}/K_m three orders of magnitude (Fig. 1). The nature of the organic solvent affected SBP catalysis; the value of V_{max}/K_m for SBP catalysis in 10% (v/v) CH_3CN was similar to that in THF, whereas the value in 10% (v/v) DMF was about 10-fold lower. Interestingly, despite the relatively low catalytic activity of SBP in 10% (v/v) DMF, SBP remained active for anthracene oxidation even in 70% (v/v) DMF (Fig. 1). This was an important observation because high concentrations of organic solvent improved PAH solubility. Note that in no case was anthracene oxidation observed in the absence of SBP or in the presence of heat-inactivated SBP (prepared by boiling the enzyme in water for 30 min prior to use).

Product analysis by GC-MS of a 1 m*M* anthracene reaction in 50% (v/v) DMF (DMF supported SBP catalysis much better than THF at this cosolvent concentration) indicated that a total conversion of 91% to a single com-



Scheme I. Proposed mechanism of anthracene oxidation catalyzed by SBP.

pound was obtained. This compound coeluted with (and had an identical mass-to-ion ratio as) authentic anthraquinone, and had a ^{13}C-NMR spectrum also consistent with that of anthraquinone. The proposed reaction mechanism is described in Scheme I, consistent with a formal six-electron oxidation of anthracene, and similar to published mechanisms for the oxidation of veratryl alcohol *(10)* and methoxybenzenes *(13)*. The initial step in anthracene oxidation is proposed to be the one-electron oxidation of the polycyclic aromatic ring structure to give a cation radical. This species can then accept water to yield a hydroxylated aromatic free radical with subsequent oxidation to give the 9-hydroxyanthracene intermediate. A similar series of steps would yield the hydroquinone. Finally, the two-electron oxidation of the hydroquinone to the anthraquinone would also be catalyzed by SBP.

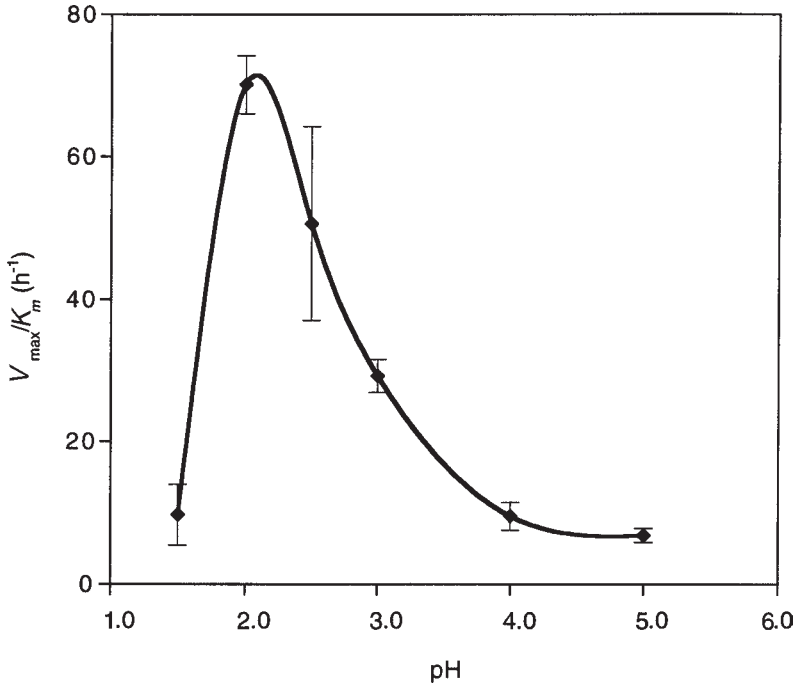


Fig. 2. The effect of pH on the catalytic efficiency of SBP-catalyzed oxidation of anthracene in 10% (v/v) THF. Error bars represent ± 2 SDs for triplicate experiments.

