Lignin Peroxidase-Catalyzed Oxidation of Sulfonated Azo Dyes Generates Novel Sulfophenyl Hydroperoxides†

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ABSTRACT: Lignin peroxidase (LiP) is an extracellular enzyme produced by the lignin-degrading fungus *Phanerochaete chrysosporium* and is involved in azo dye degradation by this organism. In this study, LiP oxidation of the sulfonated azo dyes 4-(4′-sulfophenylazo)-2,6-dimethylphenol (**I**), Orange II [1-(4′-sulfophenylazo)-2-naphthol] (**II**), a dimethyl analog of Orange II [1-(2′,6′-dimethyl-4′-sulfophenylazo)-2-naphthol] (**III**), and 4-(4′-sulfonamidophenylazo)-2,6-dimethylphenol (**IV**) was examined. Azo dye **I** was oxidized to 2,6-dimethyl-1,4-benzoquinone and 4-sulfophenyl hydroperoxide. Orange II (**II**) was oxidized to 1,2-naphthoquinone and 4-sulfophenyl hydroperoxide. The dimethyl analog of Orange II (**III**) was oxidized to 1,2-naphthoquinone and 2,6-dimethyl-4-sulfophenyl hydroperoxide. Azo dye **IV** was oxidized predominantly to 2,6-dimethyl-1,4-benzoquinone and another product, tentatively characterized as 4-sulfonamidophenyl hydroperoxide. In the ^{18}O-labeling studies with $^{18}O_2$, oxygen incorporation into the phenyl hydroperoxides from the oxidation of **I** and **III** was observed. A mechanism for azo dye degradation consistent with product identification and the ^{18}O-labeling studies is proposed. Two successive one-electron oxidations of the phenolic ring of an azo dye by the H_2O_2-oxidized forms of LiP produces a carbonium ion. Then water attacks the phenolic carbon bearing the azo linkage, producing an unstable hydroxy intermediate which breaks down to yield a quinone and a sulfo- or sulfonamidophenyldiazene. The phenyldiazene is oxidized by O_2 to generate the corresponding phenyldiazene radical, which eliminates N_2 to yield a sulfo- or sulfonamidophenyl radical. O_2 scavenges the latter to yield the corresponding hydroperoxide. This is the first report on the production and identification of a phenyl hydroperoxide in any chemical or biological system.

A multitude of synthetic dyes are used for textile dyeing, paper printing, and color photography (Zollinger, 1987). Major classes of synthetic dyes include azo, anthraquinone, and triarylmethane dyes, with azo dyes constituting more than 50% of those used in industrial applications (Zollinger, 1987). Azo dyes appear to be resistant to aerobic degradation by bacteria (Kulla et al., 1983; Michaels & Lewis, 1985; Pagga & Brown, 1986; Shaul et al., 1991); however, a consortium of aerobic and anaerobic bacteria have been demonstrated to degrade a few azo dyes (Haug et al., 1991). In mammals, the azo linkages of azo dyes are reduced to generate aromatic amines, and this reductive reaction is primarily performed by the intestinal microflora (Chung & Cerniglia, 1992; Huang et al., 1979; Rafii et al., 1990). Some of the aromatic amines generated in this process are potentially carcinogenic (McCann & Ames, 1975).

White-rot basidiomycete fungi are the only microorganisms that can mineralize the wood components, lignin, cellulose, and hemicellulose (Kirk & Farrell, 1987; Gold et al., 1989). In these fungi, lignin degradation occurs in the secondary metabolic stage in response to low levels of nutrient nitrogen in the medium (Kirk & Farrell, 1987; Gold et al., 1989). The lignin degradative system of the white-rot fungus *Phanerochaete chrysosporium* has been studied in detail and some of the extra- and the intracellular enzyme systems involved in lignin degradation in this organism have been identified. The extracellular enzyme systems include two peroxidases, lignin peroxidase (LiP)[1] and manganese peroxidase (MnP), and a H_2O_2-generating system, glyoxal oxidase (Tien & Kirk, 1984; Gold et al., 1984; Glenn & Gold, 1985; Kersten & Cullen, 1993). The intracellular enzyme systems include a quinone reductase and an aromatic ring-cleaving enzyme, 1,2,4-trihydroxybenzene 1,2-dioxygenase (Constam et al., 1991; Rieble et al., 1994).

Lignin-degrading cultures of *P. chrysosporium* degrade aromatic pollutants such as chlorophenols, nitrotoluenes, polycyclic aromatic hydrocarbons, and dioxins in addition to lignin (Hammel & Tardone, 1988; Valli & Gold, 1991; Mileski et al., 1988; Fernando et al., 1990; Valli et al., 1992a,b; Hammel et al., 1992; Haemmerli et al., 1986). Metabolic pathways for pollutant degradation have been described, and the peroxidases have been demonstrated to

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[1] Abbreviations: DMSPH, 2,6-dimethyl-4-sulfophenyl hydroperoxide; ESI-MS, electrospray ionization−mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; GC-MS, gas chromatography−mass spectrometry; HPLC, high performance liquid chromatography; LiP, lignin peroxidase; MnP, manganese peroxidase; SAPH, 4-sulfonamidophenyl hydroperoxide; SPH, 4-sulfophenyl hydroperoxide.

