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Transformation and Removal of Bisphenol A from Aqueous Phase via Peroxidase-Mediated Oxidative Coupling Reactions: Efficacy, Products, and Pathways

QINGGUO HUANG AND WALTER J. WEBER, JR*

Department of Chemical Engineering, Energy and Environment Program, 4103 Engineering Research Building, The University of Michigan, Ann Arbor, Michigan 48109-2099

A systematic investigation of the feasibility of and mechanisms for transformation and removal of bisphenol A (BPA) from aqueous phase via oxidative coupling mediated by horseradish peroxidase is described. It is demonstrated that BPA can be effectively transformed into precipitable solid products in HRP-mediated oxidative coupling reactions. A total of 13 reaction intermediates and products are identified using LC/MS and GC/MS techniques, and with the help of *ab initio* molecular modeling, detailed reaction pathways are proposed. It is postulated that two BPA radicals are coupled primarily by the interaction of an oxygen atom on one radical and propyl-substituted aromatic carbon atom on another, followed by elimination of an isopropylphenol carboncation. All intermediates or products detected can be interpreted as resulting from either coupling or substitution reactions between BPA and other intermediates or products. The efficacy of the reaction at low substrate concentrations is demonstrated using a sensitive analytical procedure involving solid-phase extractions. The results suggest that catalyzed oxidative coupling reactions may be important natural transformation pathways for estrogenic phenolic compounds and indicate their potential use as an efficient means for removal of estrogenicity from waters and wastewaters.

Introduction

Endocrine disrupting compounds are attracting increased scientific and public attention (*1*−*4*). Such compounds mimic and interfere with hormonal activities and thus adversely impact ecosystems and human health by disrupting growth, development, and reproduction (*1*, *5*, *6*). Some have also been demonstrated to have carcinogenic and mutagenic effects (*7*). Phenolic compounds comprise an important class of endocrine disruptors (*8*−*10*), many resembling natural estrogens in structural features and thus being able to bind specifically to estrogen receptors and trigger estrogenic toxicity (*11*, *12*).

Bisphenol A (2,2-bis(4-hydroxyphenyl)propane, BPA) is a compound of particular concern (*13*, *14*). It is mass-produced as a monomer of polycarbonate and epoxy plastic materials used widely in food and beverage containers, dental materials, and lacquers. Polymers made from BPA are commonly used for coating concrete and steel tanks and pipes used in water supply systems, leading to BPA releases to drinking water (*9*, *14*). Widespread occurrences of BPA in water sources and wastewaters have been identified worldwide (*8*, *9*, *15*−*19*). BPA is traditionally thought to be mildly estrogenic, exhibiting a dose-dependent response between 1 and 100 mg/L in a yeast two-hybrid estrogenicity assay (*20*). One recent study has demonstrated that laboratory mice exposed to about 20 ppb or so of BPA have developed chromosomal abnormalities in eggs (*21*), raising earlier concerns about safe levels of BPA exposure (*13*, *22*).

The removal of estrogenic phenolic compounds challenges traditional water and wastewater treatment technologies. Because phenols are relatively hydrophilic, they can easily escape physical/chemical treatment processes designed to remove hydrophobic contaminants (*4*, *23*). Many phenolic contaminants are resistant or toxic to microbes and thus cannot be readily treated by biological processes and often cause biological process malfunctions (*24*). Many estrogenic phenolic chemicals are in fact formed as incomplete degradation products in wastewater treatment plants (*2*). The greatest challenge for removal of endocrine disruptors arises from the fact that they remain biologically effective at very low concentrations (*4*), and it thus becomes economically prohibitive to remove them to levels below their respective safety levels via conventional physicochemical and biological treatment processes. More specific and efficient means for their removal must therefore be developed to address the problems associated with materials such as BPA.

It is known that most phenolic compounds are subject to oxidative coupling, a reaction that can be catalyzed by certain naturally occurring extracellular enzymes (*25*−*27*). These enzymatic reactions have been examined as potential means for removal of phenolic contaminants in wastewater treatment and soil remediation (*28*−*35*). Enzymatic reactions are generally advantaged with respect to reaction specificity and efficiency because they involve specific substrate−enzyme binding processes. These advantages make enzyme-mediated phenol coupling a potentially promising technology for dealing with removal of estrogenic phenolic chemicals. Peroxidases and laccases comprise two important classes of enzymes able to catalyze the oxidative coupling reactions of phenolic substrates (*26*, *36*−*38*). Peroxidases mediate single-electron oxidation of phenols to yield phenoxy radicals in the presence of hydrogen peroxide (*39*, *40*), and laccases do so in the presence of oxygen (*26*). Phenoxy radicals generated in these enzymatic processes can then couple with each other to form polymeric precipitates that can be readily removed from water (*28*, *41*).

