Influence of NOM and SS on the BPA removal via peroxidase catalyzed reactions: Kinetics and pathways

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ABSTRACT

In the present study, horseradish peroxidase (HRP) was employed to removal bisphenol A (BPA) with the presence of natural organic matter (NOM) and suspended solids (SS). Humic acid (HA) and kaolin have been selected as the typical NOM and SS in the experiment. According to the results, HA has shown a very interesting performance, which has both inhibitory and facilitation effects on BPA removal. For the low enzyme concentration (20 u/L and 50 u/L), small amount of HA could facilitate the BPA removal, while large amount of HA could inhibit the BPA removal. For the higher enzyme concentration (100–300 u/L), the addition of HA reduced the removal transformation of BPA and the inhibition effect of HA raised with the increase of HA concentrations. The facilitation nature of HA on enzyme catalytic processes can be attributed to the mitigation of enzyme inactivation, which has been proven by HRP inactivation rate. The inhibitory effect could be caused by the inhibition effect of HA on the enzyme activity, which has been suggested by both kinetic study and enzyme inactivation analysis. Kaolin showed a negative effect on BPA removal by HRP catalyzed reactions due to the inhibition effect of kaolin on HRP activity. The results have important implications for the engineering design by employing HRP in water and waste water treatment.

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1. Introduction

Endocrine disrupting compounds (EDCs) are attracting increasing attention because they can disturb the synthesis, secretion, transport, binding, action and elimination of the endogenous hormones which are responsible for maintaining homeostasis, reproduction, development and integrity in living organisms and their progeny [1]. Bisphenol A as a typical EDC is widely used as an intermediate material for the production of polycarbonates and epoxy resins. The discharge of BPA into the water can occur from BPA producing factories, from installations that incorporate BPA into plastic [2], from leaching of plastic wastes [3,4] and landfill sites [4]. In China, the extensive demand and production of BPA has led to its ubiquitous in the environmental media [5]. Furthermore, even low-dose BPA could cause adverse effects on the living organisms [1]. Therefore, the efficient techniques for the decomposition and removal of BPA in water are urgently required.

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As the BPA is relatively hydrophilic, therefore, it is less vulnerable to traditional physical/chemical treatment processes such as biological method [6], coagulation/flocculation [7] adsorption [8]. Advanced oxidation processes (AOPs) can efficiently oxidize BPA due to the production of hydroxyl radicals. However, some of the intermediates generated during AOPs may exhibit a higher toxicity as their parent compounds [9]. Recently, enzymatic reactions are attracting more attentions in treatment of BPA at low concentration level due to its reaction selectivity and efficiency [10]. Besides, phenolic compounds are susceptible to catalytically oxidation by enzymes, producing intermediate radicals that may subsequently trigger a series of radical reactions and polymerizations [11]. BPA oxidation has been demonstrated in reaction systems mediated by laccases [12], manganese peroxidase (MnP) [13], lignin peroxidase (LiP) [14] and horseradish peroxidase (HRP) [11]. Among these classes of enzymes, horseradish peroxidase is a commonly used peroxidase that can catalyze the oxidative coupling of a broad spectrum of phenolic compounds in the presence of peroxide [15,16]. The catalytic cycle of the HRP mediated oxidation is presented in Fig. S1.

Natural organic matter (NOM) and suspended solids (SS) are ubiquitously present in water environment. It has been proved that NOM or SS could act as co-substrates with the phenolic compounds in a few reports. Some studies showed that NOM and SS could facilitate the enzymatic reactions [10,11,17]. However, the other studies also presented a contradictive result indicating the presence of NOM or SS could also address inhibitory effect for the enzyme [18]. The removal efficiency and reaction kinetics for BPA removal through HRP catalyzed oxidation has not been fully studied for a water system containing NOM and SS, both of which are common natural water constituents. In addition, little information is available on the influence of NOM and SS for the reaction pathways for BPA removal through HRP catalyzed oxidation process. Thus it is vital to thoroughly investigate the performance and understanding the mechanism among NOM, SS and HRP catalyzed oxidation during the BPA removal.

