Use of Chitosan for Removal of Bisphenol A and Bisphenol Derivatives Through Tyrosinase-Catalyzed Quinone Oxidation

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ABSTRACT: In this study, the availability of chitosan was systematically investigated for removal of bisphenol A (BPA, 2,2-bis(hydroxyphenyl)propane) through the tyrosinase-catalyzed quinone oxidation and subsequent quinone adsorption on chitosan beads. In particular, the process parameters, such as the hydrogen peroxide (H_2O_2)-to-BPA ratio, pH value, temperature, and tyrosinase dose, were discussed in detail for the enzymatic quinone oxidation. Tyrosinase-catalyzed quinone oxidation of BPA was effectively enhanced by adding H_2O_2 and the optimum conditions for BPA at 0.3 m*M* were determined to be pH 7.0 and 40°C in the presence of H_2O_2 at 0.3 m*M* ($[H_2O_2]/[BPA] = 1.0$). Removal of BPA from aqueous solutions was accomplished by adsorption of enzymatically generated quinone derivatives on chitosan beads. The use of chitosan in the form of beads was found to be more effective because heterogeneous removal of BPA with chitosan beads was much faster than homogeneous removal of BPA with chitosan solutions, and the removal efficiency was enhanced by increasing the amount of chitosan beads dispersed in the BPA solutions and BPA was completely removed by quinone adsorption in the presence of chitosan beads more than 0.10 cm^3/cm^3. In addition, a variety of bisphenol derivatives were completely or effectively removed by the procedure constructed in this study, although the enzyme dose or the amount of chitosan beads was further increased as necessary for some of the bisphenol derivatives used. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 118: 721–732, 2010

Key words: adsorption; biopolymers; enzymes; resins; separation techniques

INTRODUCTION

Scientific and public attention has been recently focused on environmental pollutants that can mimic or antagonize the effects of endogenous hormones.[1,2] These chemicals are called endocrine disrupting chemicals and their adverse effects on human [3–7] and wildlife such as fish, amphibians, and birds have been documented in many articles.[8–13] Bisphenol A [2,2-bis(hydroxyphenyl)propane, (BPA)] is a ubiquitous substance used mainly in the production of epoxy resins and polycarbonate plastics[6] and the annual production of BPA stood at 490,000 ton in 2001 in Japan. Estrogenic activity of BPA has been widely known since 1938.[7] However, BPA is detected in effluent samples of wastewater treatment plants and also found in sediments and fish. One of the most likely sources is the leachate from hazardous waste landfill. When waste plastics produced form BPA are buried in a landfill, a hydrolytic or leaching process may occur to release BPA to the leachate.[14–16]

Recently the bisphenol derivatives such as bisphenol B (BPB),[17] bisphenol C (BPC),[18–21] bisphenol E (BPE),[22,23] bisphenol F (BPF),[24–26] bisphenol O (BPO),[27] bisphenol S (BPS),[28–30] bisphenol T (BPT),[31,32] and bisphenol Z (BPZ)[33,34] shown in Figure 1 have come into wide use for synthesis of specialized epoxy and polycarbonate resins with more high-performance compared with ordinary ones. Although these bisphenol derivatives have two phenol groups in common, the chemical structure between phenol groups is different. Some of the bisphenol derivatives in addition to BPA are reported to exhibit estrogenic activity in human breast cancer cell line MFC-7 or inhibitory effects on the androgenic activity of 5α-dihydrotestrosterone in mouse fibroblast cell line NIH3T3.[35]

Chemical procedures such as adsorption,[36–40] nanofiltration,[41] electrochemical polymerization,[42]

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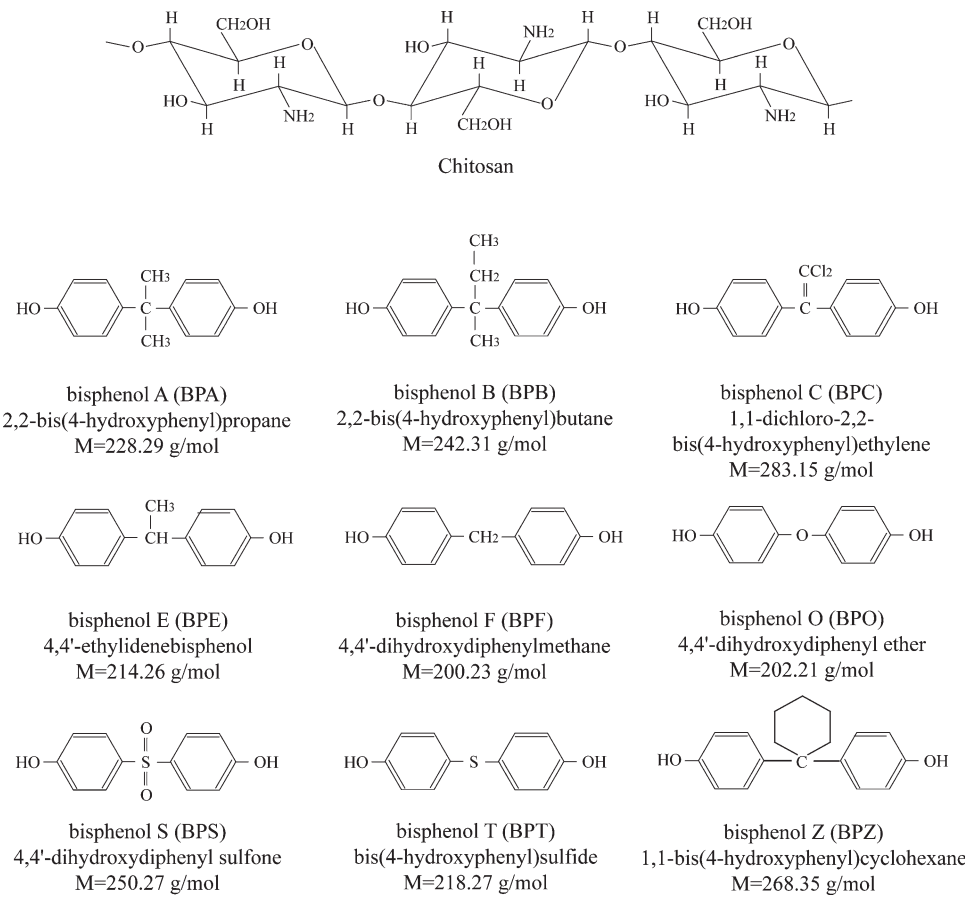


Figure 1 Chemical structures of chitosan and BPA and bisphenol derivatives used in this study.

photolysis,[43,44] photooxidation,[43] or biological procedures by use of microalgae and bacteria [45,46] have been constructed to detoxify or degrade BPA. Alternatively, much attention has been also paid to the potentials of enzymes to catalyze the transformation of phenol compounds.[47–51] The use of enzymes has many advantages over the above conventional procedures which are effective but suffer from high cost, incomplete purification, formation of hazardous by-products, and applicability to only a limit concentration range.