The enzymatic coupling of BPA has been investigated in several earlier studies. BPA transformation has been demonstrated in reaction systems mediated by laccases (*42*−*46*), manganese peroxidase (*47*, *48*), horseradish peroxidase (*49*), and soybean peroxidase (*41*). Several reaction products have been identified in these studies (*42*, *44*, *48*, *49*) but not enough to support a detailed map of reaction pathways. Most prior research on BPA has been conducted at millimolar concentration levels, with no efforts having been made to evaluate reaction effectiveness at the much lower environmentally relevant levels for such chemicals. More comprehensive understandings of reaction pathways and assessments of reaction efficacy at low substrate concentration levels are therefore desirable.

The study described here focuses on a systematic investigation of transformation behaviors and mechanisms of

* Corresponding author phone: (734)763-2274; fax: (734)936-4391; e-mail: wjwjr@engin.umich.edu.

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bisphenol A conversions in oxidative coupling reaction systems mediated by horseradish peroxidase (HRP). A total of 13 reaction intermediates and products are identified using LC/MS and GC/MS techniques, and with the help of *ab initio* molecular modeling, detailed reaction pathways are proposed. The efficacy of the reaction at low concentration levels is demonstrated using a sensitive analytical procedure involving solid-phase extraction and having a detection limit of 0.4 nM. The results suggest that catalyzed oxidative coupling reactions constitute an important natural transformation pathway for estrogenic phenolic compounds, one that has potential utility as a treatment technique for their removal.

Experimental Section

Materials. Extracellular horseradish peroxidase (type-I, RZ = 1.3), hydrogen peroxide (29.9%, ACS reagent), 2,2′-azinobis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) (98%, in diammonium salt form), and BPA (99+%) were obtained from Sigma-Aldrich Chemical (St. Louis, MO). Propyl-2-^{14}C-labeled BPA (200 mCi/mmol) was from Moravek Biochemicals (Brea, CA), and carbon-14 cocktail for Harvey Biological Oxidizer was from R. J. Harvey Instrument Co. (Hillsdale, NJ). ScintiSafe Plus 50% liquid scintillation cocktail and all other chemicals were obtained in the highest quality available from Fisher Scientific (Fairlawn, NJ).

BPA Removal under Different Reaction Conditions. A series of experiments were performed to evaluate the efficiency of BPA removal under varying reaction conditions. The reactions were conducted at room temperature in 13 × 100-mm glass test tubes. Each reactor contained 6 mL of 10 mM phosphate buffer (pH = 7.0) comprising predetermined amounts of BPA, H_2O_2, and HRP. H_2O_2 was added to the reactor as the final reagent to initiate the reaction, and the reactor was incubated on a rotary shaker table operated at 200 rpm. Triplicate experiments were conducted for each reaction condition. After 40 min of reaction, each reactor was centrifuged at 1300*g* for 35 min to separate the solid and liquid phases, and a 1-mL sample was then taken from the liquid phase for analysis of residual BPA concentration. BPA concentration was measured using an Agilent 1100 Series HPLC system equipped with a Phenomenex C18 column (250 × 2.0 mm, 5-μm particle size). The mobile phase, operated at 0.40 mL/min, comprised an acetonitrile component and an aqueous component. The acetonitrile component was initially 20%, increased to 45% at 10 min and was maintained for 5 min, and then increased to 80% at 20 min and was maintained for another 5 min. BPA concentration was determined by a fluorescence detector with excitation and emission wavelengths at 230 and 315 nm, respectively.

Time Courses of BPA Reactions. Time courses of BPA reactions were evaluated in 250-mL completely mixed batch reactors (CMBRs), each initially (before sampling) containing 100 mL of reaction solution in phosphate buffer comprised by 150 μM BPA, 150 μM H_2O_2, and a predetermined dosage of HRP, and were continuously mixed using a glass-sealed magnetic stir bar. Three-milliliter samples were taken at preselected times, and 100 μL of 0.3 M HCl was added to each sample to stop the reaction. The sample was centrifuged at 1300*g* for 35 min to separate phases, and a 1-mL aliquot was taken from the supernatant to analyze the residual BPA concentration using the HPLC method described above.

