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Aqueous Phase Toxicity Changes Resulting from Horseradish Peroxidase-Mediated Polymerization of Phenols and Hydroxylated Polynuclear Aromatic Contaminants

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Substituted phenols and hydroxylated polynuclear aromatic hydrocarbons (hPAHs) such as 1-naphthol, are widely used and are common pollutants in the effluents of many industries such as coal, petroleum, pharmaceutical, pulp and paper, dyes, and textile (Tatsumi et al, 1996). Most of these chemicals are toxic and considered to be hazardous pollutants (Caza et al., 1999). Various physical and chemical treatment approaches have been used for the removal or precipitation of these compounds from wastewater and the soil environment (Wagner and Nicell, 2001). The enzyme-mediated polymerization process has been adopted as an alternative method for the remediation of contaminated ecosystems (Bollag, 1992). Among the enzymes that have been studied, horseradish peroxidase (HRP) has been found to have several characteristics (broad substrate specificity, and stability over a wide range of pH and temperature) appropriate for application in decontaminating soils, groundwater and industrial effluents (Nicell et al., 1993; Wagner and Nicell, 2002). The HRP-catalyzed polymerization process produces oligomers that can be removed by precipitation, adsorption or filtration.

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The HRP-catalyzed oxidative polymerization process produces various free radicals and polymers according to the following reaction chemistry (Dunford, 1991):

$$E + H_2O_2 \leftrightarrow E\text{-}I + H_2O$$
$$E\text{-}I + AH_2 \leftrightarrow E\text{-}II + AH^*$$
$$E\text{-}II + AH_2 \leftrightarrow E + AH^* + H_2O$$

where E is the native peroxidase, E-I and E-II are active enzyme complexes I and II, respectively, AH_2 is a reducing substrate such as a phenolic compound and AH* is a free radical product. The overall reaction can be described as

$$2AH_2 + H_2O_2 \leftrightarrow 2AH^* + 2H_2O$$

The free radical (AH*) products readily undergo self-coupling with other radicals in solution through nucleophilic addition or radical binding, resulting in the formation of dimers, trimers and larger oligomers (Dunford, 1991). Hypothesized pathways for the formation of polymers during HRP-mediated oxidative polymerization of 1-naphthol have been reported, and suggest that naphthol polymerization occurs preferentially through C–C bonding, resulting in the production of oligomers with intact OH groups. (Xu et al, 2005). Although various characteristics of phenolic oligomers generated by polymerization are well documented, the impact of oligomerization and subsequent precipitation of the polymerized products on the residual aqueous toxicity remains unclear. Polymeric products have been reported as being more or less toxic than the parent compounds (Wagner and Nicell, 2002; Ghioureliotis and Nicell, 2000). Therefore, an assessment of the residual aqueous phase toxicity after phenol/hPAH polymerization and subsequent precipitation of the oligo-

meric products has the potential to provide valuable information relevant to the environmental applicability of HRP-mediated processes.

Various microorganisms have been utilized for environmental toxicity monitoring (Bitton, 1999; Kim and Gu, 2005). Bioluminescence-based toxicity assays appear to have several advantages over other techniques, including short exposure times, convenient signal measurements, and cost efficiency (Ren et al., 2003). In this investigation, toxicity changes were observed based on bioluminescence inhibition. One of the most thoroughly studied and used bioluminescent bacteria-based bioassay is the Microtox assay marketed by Azur Environmental (Carlsbad, CA). A freeze-dried marine bacterium *Vibrio fischeri* used in this assay is generally exposed for 5–15 minutes to aqueous samples at 15°C.

In this investigation, solutions containing a solute selected from 10 phenols and a hPAH were subjected to HRP-mediated oxidative polymerization. The effect of this process on the residual aqueous phase toxicity was examined using a bacterial bioluminescence assay. The bioluminescence bacterium used was *Escherichia coli* DH5 RB1436, which contained a spontaneously deleted pUCD 615 plasmid, resulting in the translocation of a constitutive promoter of the plasmid to the proximity of the *lux* genes and produced relevant bioluminescence for toxicity during growth period.