The purposes of this study were to systematically explore how NOM and SS affect the removal efficiency and degradation pathway of BPA through HRP catalyzed oxidation. The influence of NOM and SS on the removal efficiency of BPA, degradation kinetics and enzyme deactivation rates were studied. Furthermore, the principal reaction intermediates and products were identified by gas chromatography-mass spectrum (GC-MS) technique and attempts were made in this paper to elucidate a detailed reaction scheme.

2. Materials and methods

2.1. Chemicals

Extracellular horseradish peroxidase (type-VI, RZ = 3.3), humic acid (HA), kaolin and 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid)(ABTS) (>98%, HPLC) were purchased from Sigma-Aldrich Chemical. BPA (>99.0%) was purchased from Tokyo Chemical Industry Co., Ltd. Trimethylchlorosilane (99%) and Hexamethyldisilazane (98%) were purchased from J&K. Sinopharm Chemical Reagent Co., Ltd was the source of hydrogen peroxide (30%, GR). Methanol, acetonitrile and dichloromethane were HPLC grade and obtained from Fisher Scientific. All other chemicals were obtained in the highest quality available from Sinopharm Chemical Reagent Co., Ltd. All water solutions were prepared with Milli-Q water.

2.2. Experimental study

The reactions were conducted at room temperature in 50 mL glass tubes. Reaction mixtures were constantly stirred on a rotary shaker table of 200 rpm. Reaction solutions were maintained at a constant pH with phosphate buffers (pH = 7). Each reactor contained predetermined amounts of BPA, H₂O₂ and HRP with or without co-solutes (kaolin, HA). H₂O₂ was the final reagent added to the reactor to initiate the reaction. Triplicate experiments were conducted for each reaction condition.

The effect of initial HA concentration on BPA removal was studied by employing various HA concentrations (0, 1, 3, 5, 10, 30, 50 mg/L) with a fixed concentration of BPA (1 mg/L), H₂O₂ (0.2 mg/L) and HRP (0, 20, 50, 100, 200 u/L). The effect of kaolin on BPA removal was studied by the same process with kaolin concentration 0, 0.5, 1, 2, 3, 4 g/L. After 1 h of reaction, samples were centrifuged at 17,964g for 30 min before analysis. BPA concentration was determined using HPLC with a Waters 2487 UV Detector and a C18 column (5 μ m, 150 mm $\times$ 4.6 mm). The mobile phase was water/acetonitrile (30:70), with a flow rate of 1.0 mL/min. The wavelength was set at 275 nm.

2.3. Reaction rates and kinetics determination

The reaction rates of the catalytic experiment were performed in 250 mL completely mixed batch reactors (CMBRs). Each reactor contains 100 mL of solutions comprised by 1 mg/L BPA, 100 u/L HRP and 0.2 mg/L H₂O₂. To study the influence of HA and kaolin on the reaction rate, 5 mg/L HA and 0.5 g/L kaolin were added individually to the solution. Three-milliliter samples were taken at 0.5, 1, 3, 5, 10, 20, 30 min and the reactions were quenched by adding 100 μ L of 0.3 M HCl immediately. Samples were centrifuged at 17,964g for 30 min before analysis. BPA concentration was determined using the HPLC methods stated previously.

Regarding the kinetic study, the experimental procedures were exactly the same as stated above, except that samples were taken at 10 s intervals over the first 30 s of reaction to identify the initial reaction velocity. The initial velocity, *v*, was computed at conditions of a maximum of only 20–30% conversion of substrate to product.

2.4. Pathway determination

For reaction product identification, 100 mL of reaction solution containing 1 mg/L BPA and 20 u/L HRP with or without 500 mg/L kaolin or 5 mg/L HA was prepared. The reactions were quenched after 2 h by adding HCl, which was long enough for nearly complete removal of BPA. The productions in the reaction solution were extracted, derivatized, and subjected to analysis using an Agilent 6890GC/6973 MSD with a DB-5 MS capillary column. Before GC-MS analysis, the solution was subjected to solid-phase extraction (SPE) performed using 6-cc HLB column from Waters. The solution was dehydrated by passing through the column at a flow rate of 5 mL/min, and concentrated to dryness by an air stream. After which the column was eluted with a 5 mL methanol. The extract was evaporated to dryness under a gentle nitrogen gas flow, and 1 mL dichloromethane was added to redissolve the residue. Silylation reagents, hexamethyldisilazane (0.1 mL) and trimethylchlorosilane (0.05 mL) were quickly added into the mixture without water. The mixtures were vigorously shaken for 1 min. The GC-MS conditions were as follows: the initial column temperature was held for 20 min at 353 K, ramped at 3.5 K/min to 553 K. The byproducts derivatives were identified using the NIST library search.