Product Phase Distributions. Radiolabeling measurements were employed to evaluate the phase distribution of the BPA reaction products. The reaction was conducted in 250-mL CMBRs mounted with a center-well hydroxide CO_2 trap. Each reactor contained 50-mL phosphate buffer (10 mM, pH = 7.0) consisting of a 150 μM mix of radiolabeled and unlabeled BPA, 150 μM H_2O_2, and 2.5 unit/mL HRP, and the solution was continuously mixed by a glass-sealed magnetic stir bar. Two milliliters of 2 M hydroxide was used as CO_2 trap solution. The reaction was stopped after a predetermined time by injecting 0.5 mL of 1 M HCl into the reactor through a side sampling port covered with a rubber cap. This will lower the pH of the system to around 2, and thus the product distribution measured in this specific experiment reflects a situation at pH 2. A 0.5-mL aliquot of the CO_2 trap solution was sampled and mixed with 3 mL of the ScintiSafe Plus 50% liquid scintillation cocktail, and the ^{14}C radioactivity was then measured using a Beckman LS6500 liquid scintillation counter. After the trap solution was sampled, the trap well was dissembled from the reactor, and the product mixture was filtered using a glass fiber filter to separate solid/liquid phases. A 0.5-mL sample was taken from the filtrate and mixed with 3 mL of the ScintiSafe Plus 50% liquid scintillation cocktail to measure radioactivity remaining in the aqueous phase. The solid residue retained by the filter, after being air-dried, was combusted using a Harvey Biological Material Oxidizer (R. J. Harvey Instrument Co., Hillsdale, NJ). The resulting $^{14}CO_2$ was absorbed into a 15-mL volume of carbon-14 cocktail, and the radioactivity was then measured.

BPA Removal under Sequenced Reaction Conditions. In this experiment, radiolabeled BPA solutions were periodically resupplied with HRP and H_2O_2 to test the effectiveness of BPA removal under sequenced reagent feed reaction conditions. Because BPA concentrations were reduced significantly along each step of such sequenced treatment, this type of experiment also served to test reaction efficiencies at different BPA levels. To determine the residual BPA concentration remaining after each such sequenced treatment, solid-phase extraction (SPE) procedures were performed prior to chemical analysis, allowing BPA to be detected at the nanomolar level. The formation of dissolved products was also evaluated through radioactivity analysis.

The sequenced-feed reactions were performed in 250-mL reactors, with solutions continuously mixed by glass-sealed magnetic stir bars. Each reactor initially contained 100-mL phosphate buffer consisting of a 150 μM mix of radiolabeled and unlabeled BPA. Concentrated stock solutions of HRP and hydrogen peroxide were sequentially injected into the reactor in volumes of 0.24 and 1.5 mL, respectively, to obtain 1 unit/mL of enzyme activity and 150 μM H_2O_2 at the outset of the experiment. After the reaction had proceeded for 40 min, HRP and H_2O_2 were again added to the reactor, this time in volumes of 0.24 and 0.15 mL, respectively, to yield 1 unit/mL of enzyme activity and 15 μM H_2O_2. These reagent additions were then made after each subsequent 40-min period until completion of the experiment, after which the final product mixture was filtered using a glass fiber filter to separate solid/liquid phases. Phenanthrene was added to the filtrate at a level of 0.05 μM as an internal standard, and the solution was then subjected to solid-phase extraction (SPE) performed using 8-mL high capacity C18 columns from Alltech Chemicals (Deerfield, IL). Prior to extraction, the column was conditioned with 5-mL methanol followed by a rinse of the same amount of Milli-Q water. The solution was passed through the column at a flow rate of approximately 5 mL/min followed by an air stream for 10 min to dry the column, after which the column was eluted with 5 mL of methanol. The effluent was blown nearly dry under a gentle nitrogen gas flow and was reconstituted in a 1-mL mixture of dichloromethane and methanol (1:1, v/v). The reconstituted solution was sampled for HPLC analysis to determine BPA concentration. The method detection limit (MDL) of this SPE-assisted HPLC analysis was 0.4 nM, estimated as 3 times the standard deviation of the background noise signals. The reconstituted solution was also sampled to measure remaining radioactivity, and the total amount of dissolved products are calculated and expressed as BPA equivalent concentration.

Product Characterizations. Samples used for product characterization were prepared by a reaction performed in a 250-mL flask reactor containing 100 mL of reaction solution mixed continuously by a magnetic stir bar. The reaction, initiated with 150 μM BPA, 150 μM H_2O_2, and 1 unit/mL of HRP, was quenched after 1 min by addition of 1.6 mL of 1 M HCl, and the product mixture was then filtered using a glass fiber filter to separate the solid and liquid phases. SPE was used to concentrate the filtrate, as described above, and the concentrated sample was then subjected to GC/MS and LC/MS characterization. The solid residue retained on the membrane after filtration was rinsed with Milli-Q water several times and was then blown dry under a gentle air stream. The residue was scraped into a glass test tube and mixed with a 1-mL mixture of dichloromethane and methanol (1:1, v/v) for 30 min, leading to the dissolution of about one-third of the precipitate products. This was followed by centrifugation at 1300*g* for 35 min and by LC/MS characterization of the resulting supernatant.

GC/MS characterization was performed on a Hewlett-Packard 5890 Series II gas chromatograph equipped with an HP 5972A mass selective detector. A DB-5 column was used for GC separation, and helium was used as the carrier gas at 1 mL/min. The oven temperature was initially 50 °C for 2 min, increased to 130 °C at 20 °C/min, then increased to 300 °C at 10 °C/min, and finally was kept isothermal for 30 min. The detector temperature was 320 °C, and the electron impact energy was 70 eV. The mass spectra of the target solutes were compared with those from NIST standard library to assign structures.