SBP-catalyzed oxidation of anthracene was strongly influenced by the solution pH. As shown in Fig. 2, maximal catalytic efficiency in 10% (v/v) THF was obtained at pH 2.0, albeit measurable oxidation was obtained from pH 1.5 to 5.0. These results are in close agreement with the optimum value of pH 2.4 reported by McEldoon et al. *(11)* for SBP-catalyzed oxidation of veratryl alcohol. Presumably, the acidic conditions promote a higher oxidation potential for SBP and facilitate anthracene oxidation *(11)*. The stability of SBP was also strongly dependent on the solution pH. In the presence of 10% (v/v) THF, the half-life at 25°C was nearly 10-fold higher at pH 2.5 than at pH 1.5 (Fig. 3), providing a half-life of SBP at pH 2.5 of about 120 h. The reaction rate of anthracene oxidation catalyzed by SBP was dependent on temperature; in 10% (v/v) THF, the optimum temperature for the reaction was 50°C (data not shown).

Oxidation of Other PAHs Catalyzed by SBP

Transformations of 1 m*M* PAH solutions were performed in 50% (v/v) DMF to take advantage of the higher solubilities of the PAHs in this reaction medium. Conversions after 24 h varied widely and indicated a broad range of reactivities (Table 1). Nearly complete conversions were obtained for acenaphthene, 9-methylanthracene, and anthracene. Substantially lower conversions were obtained for pyrene and chrysene. Table 2 gives proposed major reaction products (along with MS assignments).

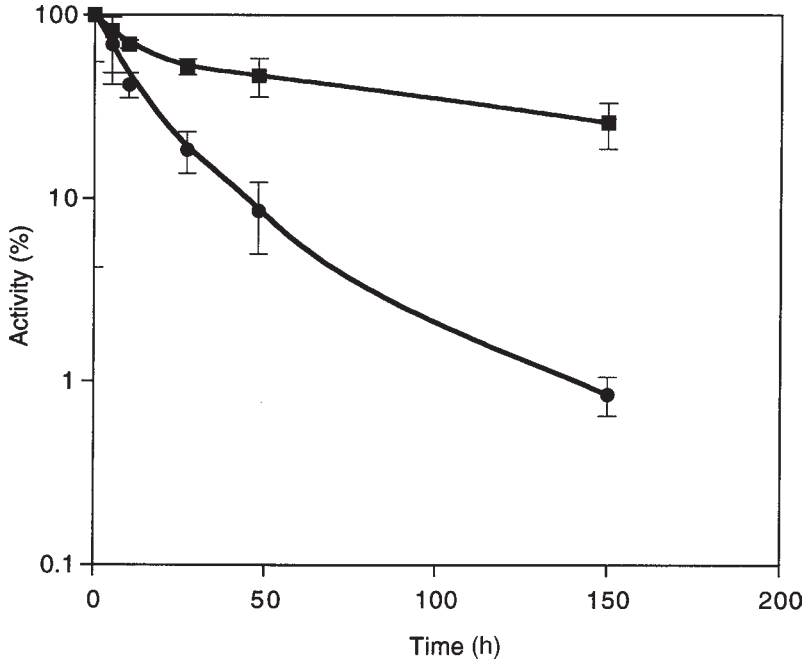


Fig. 3. Stability of SBP at 25°C for anthracene oxidation reactions in 10% (v/v) THF at pH 1.5 (●) and 2.5 (■). Error bars represent ± 2 SDs for triplicate experiments.

Table 1
Reactivity of Various PAHs in SBP-Catalyzed Oxidation

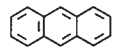
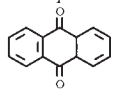
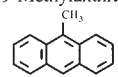
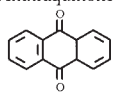
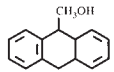
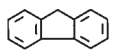
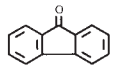
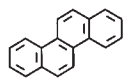
PAH	V_{max}/K_m (h^{-1})[a]	Conversion (%)[b]
Anthracene	16.5	91
9-Methylanthracene	115	97
Pyrene	1.09	23
Acenaphthene	0.37	100
Chrysene	1.38	4
Fluorene	<0.02	<1
Phenanthrene	0	0

[a]Reactions conducted in 10% (v/v) DMF using 20 µg/mL of SBP, 5–30 µ*M* PAH, and 1 m*M* H_2O_2, in aqueous buffer, pH 2.5.

[b]Reactions performed in 50% (v/v) DMF in 24 h using 1 m*M* PAH, 2 m*M* H_2O_2, and 1 mg/mL of SBP, in aqueous buffer, pH 2.5.