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play important roles (Hammel & Tardone, 1988; Valli & Gold, 1991; Mileski et al., 1988; Fernando et al., 1990; Valli et al., 1992a,b; Hammel et al., 1992; Haemmerli et al., 1986). Spadaro et al. (1992) and Paszczyński et al. (1992) demonstrated that lignin-degrading cultures of *P. chrysosporium* mineralize a number of nonsulfonated and sulfonated azo dyes to CO_2. However, the pathways for azo dye degradation are still not understood. Spadaro and Renganathan (1994) demonstrated that Disperse Yellow 3 [2-(4′-acetamidophenylazo)-4-methylphenol], a nonsulfonated dye, is oxidized to 4-methyl-1,2-benzoquinone and acetanilide by LiP, horseradish peroxidase, and Mn(III)-malonate, a manganese peroxidase mimic. A detailed mechanism involving phenyldiazene and phenyl radical intermediates was proposed to explain Disperse Yellow 3 degradation (Spadaro & Renganathan, 1994). Goszczyński et al. (1994) recently identified 2,6-dimethyl-1,4-benzoquinone, 4-nitrosobenzenesulfonic acid, 4-aminobenzenesulfonic acid, 2,6-dimethyl-4-aminophenol, 4-hydroxybenzenesulfonic acid, and benzenesulfonic acid from the oxidation of 4-(4′-sulfophenylazo)-2,6-dimethylphenol (**I**) by LiP and proposed a mechanism involving redox processes between 4-sulfophenyldiazene, 4-nitrosobenzenesulfonic acid, and quinone intermediates to explain product formation.

In this report, LiP oxidation of the sulfonated azo dyes 4(4′-sulfophenylazo)-2,6-dimethylphenol (**I**), Orange II (**I**), a dimethyl analog of Orange II (**III**), and 4(4′-sulfonamidophenylazo)-2,6-dimethylphenol (**IV**) is examined. Novel sulfophenyl hydroperoxides have been identified as products from the oxidation of sulfonated azo dyes (**I**−**III**). A mechanism for sulfonated azo dye degradation is proposed based on product identification and ^{18}O-labeling studies. Findings of this study disagree with the recent observations of Goszczyński et al. (1994) regarding sulfonated azo dye degradation by peroxidases.

MATERIALS AND METHODS

Chemicals. 2,6-Dimethylphenol, 2,6-dimethylaniline, 2,6-dimethyl-1,4-benzoquinone, 4-hydroxybenzenesulfonic acid, benzenesulfonamide, 4-aminobenzenesulfonamide, and 2-naphthol were obtained from Aldrich Chemical Co., Milwaukee, WI. Orange II, H_2O_2, and HPLC sorbent for reverse phase chromatography (particle size 40−63 μm) were purchased from Sigma Chemical Co., St. Louis, MO. Orange II was purified by crystallization from hot water.

Syntheses of Azo Dyes. 4(4′-Sulfophenylazo)-2,6-dimethylphenol (**I**) was prepared by coupling the diazonium salt of 4-aminobenzenesulfonic acid with 2,6-dimethylphenol and crystallizing the product from boiling water as described (Goszczyński et al., 1994).

The dimethyl analog of Orange II (**III**) was prepared by coupling the diazonium salt of 3,5-dimethyl-4-aminobenzenesulfonic acid with 2-naphthol (Muralikrishna & Renganathan, 1993; Furnis et al., 1989). To purify this dye, the crude product was dissolved in boiling water and then cooled to 80 °C. Approximately twice the volume of 95% ethanol was added, and the product was allowed to crystallize overnight. The crystals were separated by filtration under vacuum.

4-(4′-Sulfonamidophenylazo)-2,6-dimethylphenol (**IV**) was prepared by coupling the diazonium salt of 4-aminobenzenesulfonamide with 2,6-dimethylphenol as described (Goszczyński et al., 1994). The dye was further purified by crystallization from ethanol.

Syntheses of Substituted Benzenesulfonic Acids. 3,5-Dimethyl-4-hydroxybenzenesulfonic acid and 3,5-dimethyl-4-aminobenzenesulfonic acid were synthesized and purified as described (Muralikrishna & Renganathan, 1993). 3,4-Dihydroxybenzenesulfonic acid was prepared by sulfonating catechol at 60 °C as described (Furnis et al., 1989). 1,2-Benzoquinone-4-sulfonic acid was freshly prepared by reacting a cold aqueous solution of 3,4-dihydroxybenzenesulfonic acid (10 mmol) with an equivalent amount of sodium periodate (10 mmol). Reaction was instantaneous, and the formation of red color indicated the production of quinonesulfonic acid.

4-Azidobenzenesulfonic acid was synthesized by reacting the diazonium salt of 4-aminobenzenesulfonic acid with sodium azide. A cold solution of 4-aminobenzenesulfonic acid (5 mmol) in NaOH (4 N, 1.25 mL) was mixed with sodium nitrite solution (5 mmol, 1.25 mL). Hydrochloric acid (3 N, 3.4 mL) was added slowly, while the reaction temperature was maintained at 5 °C. The precipitated crystalline diazonium salt was filtered, washed with cold water, suspended in a sodium azide solution, and stirred. Production of a clear solution indicated that all of the diazonium salt had been converted to azidosulfonic acid. The product was obtained via lyophilization. HPLC analysis indicated >98% purity. [FAB-MS: *m/z* 198 (M^+).]

4-Azidobenzenesulfonamide was synthesized from 4-aminobenzenesulfonamide by a similar procedure. The diazonium salt of 4-aminobenzenesulfonamide was prepared by the addition of sodium nitrite (5 mmol) to a cold solution of 4-aminobenzenesulfonamide in 3 N HCl. The diazonium salt was soluble, and upon reaction with sodium azide (5 mmol), the product 4-azidobenzenesulfonamide precipitated. The product was filtered and dried.

HPLC Analyses. HPLC analyses were performed under ambient conditions using a reverse phase C-18 column (0.46 × 25 cm; Separations Group, Hesperia, CA). Compounds were eluted with a gradient consisting of a phosphate buffer (0.1 M, pH 7) and a water−methanol (1:1) mixture. Initially, the phosphate buffer concentration was maintained at 100% for 5 min. Then the methanol−water mixture was increased from 0% to 100% over 10 min and maintained at 100% concentration for an additional 10 min. The solvent flow rate was 1 mL/min. Compound elution was monitored at 254 nm.