Materials and Methods

The 10 phenols and one hPAH used as target chemicals in this study were: phenol, 2-chlorophenol (CP), 3-CP, 4-CP, 2,3-dichlorophenol (diCP), 2,4-diCP, 3,4-diCP, 3,5-diCP, 2,3,5-trichlorophenol (triCP), pentachlorophenol (PCP) and 1-naphthol. All stock solutions were prepared in pH 7.2 phosphate buffer solution. Fresh stock solutions of HRP were prepared at approximately 222 AU/mL (10mg/10mL) before polymerization. Working solutions of H_2O_2 were prepared by diluting 30% w/w (8.82 M) with deionized water. Catalase was used to terminate the polymerization. All chemicals were obtained from Sigma Chemical (St. Louis, MO) or Aldrich Chemicals (Milwaukee, MI).

Polymerization experiments were conducted at two aqueous solute concentrations, high and low, determined from the EC_{50} values of each chemical (where EC_{50} is the chemical concentration inhibiting 50% of bioluminescence activity). Thirteen milliliters of the aqueous solution were transferred into triplicate glass tubes and 130 μ*l* of HRP (2 AU/mL) and appropriate amount of H_2O_2 (1.2 times the chemical concentration) were added (AU = enzyme activity units. 1 AU of HRP was defined by Sigma-Aldrich as the amount of HRP that formed 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 at 20°C). This ratio was selected based on optimum polymerization of solutes at H_2O_2:solute ratios >1.2 (Palomo and Bhandari, 2005). Tubes were immediately capped and mixed completely on a vortex mixer, and incubated at 25°C by placing on a shaker (150 rpm) for 3 h. Following this 3 hr reaction, sufficient catalase (0.5 AU/mL) was added and incubated for 30 min to decompose all the residual H_2O_2. Tubes were centrifuged at 2,200 rpm for 45 min to separate the soluble and insoluble polymerized products, and the supernatant was evaluated for toxicity.

Acute toxicities of polymerized and unpolymerized samples were quanitified using a bioluminescent mutant strain RB1436, obtained from Dr. R.S. Burlage of the University of Milwaukee, USA. The light output of this strain is reduced (inhibited) in proportion to the degree of toxicity in solution. Strains were maintained and stored according to standard methods specified by Sambrook et al. (1989). Strains were stored at –70°C until needed, at which time they were grown overnight in LB medium at 27°C with shaking (130 rpm). A 1:30 dilution was made into the LB medium and allowed to grow until the optical density (OD_{600}) was approximately 0.6. This culture was equally diluted with minimal salt medium (MSM); the final density for the luminescence test was OD_{600} = 0.2. The composition of the medium (per liter basis) was: LB-Ka broth medium (tryptone 10 g, yeast extract 5 g, NaCl 5 g, 2N NaCl 0.5 ml, kanamycin 50 mg) and MSM medium ($MgSO_4 \cdot 7H_2O$ 0.2 g, $CaCl_2$ 0.1 g, $FeSO_4 \cdot 7H_2O$ 0.05 mg, $NaMoO_4 \cdot 2H_2O$ 0.05 mg, K_2HPO_4 0.43 g, KH_2PO_4 0.23 g). For the bioassay, 1 ml of the bacterial strain was mixed with a 9 ml sample and incubated. The average of three measurements (0.5, 1.0 and 2.0 hr) was used for the toxicity result. Bioluminescence was measured with a TD 20/20 Luminometer (Turner Design Inc., CA), where the maximum detection limit was 9,999 relative light units (RLU).

Results and Discussion

The maximum (>9,999 RLU) bioluminescence activity of RB1436 was observed for approximately 12 hrs following 3 hrs of incubation. Polymerization test was performed based on the EC_{50} value of all the tested compounds. For example, cells were exposed to various concentration of 4-CP, ranging from 0 to 10 mM, and were assayed for bioluminescence activity over time (0.5, 1.0 and 2.0 hr) (Fig. 1). No significant effects on bioluminescence activity were observed in the range of 0.001 to 0.05 mM compared to controls. Approximately 27% inhibition was observed at 0.1 mM and bioluminescence activity was completely

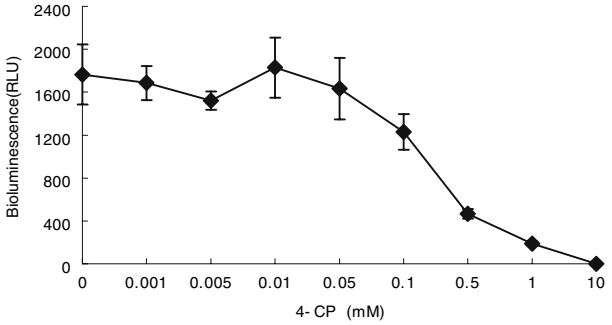

Fig. 1 The effects of 4-CP concentration on the bioluminescence activity of strain RB1436