2.5. Enzyme inactivation rates

The HRP activity was measured by the ABTS method [15]. Briefly, 0.3 mL of 20 mM ABTS is oxidized by 0.05 mL HRP sample with the addition of 0.3 mL of 10 mM hydrogen peroxide in a 3-mL volume of phosphate buffer solution (pH = 5.0). The absorbance at 405 nm was monitored over time by a 2910 UV/VIS Spectrophotometer (Hitachi). One unit of peroxidase activity is defined as the amount of enzyme that will oxidize 1 μ mol of ABTS per minute at 25 °C.

3. Results and discussion

3.1. Influence of NOM on BPA removal

In the present laboratory study, humic acid (HA) was used as the target NOM. The influence of HA on BPA removal at different HRP concentrations (from 20 u/L to 300 u/L) was investigated. As shown in Fig. 1, the addition of HA significantly influenced the removal of BPA. When HRP concentration is relatively low (20 u/L and 50 u/L), smaller amount of HA could facilitate the BPA removal and larger amount of HA could inhibit the BPA removal. At the enzyme concentration of 20 u/L, the removal efficiency of

Fig. 1. Effect of HA concentration on BPA removal at different HRP concentrations (from 20 u/L to 300 u/L).

BPA sharply increased from 55% to 82% when the HA concentration increased from 0 mg/L to 5 mg/L, and dropped from 82% to 68% when the HA concentration increased from 5 mg/L to 50 mg/L. Similar trend was also found at enzyme concentration of 50 u/L. When the HRP concentration is relatively high (100–300 u/L), the addition of HA reduced the removal transformation of BPA and the inhibition effect of HA raised with the increase of HA concentrations. The removal efficiency of BPA dropped from 99% to less than 90% at higher HRP concentrations (100–300 u/L) when the HA concentration increased from 0 to 50 mg/L. The results indicate that HA could have both facilitation and inhibition effect on BPA removal. The inhibitory effect of HA on enzyme catalytic processes has been depicted before [18]. The cause of inhibition can be resulted from the competition between HA and substrates for an active site on the enzyme surface. The facilitation nature of HA on enzyme catalytic processes can be attributed to the mitigation of enzyme inactivation. As shown in Fig. 4, the addition of HA could reduce the inactivation rate of HRP during the catalytic process, resulting in a higher removal rate of BPA. Besides, HA could also facilitate BPA removal by cross-coupling with the intermediated generated during enzymatic reaction stage. When the enzyme concentration was relatively low, the inactivation of enzyme was the dominate factor for the oxidation processes, thus moderate amount of HA could facilitate the BPA removal. While at a relatively higher enzyme concentration system, the inactivation of

Fig. 2. Effect of kaolin concentration on BPA removal at different HRP concentrations (from 50 u/L to 200 u/L).

Table 1

Experimental kinetics determination for the HRP catalyzed BPA degradation.

Conditions	K_r (mg/L s)	K_M (μ M)	V_{max} (μ M/s)
HRP only	4.315E–4	9.049	0.178
HRP + HA	4.203E–4	8.153	0.159
HRP + kaolin	3.387E–4	24.128	0.398

Fig. 3. Linear plot used in determining the catalytic parameters v_{max} and K_M of the HRP catalyzed system of BPA with or without HA or kaolin (initial HRP activity of 100 u/L).

enzyme was not critical and thus the addition of HA inhibited the BPA removal.

3.2. Influence of kaolin on removal efficiency

As shown in Fig. 2, BPA removal rates were slightly inhibited by the kaolin. For example, BPA removal efficiency marginally dropped from 95% to 91.8% when the kaolin concentration increased from 0 to 4 g/L (HRP concentration 50 u/L). It was reported that suspended solids like kaolin could adsorb and occupy the reactive surface site of enzymes. Thus the enzyme activities could be suppressed by kaolin. In this way, BPA removal could be negatively influenced by kaolin.