Mass Spectral Analyses. Organic solvent extractable products were analyzed by gas chromatography−mass spectrometry (GC-MS). GC-MS analyses were performed at 70 eV on a VG Analytical 7070E mass spectrometer fitted with an HP 5790A GC and a 15-m fused capillary column (DB-5, J&W Science). A temperature gradient was used in the GC separations. The initial temperature was 70 °C, and it was increased to 320 °C at a rate of 10 °C/min. The products were identified by comparison of their retention times and mass spectral fragmentation patterns with those of standard compounds.

Sulfonated products were analyzed by fast atom bombardment mass spectrometry (FAB-MS) and by electrospray ionization mass spectrometry (ESI-MS). These mass spectral analyses were performed in the negative-ion mode. FAB-MS analysis was accomplished using a VG Analytical 7070E mass spectrometer equipped with an Ion-Tech saddle field

atom gun operated with xenon at 6 kV. Either thioglycerol or ethanolamine served as the matrix.

ESI-MS analyses were performed at the Mass Spectrometry Facility, State University of New York, Stony Brook, NY. Mass spectra were acquired on a TRIO-2000 mass spectrometer (Fisons Instrument, Danvers, MA) equipped with an electrospray source in the negative-ion mode. Samples were initially dissolved in water and further diluted with a methanol and water mixture (9:1) for analysis. Samples were infused into the source at a flow rate of 8 μL min^{-1} using a syringe pump (Harvard Apparatus Inc., South Natick, MA) connected to a Rheodyne injector with a 10 μL sample loop. A potential of 3−3.5 keV was applied to the source capillary to produce the spray, and the lens potentials were tuned to maximize the ion beam. The instrument was calibrated in the positive-ion mode using poly(ethylene glycol). All spectra were an average of 10−12 scans with background subtraction.

LiP Reactions. LiP was purified from the lignin-degrading cultures of *P. chrysosporium* as described (Wariishi & Gold, 1990). LiP (1.2 μM) was dissolved in 20 mM sodium succinate, pH 4.5 (1 mL), containing a water-soluble sulfonated azo dye (**I, II, III**) (1 μmol), and H_2O_2 (1 μmol) was added in 10 aliquots to this mixture over a 1-h period. LiP reaction with 4-(4′-sulfonamidophenylazo)-2,6-dimethylphenol (**IV**) was performed in a similar fashion except that the reaction contained 1% dioxane to solubilize the dye.

Peroxidase reaction products were analyzed initially by HPLC as described above. These analyses were performed primarily to understand the number of products formed in the peroxidase reaction and also to achieve preliminary characterization of the products by comparison with available standard compounds. To identify the quinone products, the reaction mixture was extracted with ethyl acetate, and the extract was evaporated to dryness under a stream of nitrogen. The residue was dissolved in a minimum amount of water and reduced with sodium dithionite. The reduction products were extracted into ethyl acetate, acetylated with pyridine and acetic anhydride (1:2), and analyzed by GC-MS.

To identify the hydrophilic sulfonated products, the reactions were performed in water, in the absence of any buffer. This modification was necessary for analyzing the products by ESI-MS. Hydrophilic compounds were separated from hydrophobic compounds using a HPLC sorbent for reverse phase chromatography (particle size 40−63 μm). A slurry of the HPLC sorbent (1 g) in methanol (5 mL) was loaded on to a small glass tube, and the methanol was allowed to drain. The sorbent was then equilibrated with water, and the reaction mixture was applied to it. Under these conditions, compounds which eluted early in the HPLC analysis did not bind to the column, whereas hydrophobic degradation products and undegraded azo dyes were bound to the column. The HPLC sorbent-treated reaction mixture was lyophilized, and the residue was analyzed by ESI-MS or FAB-MS techniques.

^{18}O-Incorporation Experiments. The reaction vessel consisted of two compartments. One compartment contained LiP (4.8 μM) and an azo dye (0.5 μmol) in 0.5 mL of water, and the second compartment contained 0.25 μmol of H_2O_2 in 0.25 mL of water. The vessel was evacuated and flushed with scrubbed argon three times and then equilibrated with $^{18}O_2$ (95%, Monsanto Research Corp.). The contents were then mixed and incubated under ambient conditions for 1 h.

Table 1: Mass Spectral Characteristics of Azo Dye Degradation Products

product	technique	mass spectral peaks (rel intensity, %)
V[a]	GC-MS	222 (2), 180 (10), 138 (100)
VI	FAB-MS	189 (30), 173 (18), 157 (12)
	ESI-MS	173 (13), 157 (100)
VII[a]	GC-MS	244 (30), 202 (49), 160 (100), 131 (69), 102 (51)
VIII	FAB-MS	217 (10), 201 (95), 185 (100)
	ESI-MS	217 (14), 201 (93), 185 (100)
IX[a]	GC-MS	280 (2), 238 (6), 196 (23), 154 (100)
XI	GC-MS	157 (43), 141 (30), 93 (47), 77 (100)

[a] Products were reduced with dithionite and then acetylated before mass spectral analyses.

The reaction mixture was passed through HPLC sorbent, and the unbound compounds were lyophilized and analyzed by ESI-MS for ^{18}O incorporation.