Table 1 EC_{50} values for phenolic parent compounds

Compound	Mol. Wt. (g/mol)	EC_{50}(mM)			
		This study	Shk1[1]	Microtox[2,3,4]	
1-naphthol	144.17	0.36 ± 0.09	0.37	–	0.016
phenol	94.11	2.15 ± 0.07	5.12	0.202	0.19
2-CP	128.56	0.42 ± 0.02	1.17	0.081	0.14
3-CP	128.56	0.17 ± 0.03	–	0.021	–
4-CP	128.56	0.28 ± 0.09	0.16	0.004	0.0074
2,3-diCP	163.00	0.38 ± 0.03	–	–	–
2,4-diCP	163.00	0.27 ± 0.03	0.18	0.006	0.012
3,4-diCP	163.00	0.17 ± 0.02	–	–	–
3,5-diCP	163.00	0.057 ± 0.0001	0.25	–	0.045
2,3,5-triCP	197.45	0.04 ± 0.001	–	–	–
PCP	266.35	0.31 ± 0.04	–	–	–

[1] Ren and Frymier (2003); [2] Blum and Speece (1991), [3] Walker (1989), [4] Ghioureliotis and Nicell (2000)

inhibited at 10 mM. All the observed EC_{50} values are summarized in Table 1. The results illustrate that the EC_{50} of phenolic compounds was in the range of 0.04–0.55 mM, except for the least toxic phenol (2.15 mM) and the most toxic 2,3,5-triCP (0.04 mM). Generally, the EC_{50} values for the tested chemicals appeared to follow the order: phenol > 2-CP > 2,3-diCP, 1-naphthol > PCP > 2,4-diCP, 4-CP > 3,4-diCP, 3-CP > 3,5-diCP > 2,3,5-triCP (Table 1). Our data were comparable to the results of toxicity assays conducted by others using different bioluminescent bacteria, such as another recombinant *E. coli* and marine bacteria *V. fischeri* (Ren and Frymier, 2003; Ghioureliotis and Nicell 2000) (Table 1).

Differences in sensitivity were observed for the different bioluminescence assays, with the Microtox method being more sensitive than others. In all the methods, phenol was the least toxic, and the toxicity of 2-CP (*ortho* mono-substituted CP) was lower than other mono chlorophenols (*para* and *meta*) (Ghioureliotis and Nicell, 2000).

Table 2 Comparison of the bioluminescence activities between polymerized and unpolymerized chemicals in solution

Compound		Bioluminescence activity (RLU)	
		Unpolymerized	Polymerized
1-naphthol	0.5 mM	[a]254 ± 21.1	771 ± 75.7
	1.0 mM	1.7 ± 0.5	53 ± 24
Phenol	0.5 mM	372 ± 83.3	486 ± 267.9
	1.0 mM	189 ± 8	1003 ± 436
2-CP	0.5 mM	905 ± 110.4	1048 ± 112.8
	1.0 mM	348 ± 9.9	319 ± 43.8
3-CP	0.5 mM	748 ± 173	1332 ± 185.1
	1.0 mM	393 ± 11.5	328 ± 24.3
4-CP	0.5 mM	1040 ± 68.2	775 ± 509.1
	1.0 mM	357 ± 30.9	0.1 ± 0.05
2,3-diCP	0.5 mM	9.4 ± 1.32	428 ± 117.8
	1.0 mM	1.0 ± 0.12	5.6 ± 1.0
2,4-diCP	0.2 mM	2426 ± 107.4	326 ± 56.5
	0.4 mM	1524 ± 46.7	7.6 ± 4.95
3,4-diCP	0.1 mM	791 ± 12.2	875 ± 54.8
	0.3 mM	65 ± 7.8	38 ± 37.8
3,5-diCP	0.01 mM	82 ± 12.8	44 ± 1.5
	0.1 mM	55 ± 9.5	3.6 ± 3.56
2,3,5-triCP	0.01 mM	1185 ± 64.7	1576 ± 440.2
	0.1 mM	71 ± 4.3	943 ± 205.5
PCP	0.2 mM	351 ± 35.6	524 ± 82.5
	0.4 mM	76 ± 10	512 ± 274.2

HRP-mediated polymerization of target chemicals was evaluated at two concentrations (high and low), selected based on the EC_{50} values of the chemical. The pre-polymerization concentrations of the target chemicals were: 0.5 and 1.0 mM for phenol, 2-CP, 3-CP, 4-CP, 2,3-diCP and 1-naphthol; 0.2 and 0.4 mM for 2,4-diCP and PCP; 0.01 and 0.1 mM for 3,5-diCP and 2,3,5-triCP; and 0.1 and 0.3 mM for 3,4-diCP.