LC/MS analysis was carried out on an HP 1090 liquid chromatograph connected to an HP5989B mass spectrometer through an HP59987A electrospray interface. The HPLC separation was the same as described above except that the mobile phase program was modified as below: the acetonitrile component was initially at 20%, increased to 45% at 10 min and maintained for 5 min, and then increased to 100% at 125 min. The mass spectrometer was operated in the negative ion mode in the m/z 90−1000 range. The electrospray unit was operated with source temperature at 150 °C and the voltages for capillary entrance and exit were at 3500 and −80 V, respectively.

HRP Activity Assays. HRP stock solutions were prepared freshly on the day of each experiment, and enzyme activity was assayed by ABTS method as described earlier (*50*, *51*). Briefly, a 0.05-mL sample was added to a cuvette containing a 3-mL volume of phosphate buffer solution (pH = 6.0), followed by addition of 0.3 mL of 20 mM ABTS and 0.3 mL of 10 mM hydrogen peroxide to start the assay. The absorbance change at 405 nm was monitored by a 6405 UV/vis spectrophotomer (Jenway Inc., Princeton NJ). One unit of peroxidase activity is defined as that amount catalyzing the oxidation of 1 μmol of ABTS per minute.

Molecular Modeling. Molecular modeling was performed using a HyperChem 7.0 program. The molecular structure of a BPA free radical was first optimized by *ab initio* computation with the 3-21 G basis set, and then the 6311G** basis set was used to calculate the charge and spin densities.

Results and Discussion

BPA Removal. Data for BPA removal in HRP-mediated reaction systems with varying enzyme dosages and constant initial BPA and H_2O_2 concentrations (both at 150 μM) are presented in Figure 1, and those for varying H_2O_2 concentrations at a constant HRP dosage of 2.5 unit/mL and a constant initial BPA concentration of 150 μM are presented in Figure 2. It is evident from the data presented in Figures 1 and 2 that the residual BPA decreases with increasing HRP dosages and H_2O_2 concentrations and that nearly complete removal can be reached at the condition of 2.5 unit/mL HRP and 150 μM H_2O_2. The time courses of BPA removal at four different levels of enzyme dosages with initial BPA and H_2O_2 concentrations both at 150 μM are shown in Figure 3. It can be seen that BPA concentration decreased rapidly over the reaction time and dropped to near zero within 5 min in the 2.5 unit/mL HRP reaction system. The results displayed in Figures 1−3 indicate that BPA can be effectively removed in HRP-mediated reactions.

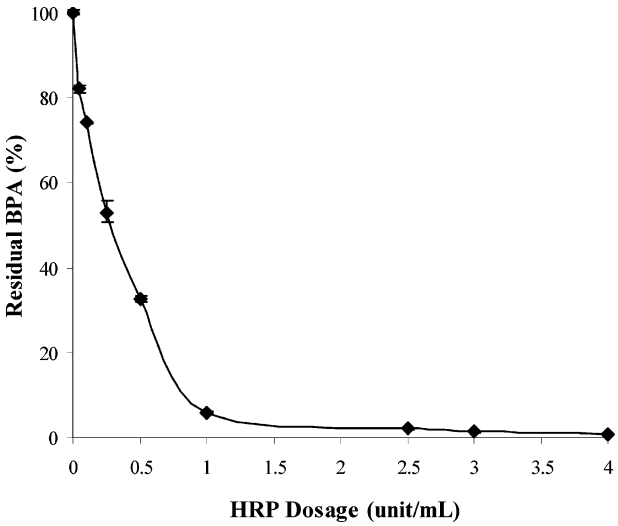


FIGURE 1. BPA removal after 40 min of reaction in a system initially containing 150 μM of BPA, 150 μM of H_2O_2, and varying amounts of HRP. Data points are mean values of triplicate experiments and represent 1-SD error bars.

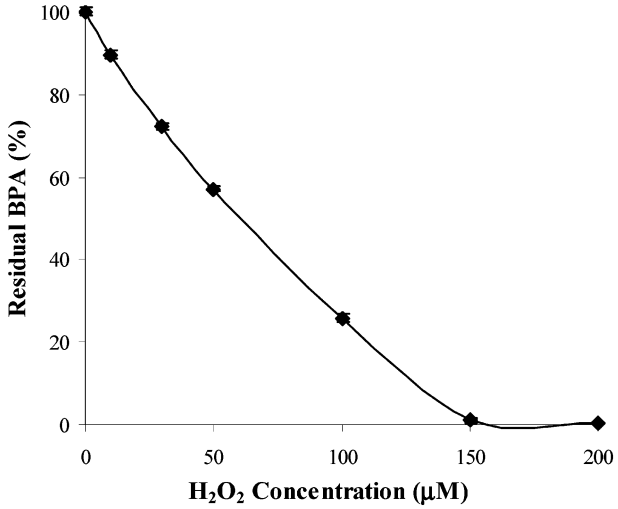


FIGURE 2. BPA removal after 40 min of reaction in a system initially containing 150 μM of BPA, 2.5 unit/mL of HRP, and varying amounts of H_2O_2. Data points are mean values of triplicate experiments and represent 1-SD error bars.