Peroxidases have been more frequently used to treat BPA in the presence of hydrogen peroxide (H_2O_2), Then, BPA is converted to phenoxy radicals, and the radicals generated simultaneously react to form water-insoluble polymers.[52] Many studies on tyrosinase-catalyzed oxidation of phenol compounds to quinone derivatives have been reported. This reaction is called "quinone oxidation," which is more frequently used than "quinone formation" in biotechnology. However, most of these studies were concerned with alkylphenols and chlorophenols. Tyrosinase has two catalytic functions, that is, *o*-hydroxylation of monophenols to *o*-diphenols in the presence of molecular oxygen (cresolase activity) and a two-electron oxidation of *o*-diphenols to *o*-quinones (catecholase activity).[53–56] In addition, Jiménez et al. reported that 4-*tert*-butylphenol (4TBP), which underwent no quinone oxidation by mushroom tyrosinase without H_2O_2, was converted to 4-*tert*-butyl-*o*-benzoquinone in the presence of H_2O_2.[57] However, according to the report by Yoshida et al., BPA undergoes no quinone oxidation by commercially available mushroom tyrosinase in the absence of H_2O_2.[58] Therefore, little was reported on tyrosinase-catalyzed treatment of BPA.

Quinone derivatives have a high reactivity and can undergo different chemical reactions. Therefore, the second step to remove phenol compounds form aqueous solutions was to use chitosan as an amino group-containing polymer.[59–63] When a chitosan solution was added to solutions of alkylphenols and chlorophenols containing tyrosinase, water-insoluble precipitates were generated through the reaction of quinone derivatives generated with chitosan's amino groups.[64] Alternatively, in our previous articles crosslinked chitosan beads were dispersed in solutions of alkylphenols and chlorophenols containing tyrosinase.[62,63] Then, alkylphenols and chlorophenols were effectively removed through chemisorption of quinone derivatives on chitosan beads. It should be noted that an increase in the amount of chitosan

beads dispersed in the solutions led to an increase in quinone adsorption and the removal efficiency was enhanced. Chitosan is produced in large amounts by deacetylation of chitin that is contained in the shells of the crustaceans such as crabs and prawns. However, the use of chitosan is limited mainly to wastewater treatment to capture heavy metal ions. Therefore, if chitosan beads are used as an adsorbent for removal of BPA, an alternative usage of chitosan will come out.

The goal of this study is to investigate the tyrosinase-catalyzed quinone oxidation of BPA and removal of BPA through quinone adsorption on the chitosan beads. First, the effects of the process parameters such as the H_2O_2 concentration, pH value, temperature, and enzyme dose were discussed to convert BPA to quinone derivatives. In particular, we closely followed the effect of the addition of chitosan in the form of beads and the capability of this two-step approach for removing BPA. In addition, removal of various bisphenol derivatives was also estimated under the optimum conditions determined for treatment of BPA.

MATERIALS AND METHODS

Chemicals

Mushroom tyrosinase (EC 1.14.18.1) of the specific activity of 2,870 U/mg-solid (activity determined by the supplier) was purchased from Sigma (St. Louis, MO). A chitosan bead, Chitopearl AL-01, from Fuji Spinning (Tokyo, Japan) (particle size, 70–210 μm; specific surface area, 70–100 m^2/g; water content, 92.5%) was used as an adsorbent, and stored in phosphate buffers at different pH values (ionic strength, 0.01 *M*). BPA and bisphenol derivatives were purchased from Wako Pure Chemicals and Tokyo Chemical Industry (Tokyo, Japan) and their chemical structures were shown in Figure 1. A 30% H_2O_2 solution was obtained from Wako Pure Chemicals.

A chitosan solution [1 (w/v) %] was prepared by adding 1.0 g chitosan flakes (chitosan 300, Wako Pure Chemicals) to 100 cm^3 distilled water and adding 2 *M* HCl to keep the pH value of 3–4. After most of the added chitosan flakes were dissolved, the solution was filtered to remove insoluble parts. Chitosan films were prepared by pipetting a chitosan solution of 5.0 g into a Petri dish of diameter 3.2 cm. The solution was allowed to dry in the oven at 50°C. The chitosan films (average thickness: 24 μm) removed from the Petri dishes were thoroughly washed with 1M NaOH and pure water to neutralize the amino groups, and then dried under reduced pressure at room temperature.

Quinone oxidation and removal of BPA

Stock solutions of BPA, H_2O_2, and mushroom tyrosinase were prepared with the phosphate buffers of pH 4.0 to 10.0 (ionic strength = 0.01 *M*, HCl/KH_2PO_4 at pH 4.0 and $NaOH/KH_2PO_4$ at pH 5.0–10.0). H_2O_2 was added to a BPA solution (30 cm^3) in a 50 cm^3 Erlenmeyer flasks. Just after the temperatures of the solutions were adjusted in a temperature-controlled bath, the enzymatic reaction was initiated by adding tyrosinase solution to the mixture solutions. The solutions were continuously stirred and the UV-visible spectra of the reaction solutions were recorded on a Shimadzu UV-visible recording spectrophotometer UV-260 at predetermined time intervals. In addition, for removal experiments, a given amount of chitosan beads stored in a buffer were added to the BPA solutions containing H_2O_2 from a bullet for titration, and then the enzymatic reaction was initiated by adding tyrosinase.

Quantitative assay of BPA

The concentration of remaining BPA was determined by a Hitachi L-7000 HPLC system equipped with a UV-spectrophotometer and an integrator. Aliquots of 0.2–0.3 cm^3 taken from the reaction solutions at given reaction times were immersed in hot water at 80–85°C for 5 min to deactivate tyrosinase. A reverse phase column, Inertsil ODS-3 (5 μm, 4.6 mm i.d.×15 cm), was used. The volume composition of aqueous acetonitrile solutions as the mobile phase was adjusted to 55 vol % of water and the mobile phase was flowed at 1.0 cm^3/min. Then, a solution of 20 mm^3 was injected and the absorption spectra were determined at 278 nm. The conversion (%) values were calculated from the area of the peaks assigned to BPA before and after the enzymatic reaction ($area_0$ and $_{area}$) using eq. (1).[62,63]

$$\text{Conversion}(\%) = \frac{\text{area}_0 - \text{area}}{\text{area}_0} \times 100 \qquad (1)$$

Instrumental analysis of chitosan/quinone reaction

A chitosan film was incubated in a BPA solution containing tyrosinase and H_2O_2 at pH 7.0 and 40°C. After incubation, the chitosan film was throughout washed with water, and then dried in an oven at 50°C. The UV-visible spectra of the incubated chitosan films were recorded on a spectrophotometer. The incubated chitosan films were placed perpendicular to the light path such that the light passed directly through the films in the spectrophotometer.