RESULTS

HPLC analysis of LiP oxidation products of 4-(4′-sulfophenylazo)-2,6-dimethylphenol (**I**) indicated the presence of two products, 2,6-dimethyl-1,4-benzoquinone (**V**) (t_R = 23.3 min) (Table 1) and another product with a t_R of 3.3 min (Figure 1). This product did not correspond to benzenesulfonic acid (t_R = 8.7 min), 4-hydroxybenzenesulfonic acid (t_R = 4.2 min), 4-aminobenzenesulfonic acid (t_R = 3.6 min), 3,4-dihydroxybenzenesulfonic acid (t_R = 3.7 min), or 1,2-benzoquinone-4-sulfonic acid (t_R = 3.9 min). HPLC sorbent treatment separated it from 2,6-dimethyl-1,4-benzoquinone (**V**) and the unoxidized dye. This product was stable at ambient temperatures (20−22 °C); however, it decomposed at higher temperatures (>60 °C). ESI-MS analysis of this product (t_R = 3.3 min) showed mass spectral peaks at m/z 157 and 173 (Figure 3). FAB-MS analysis in the negative ion electron mode indicated mass spectral peaks at *m/z* 157, 173, and 189 (Table 1). On the basis of these results, the unknown product was identified as 4-sulfophenyl hydroperoxide (SPH) (**VI**) (Figures 2 and 3). The mass peak at *m/z* 189 was due to the molecular ion peak which was presumably unstable under ESI-MS analytical conditions. The mass peak at *m/z* 173 appeared to be due to the loss of an oxygen atom from the molecular ion, whereas *m/z* 157 appeared to be due to the loss of two oxygen atoms from the SPH molecular ion. The product obtained from $^{18}O_2$-labeling experiments showed mass spectral peaks at *m/z* 157, 173, and 175, indicative of a minimum of one ^{18}O incorporation from molecular oxygen. The amount of ^{18}O incorporation was calculated to be approximately 80%. Goszczyński et al. (1994) identified 2,6-dimethyl-1,4-benzoquinone, 4-nitrosobenzenesulfonic acid, 4-aminobenzenesulfonic acid, 4-hydroxybenzenesulfonic acid, and benzenesulfonic acid from the LiP oxidation of **I**. Among these products, only 2,6-dimethyl-1,4-benzoquinone was identified in this study. Our HPLC analyses suggested that the other products either are not formed or are produced only in trace quantities.

HPLC analysis of Orange II (**II**) oxidation products indicated the presence of 1,2-naphthoquinone (t_R = 22.7 min) (**VII**) and SPH (t_R = 3.3 min) (**VI**) (Figure 2). Oxidation of the dimethyl analog of Orange II (**III**) also resulted in the formation of two products, 1,2-naphthoquinone (t_R = 22.7 min) (**VII**) and 2,6-dimethyl-4-sulfophenyl hydroperoxide (DMSPH) (t_R = 5.1 min) (**VIII**) (*m/z*: 185, 201, 217) (Figure 3, Table 1). ESI-MS analysis of DMSPH obtained from

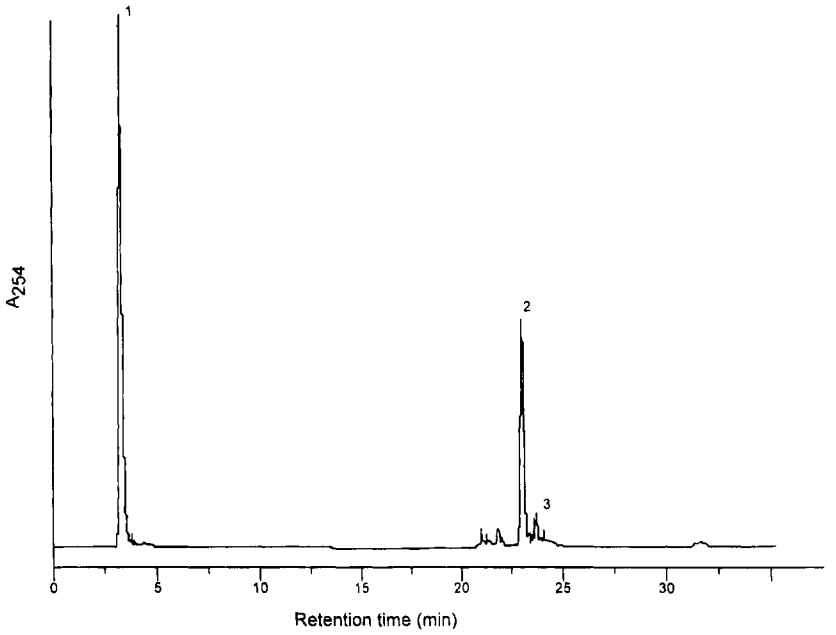

FIGURE 1: HPLC analysis of LiP oxidation products of 4-(4′-sulfophenylazo)-2,6-dimethylphenol (**I**). Peak 1, 4-sulfophenyl hydroperoxide (**VI**) (t_R = 3.3 min); peak 2, 2,6-dimethyl-1,2-benzoquinone (**V**) (t_R = 23.3 min); peak 3, 4-(4′-sulfophenylazo)-2,6-dimethylphenol (**I**) (t_R = 23.6 min).