Product Phase Distributions. The phase distributions of the BPA reaction products in the HRP-mediated systems were investigated using radiolabeling techniques, and the results are displayed in Figure 4. As described in the Experimental Section, the reactions for phase distribution study were performed with the condition of 150 μM of BPA and H_2O_2 and 2.5 unit/mL of HRP. It is evident from Figure 4 that no mineralization products were detected and that BPA was quickly converted into solid phases, with dissolved radioactivity dropping to about 10% within 1 min and leveling off thereafter. BPA was almost completely transformed within a similar time frame at this reaction condition, as indicated in Figure 3, suggesting that a portion of the products remained dissolved in the solution phase.

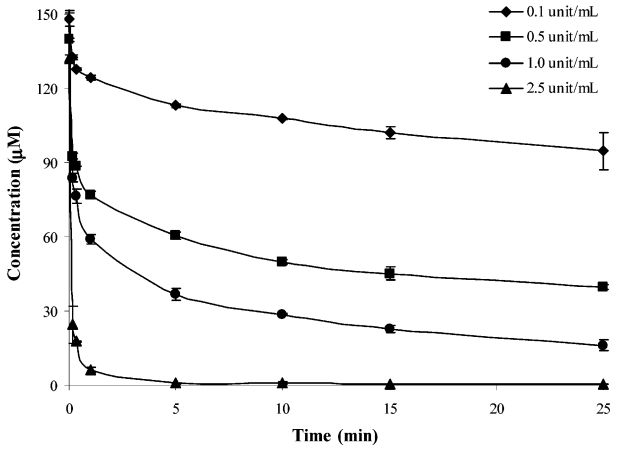


FIGURE 3. BPA removal in systems initially containing 150 μM of BPA, 150 μM of H_2O_2, and four different levels of HRP. Data points are the mean values of triplicate experiments and represent 1-SD error bars.

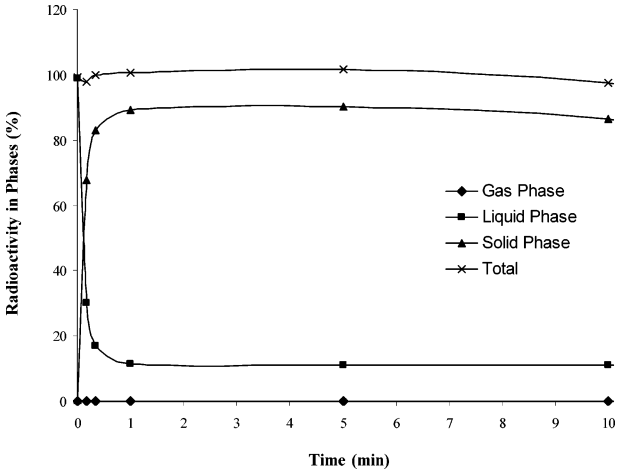


FIGURE 4. The time profile for distribution of ^{14}C radioactivity distribution among phases as the result of HRP-mediated oxidative coupling reaction of radiolabeled BPA. The reaction system initially contained a 150 μM mix of radiolabeled and unlabeled BPA, 150 μM H_2O_2, and 2.5 unit/mL of HRP.

Products and Reaction Pathways. We have attempted to identify products formed in the HRP-mediated BPA reactions using GC/MS and LC/MS techniques. For this work, a reaction with 150 μM of BPA and H_2O_2 and 1 unit/mL of HRP was quenched at 1 min. The liquid phase of the product mixture was concentrated by the SPE procedure described earlier, followed by GC/MS and LC/MS characterizations, and the results are, respectively, presented in Tables 1 and 2. The solid-phase products were cleaned and extracted by a mixture of dichloromethane and methanol (1:1, v/v) as described previously, and the extraction was characterized using LC/MS. The results are presented in Table 3. Tables 1−3 represent an exhaustive list of all species that can be identified using the respective technique.

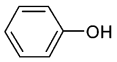
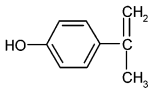
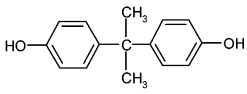
As shown in Table 1, phenol and 4-isopropenylphenol were the only two species other than BPA identified in the liquid phase of the reaction sample by the GC/MS method employed. However, the phenol peak abundance is much weaker than that of isopropenylphenol. Many more species were identified by LC/MS, as shown in Table 2, but isopropenylphenol is the only one having a molecular weight less than BPA, and phenol was not detected. As discussed in detail later, several other species shown in Table 2 are believed to result from further reactions of isopropenylphenol, but no species were identified to be a product of phenol reactions. To sum up, it can be concluded that 4-isopropenylphenol is a major intermediate involved in the reaction, but phenol is not. It can be further postulated that 4-isopropenylphenol is not produced by BPA breakdown, which would have yielded equimolar amounts of phenol, but is likely generated through concerted elimination during a coupling process.