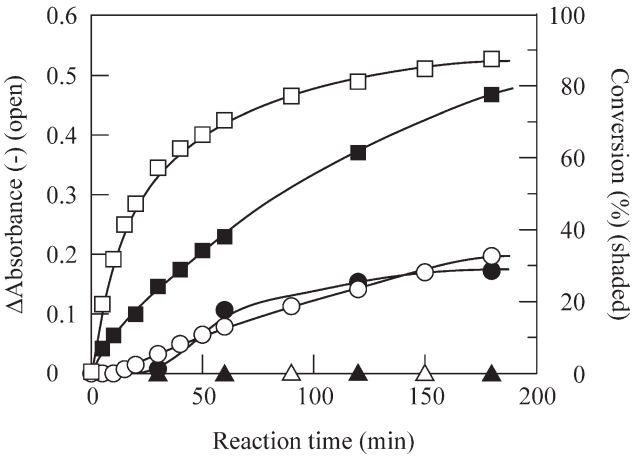


Figure 2 The time course of the absorbance at 385 nm (open) and conversion (%) value (shaded) for BPA solutions (0.3 m*M*) containing tyrosinase (200 U/cm^3) (○, ●), tyrosinase and H_2O_2 (0.3 m*M*) (□, ■), and H_2O_2 (0.3 m*M*) (△, ▲) at pH 7.0 and 40°C.

Removal of bisphenol derivatives and their quantitative assay

The experiments of tyrosinase-catalyzed quinone oxidation and quinone adsorption on chitosan beads were also applied to removal of bisphenol derivatives. In the case where quinone oxidation and/or quinone adsorption were low, the tyrosinase concentration and the amount of added chitosan beads were increased to enhance the removal efficiency. The volume composition of the mobile phases consisting of water and acetonitrile and the wavelength of the spectrophotometer for HPLC measurements depended on the type of bisphenol derivatives used. The flow rate of the mobile phases was adjusted to 1.0 cm^3/min. A solution of 20 mm^3 was injected and the absorption spectra were determined.[62,63,65]

RESULTS AND DISCUSSION

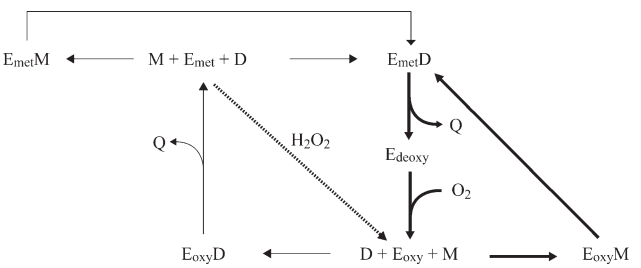
Determination of optimum condition of enzymatic reaction

Effect of H_2O_2 concentration

First, the effect of the addition of H_2O_2 on tyrosinase-catalyzed quinone oxidation was investigated. Figure 2 shows the time course of the absorbance at 385 nm and conversion (%) value for tyrosinase-catalyzed quinone oxidation of BPA under different conditions at pH 7.0 and 40°C. An absorbance at 385 nm slowly increased over the reaction time for a BPA solution containing tyrosinase without H_2O_2, and the conversion (%) value was limited to only 29% even when the reaction time was prolonged to 3 hr. An increase in the absorbance at 385 nm refers to the generation of quinone derivatives from BPA and the peak is absent for the original BPA solution containing tyrosinase. In addition, even if H_2O_2 was added to a BPA solution without tyrosinase, no quinone derivatives were generated and the reaction solution remained transparent (The symbols of △ were overlapped with those of ▲).

The peak assigned to quinone derivatives appears at 350–400 nm, and the peak position depends on the chemical structure of the phenolic substrate.[59–63] The peak position at 385 nm obtained in this study was in agreement with those assigned to quinone derivatives enzymatically generated from alkylphenols.[62] The emergence of the peak at 385 nm and color development of the solution support quinone generation from BPA by mushroom tyrosinase. Here, bisquinone derivatives can be possibly generated in addition to monoquinone derivatives with a unreacted phenolic —OH group because BPA has two phenolic groups. Yoshida et al. reported the detection of a small amount of bisquinone derivatives in addition to monoquinone derivatives by the HPLC analysis for quinone oxidation of BPA with tyrosinase from Worthington Biochemical Co.[58]

When H_2O_2 was added to a BPA solution containing tyrosinase (200 U/cm^3), both the absorbance at 385 nm and the conversion (%) value sharply increased in the first 1 hr and the solutions became yellow, then orange-red, and finally dark brown.[59] According to Payne et al., the cresolase activity of mushroom tyrosinase is rather slower than the catecholase activity for quinone oxidation of many phenol compounds.[59–61] In addition, 4TBP and 4-*tert*-pentylphenol (4TPP) undergo quinone oxidation in the presence of H_2O_2 but not in the absence of H_2O_2.[62,63] Tyrosinase has three forms, met-, oxy-, and deoxy-forms, depending on the presence or absence of H_2O_2 as shown in Scheme 1. The portion of oxy-form tyrosinase is so small in the absence of H_2O_2 that the enzymatic reaction is very low.[57] In addition, Ikehata and Nicell reported that molecular oxygen bound to deoxy-form tyrosinase, bringing it to oxy-form tyrosinase.[66,67] Some of met-form tyrosinase is also converted into oxy-form tyrosinase by adding H_2O_2. The formation of oxy-form tyrosinase



Scheme 1 Catalytic cycle for the oxidation of monophenol (M) and diphenol (D) substances to quinone derivatives (Q) by tyrosinase (E) and transformation of tyrosinase in the presence of H_2O_2.

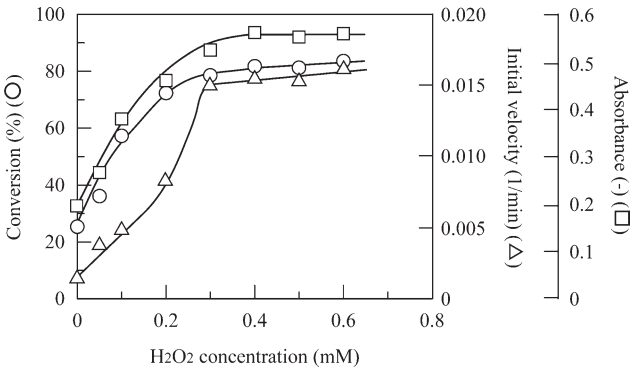


Figure 3 The effect of H_2O_2 on tyrosinase-catalyzed (200 U/cm^3) quinone oxidation of BPA (0.3 m*M*) at pH 7.0 and 40°C. The enzymatic reaction time was 3 hr.

by addition of H_2O_2 is considered to accelerate quinone oxidation of BPA.

Alternatively, the cresolase activity of mushroom tyrosinase was reported to be enhanced in the presence of 3-hydroxyanthranilic acid (HAA) for *N*-acetyl-L-tyrosine and 4TBP.[68] However, since HAA is referred to as a carcinogen, the use of HAA is not favorable in the viewpoint of environment. Therefore, On the basis of the fact that tyrosinase-catalyzed quinone oxidation of BPA was accelerated by the addition of H_2O_2, the effect of the H_2O_2 concentration on quinone oxidation of BPA was investigated at pH 7.0 and 40°C.