Values of V_{max}/K_m were determined for the oxidation of a variety of PAHs in 10% (v/v) DMF reaction solutions (Table 1). Of the seven PAHs examined, six were oxidized by the enzyme, fluorene was a very poor substrate, and only phenanthrene was unreactive; 9-methylanthracene was clearly the best substrate, having a value of V_{max}/K_m almost an order of magnitude higher than that obtained for any other compound.

Table 2
Mass Spectral Data of Products Formed
from the SBP-Catalyzed Reactions of Various PAHs in Presence of H_2O_2

Substrate	Products	Mass Spectral Ions (m/z)[a]
Anthracene 	Anthraquinone 	209(17), 208(100) [M+], 207(14), 181(16), 180(97), 153(13), 152(75), 151(37),150(19), 126(14), 76(77), 75(25)
9-Methylanthracene 	Anthraquinone 	209(17), 208(100) [M+], 207(14), 181(16), 180(97), 153(13), 152(75), 151(37), 150(19), 126(14), 76(77), 75(25)
	9-Methanol-9,10 -dihydroanthracene 	210(16) [M+], 209(100), 206(5), 178(7), 176(4), 152(22), 151(6), 77(17) 76(22)
Acenaphthene 	1-Acenaphthenone 	169(12), 168(93) [M+], 141(12), 140(100), 139(83), 113(11), 89(10)
Fluorene 	9-Flourenone 	181(19), 180(100) [M+], 152(43), 151(23), 150(15), 126(8), 98(4), 76(30), 75(13)
Pyrene 	Unknown[b]	238(36), 237(19), 236(100), 201(35), 200(39), 199(8), 118(28), 100(15)
Chrysene 	Unknown	203(14), 202(100), 201(8), 200(20), 101(28), 100(11), 88(15), 87(12)

[a]Values in parentheses are relative intensities (%). [M+] indicates the molecular ion.

[b]Mass spectral data correspond well to an unknown compound found by Vazquez-Duhalt et al. *(10)* from LiP-catalyzed oxidation of pyrene.

As previously described, anthracene was oxidized exclusively to anthraquinone. Interestingly, 9-methylanthracene was oxidized to two products in a 2:1 ratio. The minor product matched that of anthraquinone (via GC-MS), indicating removal of the methyl side chain during oxidation. The major product gave an m/z of 210 and a ^{13}C-NMR spectrum consistent with 9-methanol-9,10-dihydroanthracene; a product of similar mass was observed by Vazquez-Duhalt et al. *(10)*. Formation of both occurred under aerobic as well as anaerobic conditions (the latter performed under an argon atmosphere), indicating that the oxygen atoms in the PAH oxidation products did not originate from molecular oxygen.

The acenaphthene reaction yielded a single product with an m/z of 168, which corresponds to the expected value for 1-acenaphthenone. Similarly, oxidation of fluorene produced a single product corresponding to the expected m/z for 9-fluorenone. Pyrene oxidation yielded a single product with an m/z of 236, which may correspond to the reduced form of the 1,8-pyrenedione. The latter product was also observed in LiP-catalyzed oxidations *(10)*. Large-scale oxidation of pyrene resulted in little recoverable product. Instead, a water-insoluble precipitate was produced, possibly owing to oxidative polymerization of the 1,8-pyrenediol, which could serve as an excellent substrate of plant peroxidases *(14)*.

Conclusion

The results described here support the feasibility of using SBP as a catalyst for PAH oxidation in the presence of H_2O_2. SBP was shown to have high activity in systems consisting of 10% (v/v) water-miscible solvents such as THF, CH_3CN, and DMF. More important, SBP exhibited activity in DMF concentrations as high as 70% (v/v). Certain PAHs, such as anthracene, 9-methylanthracene, and acenaphthene, were nearly completely oxidized in as much as 50% (v/v) DMF when the concentration of SBP was 1 mg/mL. The oxidation products tend to contain hydroxyl and keto groups, which are more water soluble than PAH and presumably have fewer associated health concerns owing to their expected inability to form DNA-binding epoxides. Several approaches can be considered in this regard. For example, in aqueous-based soil systems, SBP can be used in extraction-well techniques *(15)* to catalyze the oxidation (and hence extractability) of PAHs. Conversely, the use of SBP in organic solvents may be of direct importance to the oxidation of PAHs in diesel fuels. In both scenarios, long-term use of this readily abundant enzyme could provide a safe, cost-efficient means of oxidizing PAHs into more environmentally benign materials.

Acknowledgments

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