Molecular modeling provides hints as to how phenoxy radicals tend to be coupled. Figure 5 presents the results of *ab initio* characterization of a phenoxy radical. In the insert table of Figure 5, "charge density" designates net charges associated with each atom, and "spin density" indicates the probability of the presence of a spin-unpaired electron. The computation results for spin density shown in Figure 5 reveal that the single electron is likely to associate, by almost equal probability, with atoms 1, 3, 5, and 7 of the BPA radical. This is consistent with the electron resonance shown as Scheme I in Figure 6. Among the four atoms that the single electron is likely associated with, atoms 1, 3, and 7 are partially negatively charged as indicated in Figure 5, with atom 1 having the highest charge. Atom 5 is the only one among the four atoms that possesses partial positive charge. Taking both the charge and the spin density distribution into account, two phenoxy radicals are most likely to couple between atom 1 on one radical and atom 5′ on the other (the prime is used to indicate an atom on a different radical). Such a coupling yields a sterically unstable intermediate, which is likely followed by an elimination of isopropylphenol carboncation, as indicated in reaction II in Figure 6. Such tertiary carboncation elimination would reduce the steric instability around atom 5′ and increase electronic stability of the benzene ring. Shown in reaction II of Figure 6, such a coupling and subsequent elimination process yield species A (MW = 320) and a 2-(4-hydroxyl-phenyl)-propyl carboncation. As indicated in Table 2, species A has been detected in the product solution by LC/MS.

It is known that carboncation intermediates, like the one yielded in reaction II, are subjected to a series of substitution or elimination reactions. For example, as indicated by reaction III in Figure 6, the cationic intermediate yielded in reaction II, 2-(4-hydroxyl-phenyl)-propyl cation, may substitute a proton of water to form species B (MW = 152). The carboncation intermediate may also eliminate a proton, as indicated by reaction IV in Figure 6, to form 4-isopropenylphenol (species C). This product can further react with the 2-(4-hydroxyl-phenyl)-propyl carboncation through proton substitution, as shown in reaction V in Figure 6, to yield species D (MW = 268). Such a substitution reaction can further occur with species D as indicated in reaction VI to yield E (MW = 402). The product of reaction II, species A, may be substituted at two different acidic proton positions, as indicated, respectively, in reactions VII and VIII, to form species F or G, both having the molecular weight of 454. An LC/MS characterization of a 20-s reaction sample gives a much stronger response for species A, for which the molecular ion abundance is 21 000 as opposed to 9000 for the 1-min reaction sample shown in Table 2. The quick decrease in species A supports a hypothesis that ensuing transformations have happened.

Although it is believed that BPA radical coupling happens primarily between atoms 1 and 5′ followed by elimination of the 2-(4-hydroxyl-phenyl)-propyl carboncation, as discussed above, it is not ruled out that two radicals may also be coupled at other positions in minor pathways, for example, between atoms 1 and 3′ or between 3 and 5′. Such coupling processes will also generate products having the molecular weight of 454. As indicated in Table 2, a species having the MW of 454 (retention time = 26.9 min) was identified in the product solution; however, without further structural characterization, it is not certain whether it is a substitution reaction product (F or G) or a BPA coupling product. However,

TABLE 1. GC/MS Characterization of SPE Extracts of Solution-Phase Samples Taken after 1 Min of Reaction[a]

Retention Time (GC min)	m/z (% abundance)	Base Peak Abundance	Structure Assignment
11.3	94(100), 66(81), 65(55), 55(36)	5,677	 Phenol
16.5	134(92), 119(100), 105(13), 94(20), 91(62), 77(33), 65 (41), 51(32)	105,128	 4-isopropenylphenol
30	228 (32), 213(100), 119(47), 107(16), 99(12), 91(33), 77(16), 65(23)	5,174,590	 BPA

[a] The reaction condition, SPE procedure, and GC/MS condition are described in the Experimental Section.