Figure 3 shows the changes in the absorbance and conversion (%) value at the reaction time of 3 hr and the initial velocity for BPA solutions containing H_2O_2 of different concentrations at pH 7.0 and 40°C. The initial velocity was determined from the slope of the absorbance against the reaction time in the initial stage. The increase in the H_2O_2 concentration led to the increase in the absorbance at 385 nm in the range of H_2O_2 concentrations below 0.3 m*M* and the conversion (%) value increased with the H_2O_2 concentration. However, the increment of the absorbance and the conversion (%) value had a tendency to level off at further increased H_2O_2 concentrations. Here, there was a distinct difference in the H_2O_2-dependent tyrosinase-catalyzed quinone oxidation between BPA and branched alkylphenols such as 4TBP and 4TPP. 4TBP and 4TPP underwent no tyrosinase-catalyzed quinone oxidation in the absence of H_2O_2.[57] On the other hand, BPA was slowly converted into quinone derivatives by tyrosinase even in the absence of H_2O_2 as shown in Figure 2. The catalytic cycle of tyrosinase-catalyzed quinone oxidation of phenol, *p*-alkylphenols, and chlorophenols has been deeply discussed in many articles.[50,53,66–68] According to the enzymatic reaction mechanism shown in Scheme 1, tyrosinase-catalyzed quinone oxidation of BPA can be also explained like other phenol compounds aforementioned.

Mushroom tyrosinase has the catecholase activity as well as the peculiar characteristics of cresolase activity. Therefore, phenol, *p*-*n*-alkylphenols, and chlorophenols were successfully quinone-oxidized through formation of the corresponding diphenols even in the absence of H_2O_2. Although BPA gradually underwent quinone oxidation by mushroom tyrosinase in the absence of H_2O_2 as shown in Figure 2, tyrosinase-catalyzed quinone oxidation of BPA was considerably slower than that of *p*-*n*-alkylphenols. The binding of branched alkylphenols such as 4TBP and 4TPP to met-form tyrosinase is considered to scavenge a portion of tyrosinase from the catalytic turnover as a dead-end complex in the cresolase activity.[50,53,57,67] On the other hand, oxy-form tyrosinase can convert a BPA molecule into the corresponding quinone derivative. The optimum H_2O_2 concentration was determined to be 0.3 m*M*, since the concentration of remaining H_2O_2 should be as low as possible in the viewpoint of environmental conservation.

Effects of pH and temperature

Here, the effects of the process parameters such as the pH value and temperature on enzymatic quinone oxidation of BPA were investigated because the optimum pH and temperature of the tyrosinase activity are slightly different from one article to another, and depend on the origin of enzymes and the experimental conditions such as the kind and concentration of the substrates, kind and salt concentration of buffers, and the presence or absence of H_2O_2.[62,66,69] First, the effect of the pH value on quinone oxidation of BPA at 0.3 m*M* was investigated in the presence of H_2O_2.

Figure 4 shows the effect of the pH value on tyrosinase-catalyzed quinone oxidation of BPA in the presence of H_2O_2 of 0.3 m*M* at 40°C. Tyrosinase was deactivated in a short reaction time at pH 4.0 and ~ 75% of BPA was left unreacted in the solution. The initial velocity was sharply increased at pH 5.0 and 6.0, but the absorbances 3 hr after the enzymatic reaction was initiated were relatively low. On the other hand, although the initial velocity at pH 7.0 was lower than those at pH 5.0 and 6.0, the absorbance increased over the reaction time and the absorbance at pH 7.0 was higher than those at pH 5.0 and 6.0. When the pH value was further increased, the activity of tyrosinase gradually decreased. It was found that the activity of tyrosinase was successfully kept going and a high conversion efficiency was obtained at pH 7.0 compared with at other pH values. From the above results, the optimum pH value was determined to be 7.0. Subsequently, the effect of the temperature on the tyrosinase-catalyzed quinone oxidation was investigated at pH 7.0. As shown in

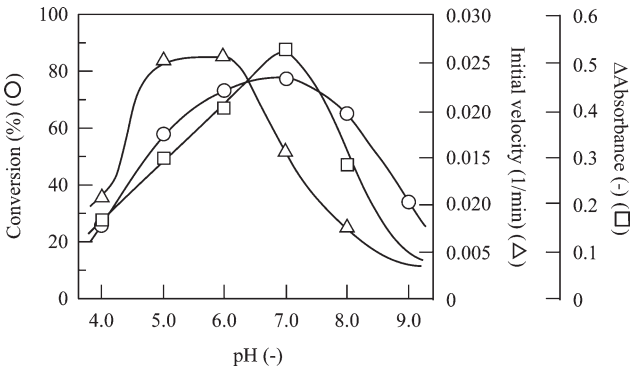


Figure 4 The effect of pH value on tyrosinase-catalyzed (200 U/cm^3) quinone oxidation of BPA (0.3 m*M*) in the presence of H_2O_2 of 0.3 m*M* at 40°C. The enzymatic reaction time was 3 hr.

Figure 5, quinone oxidation of BPA increased by increasing the reaction temperature and the activity of tyrosinase passed through the maximum value at 40°C. The activity of tyrosinase sharply decreased at higher than 40°C probably due to thermal denaturation as observed for other enzymes.[70,71]

It was found from the above results that tyrosinase-catalyzed quinone oxidation of BPA was accelerated by the addition of H_2O_2, and the optimum conditions for quinone oxidation of BPA at 0.3 m*M* were determined to be 0.3 m*M* for H_2O_2 ($[H_2O_2]/[BPA] = 1.0$) at pH 7.0 and 40°C.

Effect of enzyme dose on quinone oxidation

On the basis of the optimum conditions determined in the above section, the enzymatic reaction was initiated for BPA at 0.3 m*M* with tyrosinase of different concentrations in the presence of H_2O_2 at 0.3 m*M*. The specific initial velocity was calculated from the initial velocity obtained from the slope of the absorbance against the reaction time and the tyrosinase concentration.

Figure 6 shows the effect of the tyrosinase concentration on quinone oxidation of BPA at pH 7.0 and 40°C. The quinone oxidation of BPA was proportional to the tyrosinase concentration in the tyrosinase concentration range below 200 U/cm^3. The quantity of 78% of BPA was converted into quinone at 200 U/cm^3. In addition, the specific initial velocity reached the maximum value at 200 U/cm^3 and sharply decreased at further increased tyrosinase concentrations. We empirically demonstrated that quinone oxidation of BPA was accelerated by increasing the tyrosinase concentration up to 200 U/cm^3. An increase in the tyrosinase concentration at a constant H_2O_2 concentration led to an increase in the specific initial velocity. This behavior is considered to be attributed to an increase in the amount of oxy-form tyrosinase. However, it is our upcoming challenge to estimate the enzymatic quinone oxidation quantitatively. In addition to the above results, from the fact that the conversion (%) value was negatively deviated downward from the linear relationship at higher than 200 U/cm^3, the optimum tyrosinase concentration was determined to be 200 U/cm^3. The importance to determine the optimum conditions discussed in the sections of 3.1.1 to 3.1.3 is derived from our consideration that the activities of many enzymes decreased due to the presence of enzymatically generated chemical species or under unfavorable conditions such as high temperatures and lower and higher pH values. The enzymatic activity can be effectively developed by determining the aforementioned process parameters, leading to a reduction in the time required for the enzymatic reaction.