3.3. Influence of HA and kaolin on reaction rates and kinetics

A kinetic study was performed to find out the influence of HA and kaolin on reaction rates and kinetics. The residual BPA concen-

Fig. 4. Enzyme activity for system with or without HA and kaolin. The initial enzyme concentration is 100 u/L, peroxide concentration is 0.1 mg/L.

tration was measured at the different reaction time. Based on the results of these experiments, the time course of BPA degradation was found to be reasonably well described by an apparent second order rate expression, i.e.

$$r = dc/dt = k_r \cdot c^2 \quad (1)$$

This can be turned into the following equation after integration:

$$1/c - 1/c_0 = k_r \cdot t \quad (2)$$

In which r represent the BPA degradation rate, c represent the residual BPA concentration after time t , and c_0 represents the initial BPA concentration, and k_r represents the rate coefficient for second order reaction. Value of k_r obtained from the fitting of Eq. (2). Pseudo second-order rate fitting result for the HRP catalyzed system of BPA is shown in Fig. S2.

The reaction constants are listed in Table 1. As can be seen from Table 1, after adding HA, the reaction rate constants k_r are barely changed. While after adding kaolin, the reaction rate constant k_r significantly reduced.

The kinetic study of the BPA removal process can be well described by the Michaelis-Menten equation:

$$v_A = v_{\max} \cdot [A]/(k_M + [A]) \quad (3)$$

where v_A stands for the initial rate of conversion ($\mu\text{M/s}$) for each initial substrate concentration, $v_{\max}$ stands for the maximum reaction velocity the reaction can achieve when the enzyme is saturated in substrate, and k_M stands for the Michaelis constant under the conditions used. A lower k_M value corresponds to a higher affinity of the enzyme for a substrate.

The k_M and $v_{\max}$ values could be determined graphically using the Lineweaver-Burk method

(a) Mitigation of HRP activity loss by HA

(b) Uncompetitive inhibition of HA

(c) Inhibition of Kaolin

Fig. 5. Proposed roles of HA and kaolin in HRP activity.

radical is susceptible to interchange to other transition forms via resonance, forming transition form R2. R1 and R2 are likely to coupling with each other, which yields a sterically unstable intermediate and subsequently eliminates cationic isopropyl phenol R3 and release 1 (Fig. S3, E) and reduce the steric instability. The cationic carbon R3 is known to undergo substitution or elimination reactions [21]. This is also consistent with the reaction schemes of HRP catalyzed BPA removal. As shown in Fig. 6, R3 may be subject to deprotonation, yielding 2 (Fig. S3, A); or substitution of a proton can occur with water or methanol, forming 3 and 4 (Fig. S3, B and C). Such product can further with the R3 through proton substitution to yield 5 (Fig. S3, D). The cationic carbon could also act with the monomers of humic acid and forming products such as 6 (Fig. S3, F).

4. Conclusion

In this paper, the influence of NOM and SS on the removal of BPA by HRP catalyzed oxidation has been thoroughly investigated. Based on the analytical and experimental results, the following conclusions may be obtained:

1. Natural water constitutes such as HA and kaolin exist a significant effect on HRP-catalyzed BPA removal.
2. HA could have both facilitation and inhibition effect on HRP-catalyzed BPA removal. HA could bind with the phenoxy radicals and mitigate the enzyme inactivation, thus moderate amount of HA could facilitate the BPA removal. On the other hand, HA is also an uncompetitive inhibitor for HRP, which could inhibit the BPA removal by HRP catalyzed oxidation.
3. The effect of HA on BPA removal is dependent on both of the HRP concentration and HA concentration. At a relatively lower HRP concentration, the inactivation of enzyme was the dominate factor for the oxidation processes, thus moderate amount of HA could facilitate the BPA removal. While at a relatively higher enzyme concentration system, the inactivation of enzyme was not critical and the uncompetitive inhibition effect of HA dominates the HRP-catalyzed BPA removal process.
4. Kaolin is not involved in the product generation processes, but kaolin could inhibit HRP activity by shielding the enzyme active sites. Thus the addition of kaolin could negatively affect BPA removal by HRP catalyzed oxidation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.seppur.2016.09.029>.

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