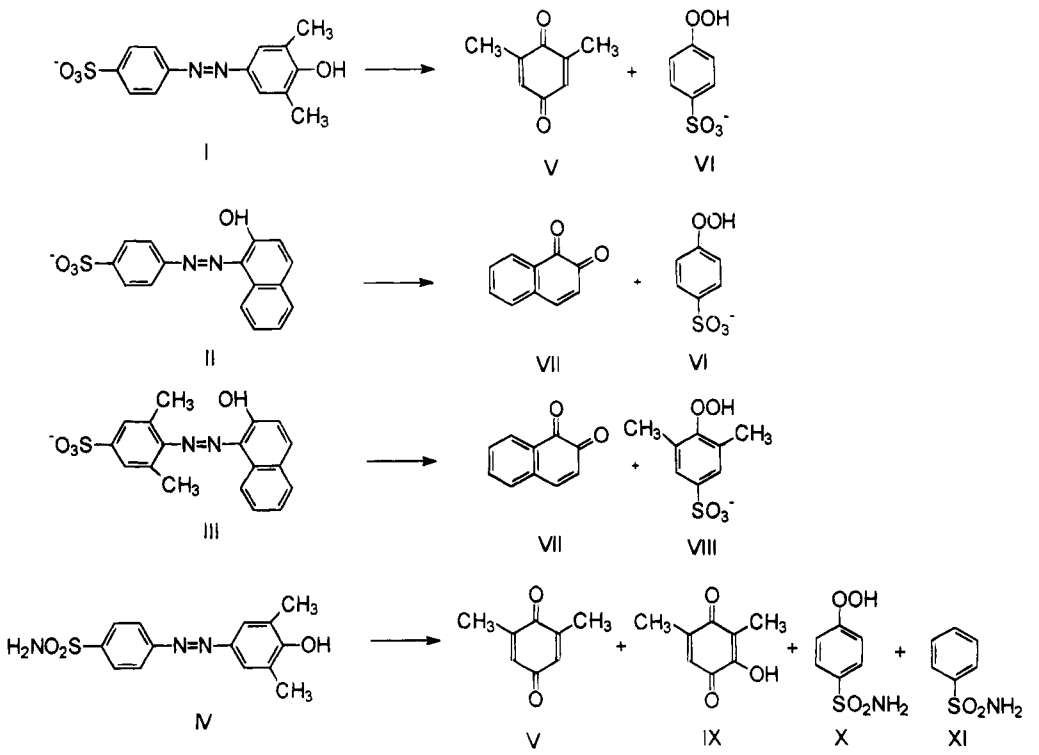

FIGURE 2: Products identified from LiP-catalyzed oxidation of 4-(4′-sulfophenylazo)-2,6-dimethylphenol (**I**), Orange II (**II**), a dimethyl analog of Orange II (**III**), and 4-(4′-sulfonamidophenylazo)-2,6-dimethylphenol (**IV**).

$^{18}O_2$-labeling experiments exhibited mass spectral peaks at *m/z* 185, 201, 203, 219, and 221, indicative of one and two atoms of molecular O_2 incorporation (Figure 3). The ^{18}O-incorporation pattern can be explained as follows (Figure 4). We propose that two ^{18}O oxygens are incorporated initially into DMSPH (*m/z* 221). A portion of DMSPH then exchanges the ^{18}O label in the end oxygen with water, resulting in the loss of one ^{18}O label (*m/z* 219). It appears that, in ESI-MS analysis, DMSPH can either lose the oxygen bonded to the phenyl ring to generate a hydroxybenzenesulfonic acid or eliminate molecular oxygen to produce a benzenesulfonic acid. Thus, when a doubly ^{18}O-labeled DMSPH loses one oxygen atom, one ^{18}O label will remain with the fragment ion (*m/z* 203), whereas the corresponding fragment ion from a DMSPH, which exchanged one of its ^{18}O labels with ^{16}O water, will have no ^{18}O label (*m/z* 201). However, when a molecular oxygen is lost from either singly or doubly ^{18}O-labeled DMSPH, the same fragment ion (*m/z* 185) will be generated (Figure 4).

HPLC analysis of the sulfonamide dye (**IV**) degradation products suggested the formation of two major (t_R = 5.6, 23.3 min) and several minor products (Figure 5). Products