TABLE 2. LC/MS Characterization of SPE Extracts of Solution-Phase Samples Taken after 1 Min of Reaction[a]

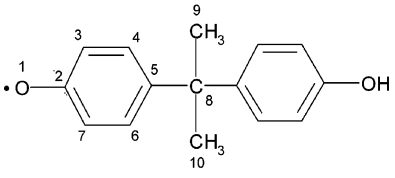
retention time (LC min)	molecular ion	abundence	molecular weight	possible formation pathway[b]	possible structure[b]
5.2	151	180 000	152	III	B
9.8	133	28 000	134	IV	C
14.5	227	180 000	228		BPA
21.9	267	450 000	268	V	D
25.9	319	9000	320	II	A
26.9	453	60 000	454	VII, VIII, or BPA coupling	F, G, or BPA dimer
29.4	359	14 000	360	coupling between C and BPA	oligomer (BPA + C)
33.1	401	60 000	402	VI	E
35.8	493	40 000	494	coupling between D and BPA	oligomer (BPA + D)

[a] Reaction condition, SPE procedure, and LC/MS condition are described in the Experimental Section. [b] Greek numbers and English alphabet letters, respectively, indicate reaction pathways and chemical species that are shown in Figure 6.

TABLE 3. LC/MS Characterization of Solvent Extracts of Precipitate Samples Taken after 1 min of Reaction[a]

retention time (LC min)	molecular ion	abundance	molecular weight	possible structure[b]
26.9	453	56 000	454	F, G, or BPA dimer
28.1	679	35 000	680	BPA trimer or oligomers (BPA + F or G)
34.9	453	30 000	454	E, F, or BPA dimer
44.8	545	4000	546	oligomer (BPA + A)
50.4	679	10 000	680	BPA trimer or oligomers (BPA + F or G)
55.8	679	8000	680	BPA trimer or oligomers (BPA + F or G)

[a] Reaction condition, solvent extraction procedure, and LC/MS condition are described in the Experimental Section. [b] English alphabet letters indicate chemical species shown in Figure 6.



	Charge Density ($e/Å^3$)	Spin Density ($e/Å^3$)
1	-0.47	0.87
2	0.34	-0.78
3	-0.21	0.84
4	-0.25	-0.77
5	0.027	0.87
6	-0.22	-0.77
7	-0.22	0.82
8	-0.31	-0.1
9	-0.52	0.02
10	-0.52	0.008

FIGURE 5. Distributions of charge and spin densities of a BPA radical obtained by *ab initio* calculation.

the products having MWs of 360 (retention time = 29.4 min) and 494 (retention time = 35.8 min) can be assigned for certain as products resulting from coupling reactions, respectively, between BPA and C (227 + 133) and between BPA and D (227 + 267), because no other substitution or coupling pathways would yield molecules of these sizes.

Certain species that might be predicted to form were not detected. For example, a product of 266 amu would have been yielded from self-coupling of species C (133 + 133) and a product of 362 amu would have been yielded by the carboncation substitution of BPA (228 + 135 − 1). Neither of these species can be identified in the LC/MS spectra, suggesting that these reaction pathways are kinetically less favored.

A total of six species were identified in the solvent extract of the precipitate product as displayed in Table 3. Two of them are of the size of a BPA dimer (MW = 454), three of a BPA trimer size (MW = 680), and one of 546 amu, indicating that a product near or above 500 amu tends to precipitate and little polymers are formed beyond the trimer size. The species of 546 amu can be assigned to be a coupling product between BPA and A (227 + 319). One of the 454 amu species was also detected in the solution phase (retention time = 26.9 min), but the other one was not, indicating their different solubility. Formation of multiple species of BPA dimer and trimer sizes confirms that parallel reaction pathways have occurred as discussed above, for example, proton substitution or free-radical coupling at different positions.