Instrumental estimation of quinone adsorption on chitosan

First, the reactivity of quinone derivatives enzymatically generated from BPA with chitosan's amino groups was estimated with chitosan films. Figure 7 shows the UV-visible spectra of chitosan films incubated in BPA solutions containing both tyrosinase and H_2O_2 and containing either tyrosinase or H_2O_2. The absorbance at 460 nm emerged for the chitosan film incubated in a BPA solution containing both tyrosinase and H_2O_2. No this peak appeared for chitosan films incubated in BPA solutions containing either tyrosinase or H_2O_2. In addition, this peak was not also observed for a control cellulose film incubated with a BPA solution containing both tyrosinase and H_2O_2.[62] This difference reveals that an amino group is involved in quinone binding.

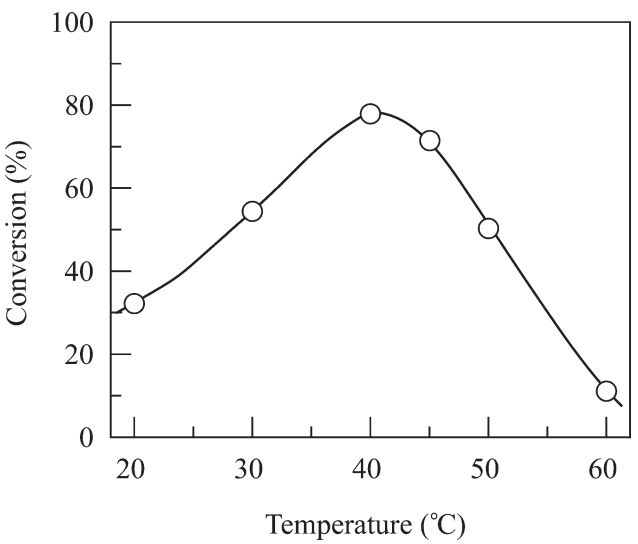


Figure 5 The effect of the temperature on tyrosinase-catalyzed (200 U/cm^3) quinone oxidation of BPA (0.3 m*M*) in the presence of H_2O_2 of 0.3 m*M* at pH 7.0. The enzymatic reaction time was 3 hr.

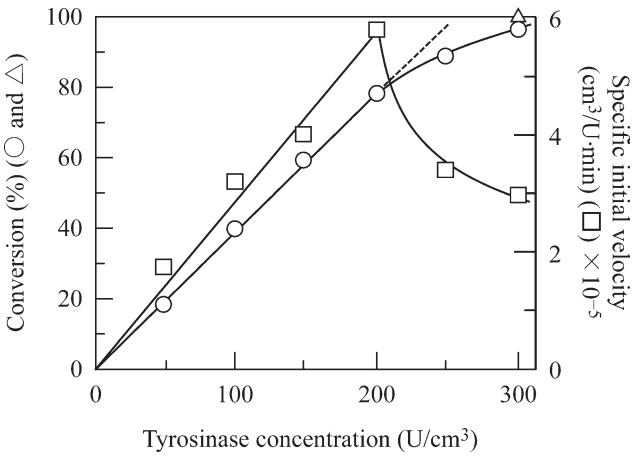


Figure 6 The effect of the tyrosinase concentration on quinone oxidation of BPA (0.3 m*M*) in the presence of H_2O_2 of 0.3 m*M* at pH 7.0 and 40°C. Enzymic reaction time (hr)— ○: 3, △: 5.

According to some articles, this reaction is considered to occur through either Schiff base or Michael-type addition.[60,62,72]

The position of the peak at 460 nm is assigned to the binding of quinone derivatives generated from BPA to amino groups. This value was in good agreement with the position of the peak assigned to amino groups reacted with quinone derivatives generated from *p*-cresol under a similar condition.[62] These results reveal that quinone derivatives generated from BPA successfully reacted with chitosan's amino groups under a mild condition. Therefore, we estimated quinone adsorption on chitosan beads in the next section.

Removal of BPA through quinone adsorption

Figure 8(a) shows the time course of the absorbance of the BPA solutions containing tyrosinase (200 U/cm^3) and H_2O_2 (0.3 m*M*, $[H_2O_2]/[BPA] = 1.0$) in the presence of different amounts of chitosan beads at pH 7.0 and 40°C. The increase in the absorbance at 385 nm was considerably depressed by addition of chitosan beads because quinone derivatives generated were chemically adsorbed on chitosan beads. However, the portion of 2.3% of BPA was left unreacted, even when the reaction time was prolonged to 5 hr at the amount of chitosan beads of 0.025 cm^3/cm^3. In addition, a small portion of the quinone derivatives were left in the solution and the solution remained a little colored. The conversion (%) value increased up to 95.8% at 3 hr with almost complete quinone conversion at 5 hr in the presence of chitosan beads at 0.050 cm^3/cm^3 as shown in Figure 8(b). In the presence of chitosan beads at 0.10 cm^3/cm^3, BPA was completely removed for 5 hr through the quinone oxidation and subsequent quinone adsorption on chitosan beads. Here, the concentrations of components related with the quinone oxidation and subsequent quinone adsorption were calculated as a function of the reaction time from the conversion (%) value and the absorbances in the presence of chitosan beads. The concentration of BPA enzymatically converted into quinone derivatives, $[BPA]_{converted}$, corresponding to the concentration of quinone derivatives enzymatically generated, $[Q]_{generated}$, was calculated from the peak area at time t obtained by the HPLC measurements using eq. (2).

$$[BPA]_{converted} = [Q]_{generated} = 0.30 \times \frac{area_0 - area_t}{area_0} \quad (2)$$

In addition, since the solution had the absorbance of 0.827 for the complete conversion of BPA into quinone derivatives at the enzymatic reaction of 5 hr at 300 U/cm^3 as shown in Figure 6, the apparent concentration of remaining quinone derivatives, $[Q]^{app}_{remained}$, at time t was estimated from eq. (3).

$$[Q]^{app}_{remained} = 0.30 \times \frac{Abs_t}{0.827} \quad (3)$$

where Abs_t is the absorbance at time t in the presence of chitosan beads. Since the difference between the concentration of quinone derivatives enzymatically generated and apparent concentration of remaining quinone derivatives, $[Q]_{generated} - [Q]^{app}_{remained}$, results

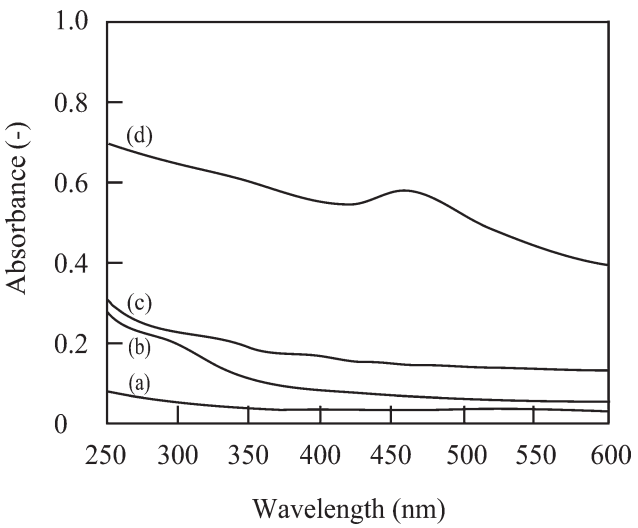


Figure 7 UV-visible spectra of chitosan films incubated in a BPA solution containing H_2O_2 and tyrosinase and in various control solutions. Incubation conditions- (a) unreacted chitosan film (b) cellulose film in BPA with H_2O_2 and tyrosinase (c) chitosan film in BPA with H_2O_2 (d) chitosan film in BPA with H_2O_2 and tyrosinase.