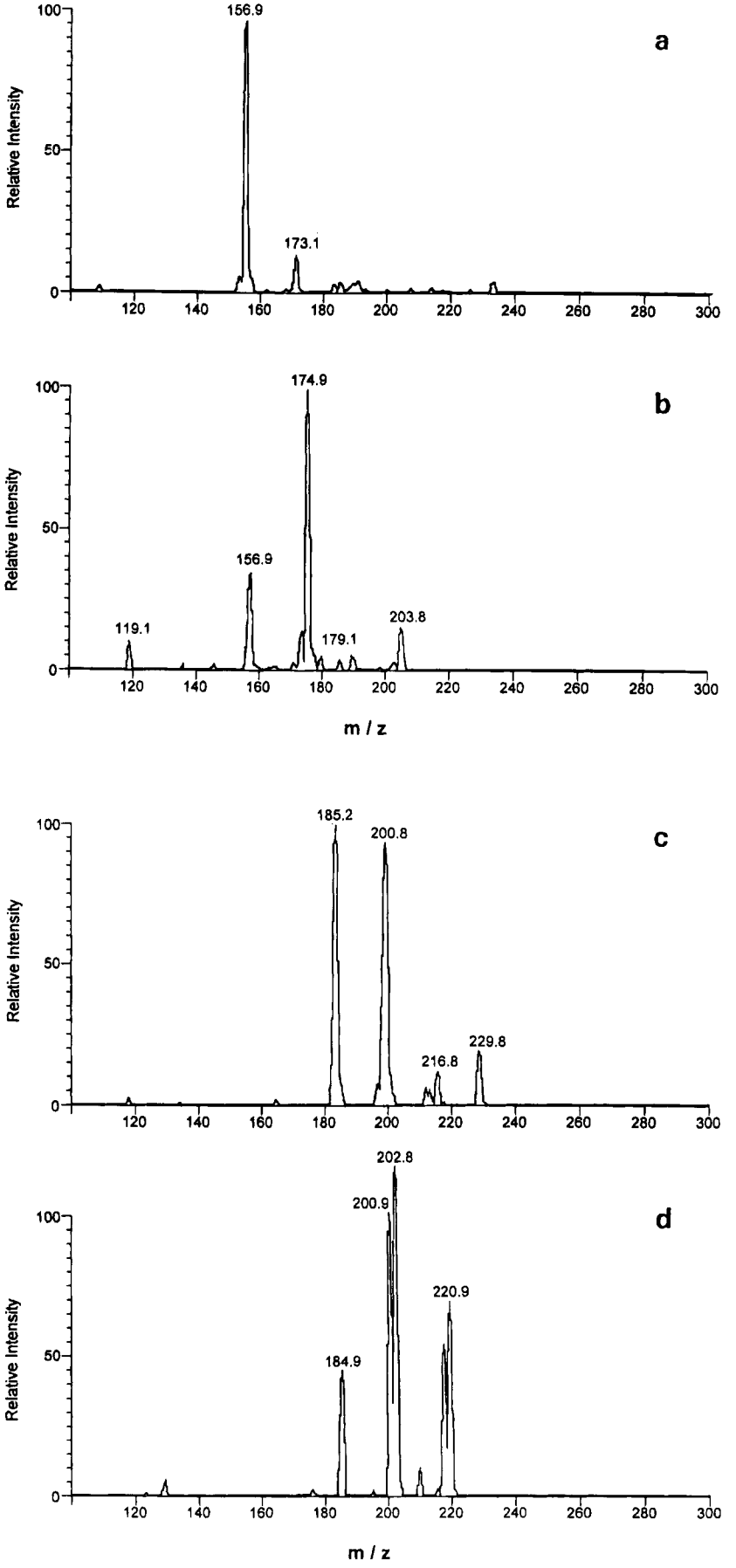

FIGURE 3: Oxygen isotopic shifts obtained by the ESI-MS for the sulfophenyl hydroperoxides. 4-Sulfophenyl hydroperoxide (SPH) (**VI**) obtained from LiP oxidation under $^{16}O_2$ (a) and $^{18}O_2$ (b). 2,6-Dimethyl-4-sulfophenyl hydroperoxide (DMSPH) (**VIII**) obtained from LiP oxidation of **III** in $^{16}O_2$ (c) and $^{18}O_2$ (d).

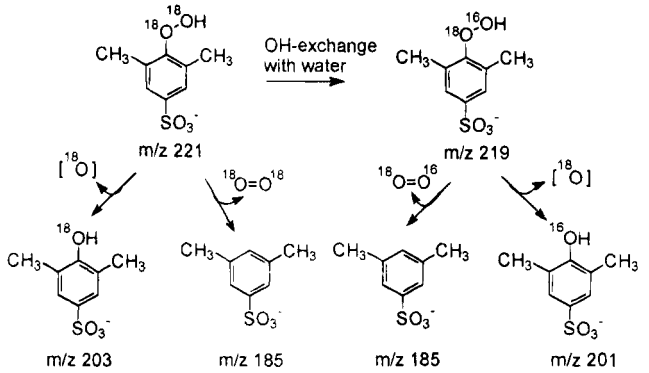

FIGURE 4: Possible mass spectral fragmentation pattern of ^{18}O-labeled DMSPH (**VIII**) under ESI-MS analytical conditions.

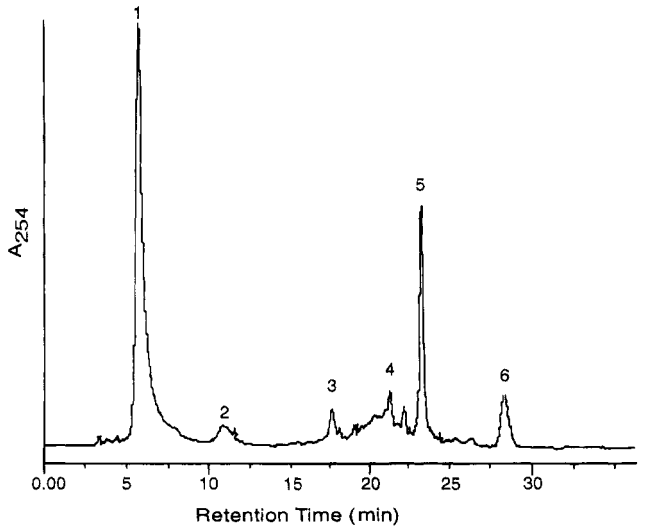

FIGURE 5: HPLC analysis of LiP oxidation products of 4-(4′-sulfonamidophenylazo)-2,6-dimethylphenol (**IV**). Peak 1, 4-sulfonamidophenyl hydroperoxide (**X**) (t_R = 5.6 min); peak 2, unknown (t_R = 10.9 min); peak 3, benzenesulfonamide (**XI**) (t_R = 17.8 min); peak 4, 3-hydroxy-2,6-dimethyl-1,4-benzoquinone (**IX**) (t_R = 21.1 min); peak 5, 2,6-dimethyl-1,4-benzoquinone (**V**) (t_R = 23.1 min); peak 6, undegraded **IV** (t_R = 28.2 min).

were purified using HPLC, and fractions containing products were extracted with ethyl acetate and analyzed by GC-MS. On the basis of this analysis, 2,6-dimethyl-1,4-benzoquinone (t_R = 23.3 min) (**V**), 2,6-dimethyl-3-hydroxy-1,4-benzoquinone (t_R = 21.3 min) (**IX**), and benzenesulfonamide (t_R = 17.6 min) (**XI**) were identified as degradation products of the sulfonamide dye (**IV**) (Table 1). The product with an t_R of 5.6 min was not extractable into ethyl acetate; hence, the HPLC fractions were lyophilized, and the residue was analyzed by GC-MS and FAB-MS techniques. No mass spectrum could be obtained for this compound by these techniques. However, similar to SPH (**VI**), this compound was polar and stable at ambient temperatures but unstable at higher temperatures. We tentatively characterize this product as 4-sulfonamidophenyl hydroperoxide (SAPH) (**X**). Goszczyński et al. (1994) identified 2-methoxybenzoquinone, benzenesulfonamide, 4-aminobenzenesulfonamide, 4-hydroxybenzenesulfonamide, and 2-methoxy-4-aminophenol from the oxidation of 4-(4′-sulfanoamidophenylazo)-2-methoxyphenol. The latter is structurally similar to the sulfonamide dye (**IV**) used in this study; however, we could identify only 2,6-dimethylbenzoquinone (**V**), benzenesulfonamide (**XI**), and SAPH (**X**) from the oxidation of **IV**. HPLC analysis did not suggest the formation of reduction products such as 4-aminobenzenesulfonamide or an aminophenol.