Parent chemicals were classified into three groups according to the residual toxicity in solution after polymerization and precipitate removal: group A consisted of the parent chemicals 1-naphthol, phenol, 3-CP, 2,3-diCP, 2,3,5-triCP, and PCP, whose enzymatic polymerization and precipitate removal produced a solution with reduced toxicity; group B consisted of 4-CP, 2,4-diCP, 3,5-diCP, solutes whose polymerization resulted in increased toxicity in the supernatant; and group C consisting of 2-CP and 3,4-diCP, whose residual aqueous phase toxicity was not significantly different from the solutions containing the parent solutes.

The changes in bioluminescence observed during HRP-mediated oxidative polymerization of the target solutes are summarized in Table 2. Note that higher values of

bioluminescence are indicative of lower toxicity. No significant change in residual aqueous phase toxicities was observed for 2-CP and 3,4-diCP despite the removal of polymerized products in the precipitate. The soluble oligomers produced from these chemicals are likely to have been more toxic on a mass basis than the parent solutes. In an earlier study, Xu et al. (2005) noted that products of 1-napthol polymerization mediated by HRP included oligomers that were more polar than the parent naphthol. This was highlighted as a cause for concern because polymerization products with a higher polarity could be transported longer distances in soil and groundwater systems. However, in the case of 1-naphthol, the authors argued that enhanced transport was not an environmental concern since the soluble polymerization products had significantly lower toxicity than the parent solute.

The most significant toxicity reductions occurred for 1-naphthol and 2,3-diCP. For example, the bioluminescence intensity increased from 254 and 1.7 RLU to 771 and 53 RLU, at initial 1-naphthol concentrations of 0.5 and 1.0 mM, respectively.

The toxicity reductions observed in polymerized 1-naphthol solutions are likely to have resulted from precipitation of the oligomers produced during enzymatic oxidative coupling reactions. In contrast, the most significant toxicity increases occurred for 4-CP and 2,4-diCP. For example, the toxicity increased in supernatants containing 2,4-diCP polymerization products, represented by the decrease in bioluminescence from 2426 and 1524 RLU to 326 and 7.6 RLU for solutions having initial 2,4-diCP concentrations of 0.2 and 0.4 mM, respectively.

The reasons for these observations include the possibilities that the soluble 2,4-diCP polymerization products were: (i) more toxic than the parent solute, although equally effectively removed by centrifugation, or (ii) equally or less toxic than the parent chemicals but less effectively removed by centrifugation. Nevertheless, in the case of 2,4-diCP, the gross aqueous-phase toxicity increased as a result of HRP-mediated oxidative polymerization. Polymerization of 2,4-diCP by HRP has been shown to result in the formation of trace quantities of polychlorinated dibenzo-*p*-dioxins and furans (PCDD/F), including the tetrasubstituted congeners, which have been reported to be the most toxic (Ghioureliotis and Nicell, 2000). Increase of aqueous phase toxicity after 4-CP polymerization can be explained by *ortho* coupling mechanisms (''the *ortho* effect''). This compound has two substituted *ortho* carbons, which could lead to a greater probability of dioxin production compared to 2-CP (Ghioureliotis and Nicell, 2000). However, previous investigators have not reported consistent results. For example, in the case of 4-CP, a toxicity increase was reported by Aiken et al. (1994), but a decrease was found be Ghioureliotis and Nicell (2000) and Maloney et al. (1986) after polymerization. Wagner and Nicell (2002) also reported that the use of chitosan as an additive reduced toxicity over time.

This study shows that the residual aqueous-phase toxicities after HRP-mediated oxidative polymerization of phenols and hPAHs can vary widely depending on the type of parent compound. Although HRP-mediated polymerization appears to be a promising technology to treat water containing certain types of phenols, generalizations about the effectiveness of this approach to all types of phenolic contaminants should be made with caution.

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