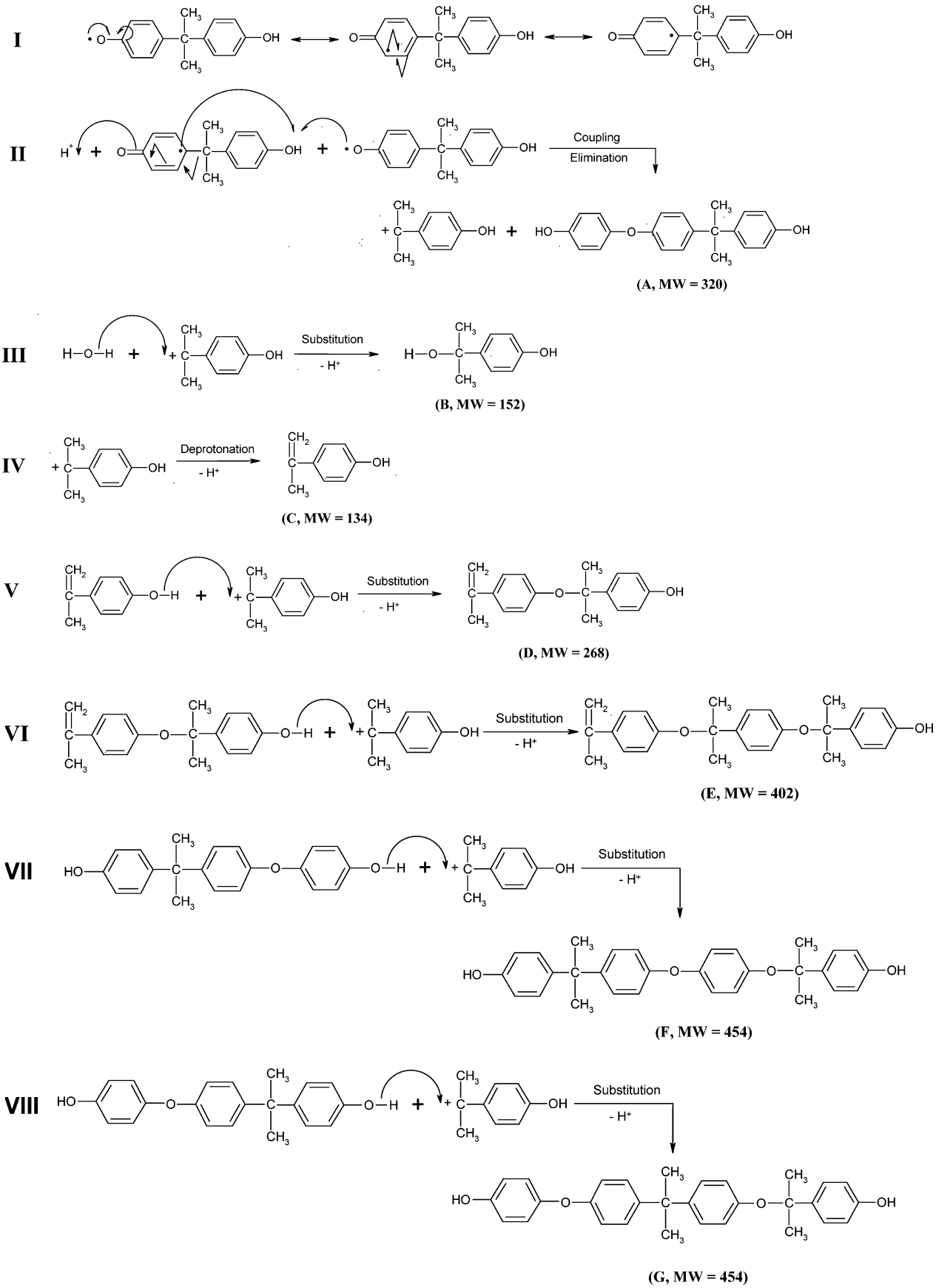


FIGURE 6. Possible reaction pathways.

BPA Removal under Sequenced Reaction Conditions. As noted earlier, sequenced reagent feed experiments were performed by repeatedly supplying HRP and H_2O_2 to the reaction system to test the effectiveness of BPA removal in sequential reactions. The results, shown in Figure 7, indicate that the reaction was effective in removing both BPA and

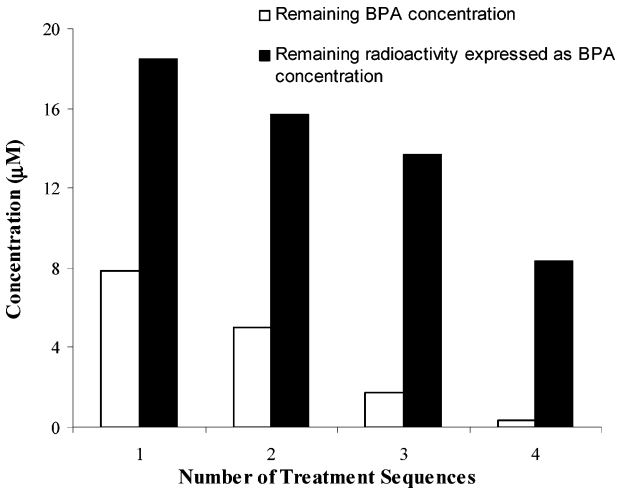


FIGURE 7. Concentrations of BPA and dissolved products remaining after sequenced reaction treatments. The reaction system initially contained a 150 μM mix of radiolabeled and unlabeled BPA. 150 μM of H_2O_2 and 1 unit/mL of HRP were added in the first reaction treatment, and 15 μM of H_2O_2 and 1 unit/mL of HRP were added for all subsequent treatments. Each treatment lasted 40 min.

dissolved products along the process of successive treatments, with the final BPA concentration being lowered to submicromolar levels. It appears to be more difficult to achieve a complete removal of dissolved products other than just BPA. As described in Figure 6, most BPA coupling products still contain phenolic groups and may thus still be estrogenic. Precautions must therefore be taken in potential engineering applications to ensure sufficient removal not only of BPA but also of its dissolved products. Investigation currently underway in our laboratory is being conducted to evaluate reaction efficiency and product distributions in semicontinuous flow reactor systems, along with an evaluation using a recombinant cell assay of overall estrogenicity removal. The results are expected to provide more information with respect to process optimization for the potential use of catalyzed oxidative coupling reactions in the removal of estrogenic phenolic contaminants from water and wastewaters.

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