Preliminary studies suggest that the phenyl hydroperoxides are not reduced by LiP; however, in the presence of H_2O_2, LiP appears to degrade them slowly. Also, unlike other hydroperoxides, the sulfophenyl hydroperoxides are stable in the presence of transition metal ions, Fe^{3+}, Fe^{2+}, Mn^{2+}, and Mn^{3+}. Surprisingly, the phenyl hydroperoxides are susceptible to nucleophilic substitution. For example, SPH (t_R = 3.3 min) (**VI**) and SAPH (t_R = 5.6 min) (**X**), upon reaction with sodium azide (1 mM), were totally converted to 4-azidobenzenesulfonic acid (t_R = 18.3 min) and 4-azidobenzenesulfonamide (t_R = 21.7 min), respectively. In this reaction, the hydroperoxide substitution of SPH (**VI**) and SAPH (**X**) was presumably eliminated as H_2O_2. The azido derivatives had the same HPLC retention times as the standard compounds. Further studies on the reactivity of phenyl hydroperoxides are planned.

DISCUSSION

Formation of quinones (**V**, **VII**), 4-SPH (**VI**), DMSPH (**VIII**), and SAPH (**X**) from LiP-catalyzed oxidation of azo dyes **I**–**IV** could be explained by the following mechanism. Oxidation of 4-(4′-sulfophenylazo)-2,6-dimethylphenol (**I**) is shown as an example (Figure 6). Two successive one-electron oxidations of the phenolic ring of **I, II, III,** or **IV** by the H_2O_2-oxidized forms of LiP produces a carbonium ion (Figure 6) (Dunford, 1982; Renganathan & Gold, 1986; Spadaro & Renganathan, 1994). Nucleophilic attack by water on the carbon bearing the azo linkage will lead to the formation of a quinone and the corresponding (4-sulfophenyl)diazene (Spadaro & Renganathan, 1994; Goszczyński et al., 1994). Previous studies suggest that the O_2 and the H_2O_2 forms of peroxidase can oxidize phenyldiazene by one electron to produce the corresponding phenyldiazene radical (Ator & Ortiz de Montellano, 1987; Huang & Kosower, 1968; Kosower et al., 1969). The latter is unstable and readily eliminates the diazo linkage as molecular nitrogen to generate a phenyl radical (Ator & Ortiz de Montellano, 1987; Huang & Kosower, 1968; Kosower et al., 1969). Similar oxidation of sulfo- or sulfonamidophenyldiazene will lead to the formation of a sulfo- or sulfonamidophenyl radical. We propose that these radicals are scavenged by oxygen to yield the corresponding phenyl hydroperoxides (Figure 6). Incorporation of ^{18}O label from oxygen into SPH (**VI**) and DMSPH (**VIII**) supports the proposed mechanism. This observation is novel because this is the first time phenyl radicals have been proposed to react with O_2. Phenyl radicals are known only to abstract a hydrogen radical from its surroundings to yield a stable aromatic compound (Huang & Kosower, 1968; Kosower et al., 1969; Russell & Bridger, 1963). For example, the acetamidophenyl radical formed in the peroxidase-catalyzed degradation of Disperse Yellow 3 was reduced to acetanilide, and no oxygenated product was formed (Spadaro & Renganathan, 1994). The sulfonamidophenyl radical, like the acetamidophenyl radical, appears to be reduced to benzenesulfonamide (Spadaro & Renganathan, 1994); however, low yield of this product from LiP reactions (Figure 5) suggests that reduction is not a preferred pathway for this radical. Benzenesulfonic acid, expected from the reduction of the sulfophenyl radical, is not formed from the oxidation of **I, II,** or **III**, suggesting that this radical prefers to react only with oxygen.

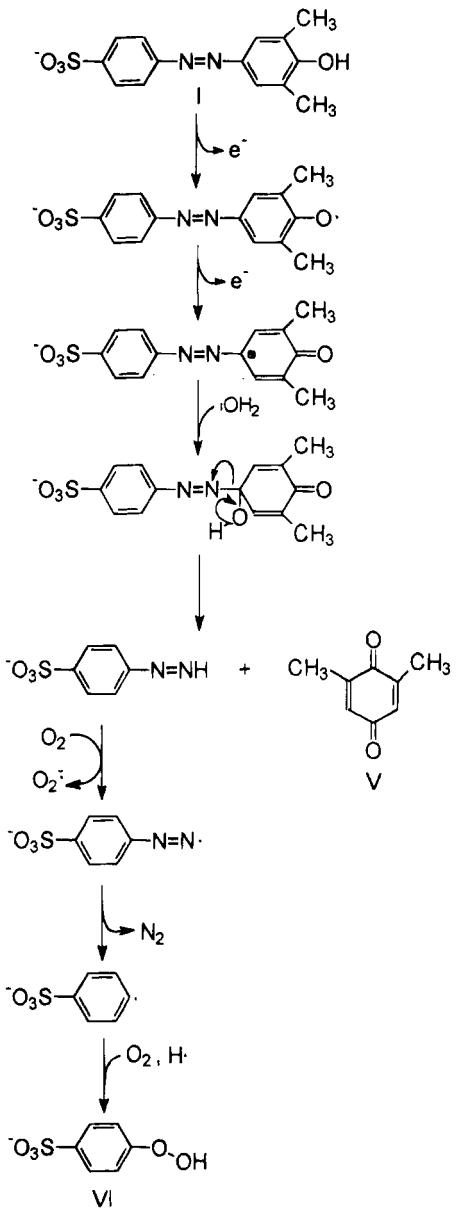

FIGURE 6: Proposed mechanism for the degradation of 4-(4′-sulfophenylazo)-2,6-dimethylphenol (**I**) by LiP.