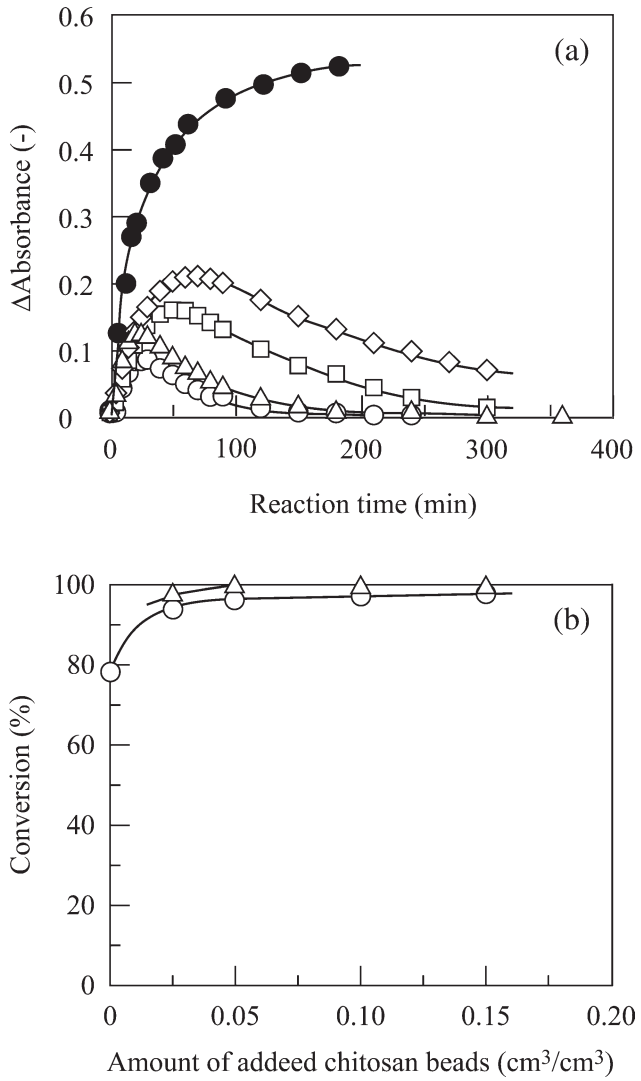


Figure 8 Removal of BPA (0.3 m*M*) through quinone oxidation by tyrosinase (200 U/cm^3) and subsequent quinone adsorption on chitosan beads in the presence of H_2O_2 of 0.3 m*M* at pH 7.0 and 40°C. (a) Amount of added chitosan beads (cm^3/cm^3)- ●: without, ◇: 0.025, □: 0.050, △: 0.10, ○: 0.15. (b) Enzymatic reaction time (hr)- ○: 3, △: 5.

from quinone adsorption on chitosan beads, the % adsorption value can be calculated by

$$\text{Adsorption}(\%) = \frac{[\text{Q}]_{\text{generated}} - [\text{Q}]^{\text{app}}_{\text{remained}}}{[\text{Q}]_{\text{generated}}} \times 100 \quad (4)$$

Finally, the removal (%) value was calculated form both conversion (%) and adsorption (%) values using eq. (5).

$$\text{Removal}(\%) = \frac{\text{conversion}(\%) \times \text{adsorption}(\%)}{100} \quad (5)$$

Figure 9 shows the time course of the concentration of quinone derivatives enzymatically generated, the apparent concentration of remaining quinone derivatives, and the values of conversion (%), adsorption (%), and removal (%). The BPA concentration decreased over the reaction time and BPA was completely converted into quinone derivatives at 5 hr in the presence of chitosan beads at 0.10 cm^3/cm^3. In the range of this reaction time, the removal (%) value also continuously increased. In addition, the initial velocities were calculated from a slope in the concentration of remaining BPA against the enzymatic reaction time in the initial stage in the presence and absence of chitosan beads. The initial velocity was calculated to be 4.32×10^{-3} mM/min in the presence of chitosan beads from Figure 9(a). This value was 2.45 times higher than the initial velocity of 1.77×10^{-3} mM/min in the absence of chitosan beads determined from Figure 2. This indicates that the decrease in the quinone concentration in the reaction solution through quinone adsorption on chitosan beads leads to the increase in the enzymatic quinone oxidation. It was noted that although the apparent concentration of remaining quinone derivatives vanished at 4 hr, removal of BPA was still uncompleted because there remained a small amount of unreacted BPA in the solution. Then, the absorbance disappeared and the complete conversion was reached at 5 hr. This shows complete removal of BPA from the solution. In addition, when the amount of chitosan beads was increased up to 0.15 cm^3/cm^3, the time required to remove BPA completely was shortened to 3 hr. These results mean that an increase in the amount of chitosan beads dispersed in the solution led to a reduction in the time required to remove BPA.[58,62,63,72] Alternately, the BPA concentration was estimated for a BPA solution without tyrosinase but in the presence of chitosan beads (0.10 cm^3/cm^3) at pH 7.0 and 40°C as a control experiment. The BPA concentration gradually decreased probably due to physical adsorption on chitosan beads. Then, even if the reaction time was prolonged to 5 hr, the adsorption (%) was limited to be only 15%.

From the above results, we can note that the combined use of tyrosinase-catalyzed quinone oxidation in the presence of H_2O_2 and quinone adsorption on chitosan beads was a very effective means to remove BPA from solutions. As shown in Figure 8(b), tyrosinase-catalyzed quinone oxidation of BPA was enhanced by increasing the amount of added chitosan beads. In other words, a decrease in the quinone concentration through chemical adsorption on chitosan beads accelerated the enzymatic quinone oxidation of BPA. This means that quinone adsorption on chitosan beads would suppress the unfavorable quinone-related inactivation of tyrosinase such as contact between quinone derivatives and tyrosinase molecules or their active sites.