There is conflict in the findings of this study and that of Goszczyński et al. (1994). This study suggests that only two major products, 2,6-dimethyl-1,4-benzoquinone (**V**) and SPH (**VI**), are formed from LiP oxidation of I. However, the previous report suggests that 2,6-dimethyl-1,4-benzoquinone, 4-nitrosobenzenesulfonic acid, 4-aminobenzenesulfonic acid, 4-hydroxybenzenesulfonic acid, and benzenesulfonic acid are formed from LiP oxidation of **I**. The reason for this discrepancy is in the conditions used for LiP reactions and product analyses. In this study, LiP reactions were performed in 20 mM succinate, pH 4.5 buffer, whereas, in the previous study, LiP reactions were conducted in 50 mM tartrate, pH 2.5. In succinate pH 4.5 buffer reactions, approximately 75% of **I** was oxidized in 1 h, whereas in tartrate pH 2.5 only 15% of **I** was oxidized in 1 h. Decreased oxidation appears to be primarily due to the pH rather than the buffer because in tartrate pH 4.5 buffer 75% of **I** was oxidized by LiP. In the HPLC analysis of tartrate pH 2.5 buffered reactions, the peak corresponding to SPH (**VI**) was insignificant, indicating low yield. In characterizing SPH,

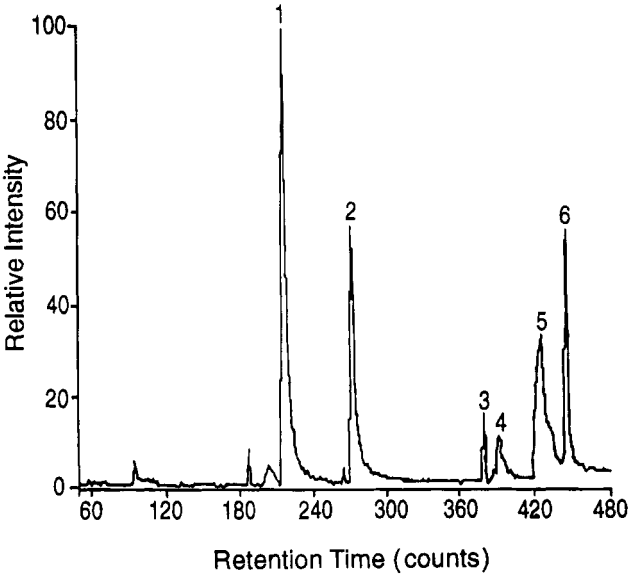

FIGURE 7: A GC profile of the thermal degradation products of 4-(4′-sulfonamidophenylazo)-2,6-dimethylphenol (**IV**). Peak 1, 4-amino-2,6-dimethylphenol; peak 2, benzenesulfonamide; peak 5, 4-aminobenzenesulfonamide; peaks 3, 4, and 6, unknown. **IV** was incubated with LiP (50 μg) in 20 mM succinate, pH 4.5 (1 mL), in the absence of H_2O_2. The incubation mixture was extracted with methylene chloride and analyzed by GC-MS.

we completely avoided any heat treatment during product isolation and subsequent mass spectral analytical procedures. However, in the previous study, products were heat-treated during isolation, derivatization, and mass spectral (GC-MS, HPLC-MS) analyses which could have potentially decomposed SPH (**VI**).

Furthermore, Goszczyński et al. (1994) identified 2-methoxybenzoquinone, benzenesulfonamide, 4-hydroxybenzenesulfonamide, and 2-methoxy-4-aminophenol from the oxidation of 4-(4′-sulfonamidophenylazo)-2-methoxyphenol. The latter is structurally similar to the sulfonamide dye (**IV**) used in this study; however, we could identify only 2,6-dimethyl-1,4-benzoquinone (**V**), benzenesulfonamide (**XI**), and SAPH (**X**) from the oxidation of **IV**. HPLC analysis did not suggest the formation of reduction products such as 4-aminobenzenesulfonamide or an aminophenol. This study revealed that azo dyes are susceptible to thermal degradation during GC analysis, leading to the generation of reduction products such as aminophenol and aminobenzenesulfonamide. In a control experiment, sulfonamide dye (**IV**) was incubated with LiP in 20 mM succinate, pH 4.5, in the absence of H_2O_2. Subsequent HPLC analysis of this control reaction mixture did not indicate any dye degradation; however, GC-MS analysis indicated the presence of 4-aminobenzenesulfonamide, 2,6-dimethyl-4-aminophenol, benzenesulfonamide, and another unidentified compound (Figure 7). A GC-MS peak corresponding to **IV** was not observed. In the previous study, the enzyme reaction mixture was extracted with methylene chloride and analyzed by GC-MS. If the reaction mixture contained undegraded dye, it could have degraded thermally in the GC to generate reduction products.

In summary, a mechanism for sulfonated azo dye degradation by LiP which is consistent with product identification is reported. Salient features of this mechanism are the release of azo linkage as nitrogen and the generation of novel sulfophenyl hydroperoxides (**VI**, **VIII**), which have not been

identified previously in any chemical or biological system. Chemical studies suggest that phenyl radicals only can abstract a hydrogen radical from the medium and that they do not react with oxygen (Russell & Bridger, 1963); however, this study suggests that the introduction of sulfonic acid or a sulfonamide substituent completely changes the reactivity of a phenyl radical toward oxygen. The proposed mechanism for sulfonated azo dye degradation is fully consistent with the mechanism for Disperse Yellow 3 degradation suggested by this laboratory (Spadaro & Renganathan, 1994). In the microbial degradation of aromatic compounds, oxygenation of the aromatic ring prepares the ring for further degradation. Only a few bacteria produce dioxygenases which hydroxylate the sulfonated aromatic ring and, in the process, also eliminate the sulfonic acid substituent (Hansen et al., 1992; Locher et al., 1989a,b). The adventitious incorporation of oxygen into the aromatic ring in peroxidatic degradation of sulfonated azo dyes could help in the subsequent degradation of the aromatic ring. Further studies to understand the fate of sulfophenyl hydroperoxides are in progress.

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