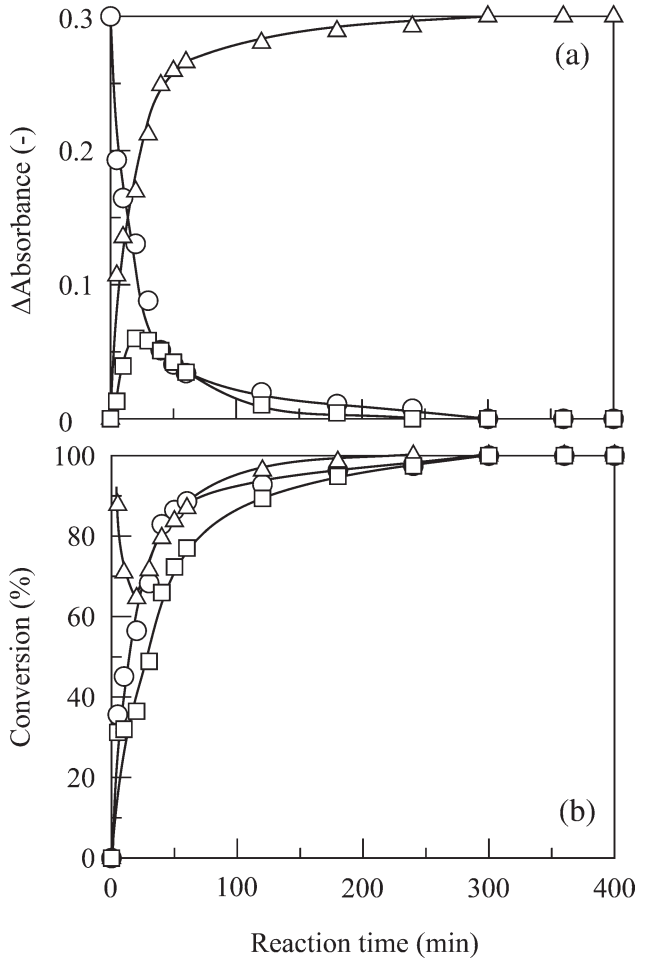


Figure 9 The time course of (a) the concentrations of unreacted BPA (○) and enzymatically generated quinone (△) and apparent concentration of remaining quinone (□) and (b) the value of conversion (%) (○), adsorption (%) (△), and removal (%) (□) for removal of BPA (0.3 m*M*) through tyrosinase-catalyzed quinone oxidation (200 U/cm^3) and subsequent quinone adsorption on chitosan beads (0.10 cm^3/cm^3) in the presence of H_2O_2 of 0.3 m*M* at pH 7.0 and 40°C.

As another control experiment, removal of BPA by adding chitosan solutions was also estimated. Chitosan solutions were added to a BPA solution containing tyrosinase (200 U/cm^3) and H_2O_2 (0.3 m*M*) so as to reach final amino group concentration from 0.2 to 10 m*M*, and then the solutions were moderately stirred for 24 hr at pH 6.0 and 40°C. Here, the pH value was adjusted to 6.0 because chitosan was insoluble in a pH 7.0 buffer.

When tyrosinase was added to a BPA solution containing H_2O_2 without chitosan, the color was developed by enzymatic quinone generation. In this case, even when the stirring was continued for 24 hr, the BPA solution was kept brown and no precipitates were generated. Contrary to this, when both tyrosinase and chitosan were added to a BPA solution containing H_2O_2, water-insoluble aggregates were generated and the solutions were gradually decolorized irrespective of the amino group concentration. The aggregates generated were filtered out with a 5C filter paper, and then the absorbance of the filtrates was measured at 385 nm. As shown in Figure 10(a), the absorbance at 385 nm sharply decreased with an increase in the chitosan's amino group concentration. The decrease in the absorbance indicates that water-soluble quinone derivatives were decreased in solutions. The reaction solution was fairly decolorized by stirring for 24 hr at the amino group concentration of 0.75 m*M*, or [—NH_2]/[BPA] = 2.5. However, when the amino group concentration further increased, the absorbance

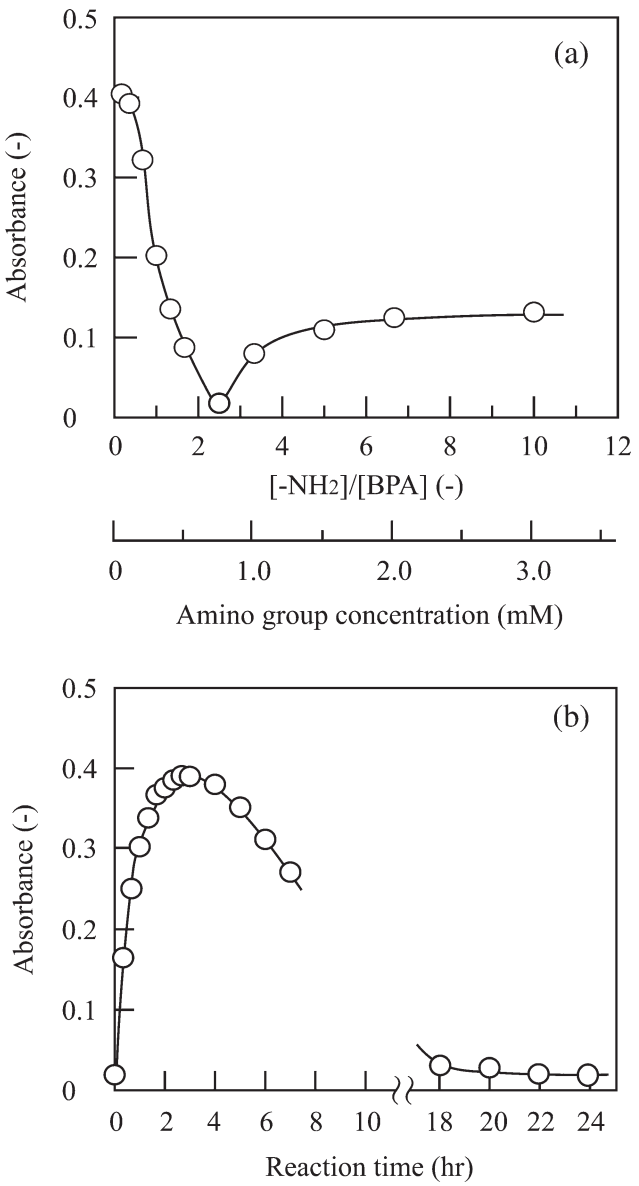


Figure 10 Removal of BPA (0.3 m*M*) through tyrosinase-catalyzed quinone oxidation (200 U/cm^3) and subsequent homogeneous quinone reaction with chitosan in the presence of H_2O_2 of 0.3 m*M* at pH 6.0 and 40°C.

TABLE I
Removal of BPA and Bisphenol Derivatives Through Tyrosinase-Catalyze Quinone Oxidation and Subsequent Quinone Adsorption on Chitosan Beads in the Presence of H_2O_2 of 0.3 m*M* at pH 7.0 and 40°C

Bisphenol derivatives	Initial concentration (m*M*)	Tyrosinase concentration (U/cm^3)	Chitosan beads (cm^3/cm^3)	Conversion (%)	Conversion time (hr)	Adsorption (%)	Adsorption time (hr)	Removal (%)
BPA	0.3	200	0.10	100	5	100	4	100
BPB	0.3	200	0.10	72.4	5	85.0	5	61.5
		200	0.20	78.4	5	92.0	5	72.6
		300	0.20	87.9	5	98.8	3.5	86.8
		400	0.20	96.9	5	100	5	96.9
BPC	0.05	200	0.10	57.9	5	100	5	57.8
		200	0.20	60.8	5	100	2	60.8
		300	0.20	63.1	5	100	5	63.1
BPE	0.3	50	0.10	100	5	98.9	5	98.9
		50	0.20	100	5	100	5.5	100
BPF	0.3	20	0.10	100	2	100	2	100
BPO	0.3	200	0.10	100	1	94.0	5	94.0
		200	0.20	100	1	100	4	100
BPS	0.3	200	0.10	3.4	5	6.0	5	0.2
BPT	0.3	150	0.10	100	2	100	0.25	100
BPZ	0.02	200	0.10	64.3	5	67.8	5	43.4
		200	0.20	66.3	5	80.4	5	53.3
		300	0.20	86.0	5	100	5	86.0

gradually increased and the generation of aggregates decreased because chitosan chains with unreacted free amino groups were soluble in the solutions.[64] Here, it is considered that the presence of H_2O_2 has an insignificant influence on chitosan, for example chain cleavage, because H_2O_2 was consumed by the transformation of tyrosinase into oxy-form as shown in Scheme 1 and a considerable change wasn't observed for a BPA solution containing H_2O_2 and chitosan.

Figure 10(b) showed the time course of the absorbance at $[-NH_2]/[BPA] = 2.5$ where the minimum absorbance was obtained. The absorbance was gradually decreased after passing through the maximum value, which indicates that quinone derivatives reacted with amino groups of chitosan dissolving in the solution. Most of BPA was removed from solution by filtering out water-insoluble aggregates generated, when the solution was stirred for 24 hr. Unfortunately, the absorbance of 0.02 at 24 hr showed that a small amount of quinone derivatives remained in the solution. For the homogeneous reaction with chitosan solutions, it was found that the generation of water-insoluble precipitates was considerably slow beyond our expectation. The absorbance of 0.386 was obtained and no water-insoluble aggregates were generated at the reaction time of 3 hr. On the other hand, BPA was completely removed from solutions at 3–5 hr by the procedure developed in this study as shown in Figure 7, although the time required to remove BPA completely depended on the amount of added chitosan beads. This comparison insists that heterogeneous procedure with chitosan beads constructed in this study is much effective in removing BPA from aqueous solutions.

Removal of bisphenol derivatives

Removal of bisphenol derivatives shown in Figure 1 was carried out to enhance the potential for the procedure with chitosan beads. The results of removal of bisphenol derivatives carried out at pH 7.0 and 40°C as the optimum conditions determined for quinone oxidation of BPA was summarized in Table I. Since BPC and BPZ had lower solubility in a pH 7.0 buffer, the tyrosinase-catalyzed treatment of BPC and BPZ was carried out at the initial concentrations of 0.05 and 0.02 m*M*, respectively. Of seven kinds of bisphenol derivatives used in this study, BPS underwent little tyrosinase-catalyzed quinone oxidation. Therefore, the experiments of removal of other six kinds of bisphenol derivatives were examined by varying the tyrosinase concentration and amount of chitosan beads. BPE, BPF, and BPT were effectively converted into the corresponding quinone derivatives at lower tyrosinase concentrations than BPA.

Since quinone adsorption of quinone derivatives generated from BPE and BPO on chitosan beads was a little low, we tried to remove both bisphenol derivatives by increasing the amount of chitosan beads to 0.20 cm^3/cm^3. On the other hand, either tyrosinase-catalyzed quinone oxidation or quinone adsorption on chitosan beads for BPB, BPC, and BPZ was lower compared with BPA. So, the tyrosinase concentration and amount of chitosan beads were increased so as to enhance the quinone oxidation and quinone adsorption. Consequently, in the presence of

chitosan beads at 0.20 cm^3/cm^3, BPB was almost completely removed at the tyrosinase concentrations of 400 U/cm^3 and the removal (%) value increased up to 98.9% by prolonging the reaction time to 20 hr. Compared with these bisphenol derivatives, the removal (%) value for BPC and BPZ was limited to 63.1 and 86.0%, respectively, even if the tyrosinase concentration was increased to 350 U/cm^3 in the presence of chitosan beads at 0.20 cm^3/cm^3. It was found from the above results that bisphenol derivatives except for BPC, BPS, and BPZ, were completely or effectively removed by increasing either the tyrosinase concentration or the amount of chitosan beads at pH 7.0 and 40°C.

For bisphenol derivatives which had lower removal efficiency or little underwent tyrosinase-catalyzed quinone oxidation, one of the solutions is the estimation of the pH dependence of the tyrosinase-catalyzed quinone oxidation. Alternatively, the usage of tyrosinases derived from different origins, for example *Aspergillus oryzae* tyrosinase, is also advisable because the substrate selectivity of enzymes varies by their origin to a greater or lesser extent.

CONCLUSIONS

In this study, the availability of chitosan was investigated for removal of bisphenol A through the tyrosinase-catalyzed quinone oxidation and subsequent nonenzymatic quinone adsorption on chitosan beads. First, the tyrosinase-catalyzed quinone oxidation of BPA was systematically estimated as a function of the H_2O_2-to-BPA ratio, pH value, temperature, and tyrosinase dose. Tyrosinase-catalyzed quinone oxidation was accelerated by the addition of H_2O_2. and its optimum conditions for BPA at 0.3 m*M* were determined to be the presence of H_2O_2 of 0.3 m*M* at pH 7.0 and 40°C.

When chitosan beads were dispersed in BPA solutions in the presence of tyrosinase and H_2O_2, quinone derivatives enzymatically generated were effectively chemisorbed on chitosan beads. The removal efficiency increased by increasing the amount of chitosan beads added to BPA solutions, and BPA was completely removed in the presence of chitosan beads at 0.10 cm^3/cm^3. The time required to completely remove BPA was shortened by increasing the amount of chitosan beads. It was found from the control homogeneous removal experiments with chitosan solutions that the removal time for the heterogeneous systems with chitosan beads was considerably shortened compared with the homogeneous systems with chitosan solutions. In addition, for the homogeneous systems with chitosan solutions, quinone derivatives enzymatically generated form BPA were a little left in the reaction solutions even at the optimum amino group concentration of 0.75 m*M*, that is, $[-NH_2]/[BPA] = 2.5$. At least the reaction times of 20 to 24 hr were required to effectively remove BPA from solutions because the formation of precipitates through the reaction of quinone derivatives with chitosan's amino groups was rather slow. Contrary to this, BPA was completely removed only for 3 to 5 hr through the tyrosinase-catalyzed quinone oxidation and subsequent nonenzymatic quinone adsorption on chitosan beads. In addition, bisphenol derivatives, which are suspected as endocrine disrupting chemicals, were completely or effectively removed by this procedure, although either the tyrosinase concentration or the amount of added chitosan beads is required to be increased for some bisphenol derivatives that had low removal efficiency. In addition, we can safely say from the above results that an alternative usage of chitosan came out. Our study on removal of BPA provides the results comparable to or better than studies reported previously because alternative separation techniques for removing colored quinone derivatives enzymatically generated from BPA would be operationally complex and would require capital investments. Although we focused on the technical feasibility in this study, the practical application of this procedure may require the immobilization of tyrosinase.

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