Removal of Alkylphenols by the Combined Use of Tyrosinase Immobilized on Ion-Exchange Resins and Chitosan Beads

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ABSTRACT: Mushroom tyrosinase was covalently immobilized on a poly(acrylic acid)-type, weakly acidic cation-exchange resin (Daiaion WK10, Mitsubishi Chemical Corp., Tokyo, Japan) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride salt as a water-soluble carbodiimide. Ion-exchange resins immobilized with tyrosinase were packed in one column, and crosslinked chitosan beads prepared with epichlorohydrin were packed in another column. The enzymatic activity was modified by covalent immobilization, and the immobilized tyrosinase had a high activity in the temperature range of 30–45°C and in the pH range of 7–10. When solutions of various alkylphenols were circulated through the two columns packed with tyrosinase-immobilized ion-exchange resins and crosslinked chitosan beads at 45°C and pH 7 (the optimum conditions determined for *p*-cresol), alkylphenols were effectively removed through quinone oxidation with immobilized tyrosinase and subsequent quinone adsorption on chitosan beads. The use of chemically crosslinked chitosan beads in place of commercially available chitosan beads was effective in removing alkylphenols from aqueous solutions in shorter treatment times. The removal efficiency increased with an increase in the amount of crosslinked chitosan beads packed in the column because the rate of quinone adsorption became higher than the rate of enzymatic quinone generation. The activity of tyrosinase was iteratively used by covalent immobilization on ion-exchange resins. One of the most important findings obtained in this study is the fact that linear and branched alkylphenols suspected of weak endocrine-disrupting effects were effectively removed from aqueous solutions. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 115: 137–145, 2010

Key words: adsorption; biopolymers; crosslinking; enzymes

INTRODUCTION

Nowadays, water pollution is one of the environmental problems facing our societies. Persistent chemicals discharged into surface water can have adverse influences on human life and aquatic animals and plants. Chemical treatments such as ozone oxidation,[1,2] UV irradiation,[3,4] and adsorption on activated carbon[5–7] have been used for the treatment of effluents containing persistent chemicals. However, there are problems to be solved, such as insufficient decreases in dissolved organic carbon, large-scale equipment, and high expenses. Alkylphenols are some of the organic pollutants detected in the environment. Discharge of alkylphenols into surface water can be responsible for unpleasant odors due to the formation of chlorophenols through a reaction with residual chlorine in water.[8,9]

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Recently, many studies have been extensively carried out on the construction of sensing systems,[10,11] quantification,[12] and degradation and detoxification of various phenolic compounds[13–17] through the use of oxidoreductases such as tyrosinases[10,11,13–15] and peroxidases.[12,16,17] Among them, mushroom tyrosinase (EC 1.14.18.1) has been widely used for the treatment of alkylphenols and chlorophenols. Payne and coworkers[18–20] discovered a process for removing alkylphenols through tyrosinase-catalyzed quinone oxidation and a subsequent reaction with chitosan. Alternatively, Wada et al.[21] reported that amino-containing polymers such as chitosan and polyethylenimine could be used as coagulants to remove enzymatically generated quinone derivatives from aqueous solutions. These investigations showed that the combined use of tyrosinase and amino-containing polymers is an effective procedure for removing alkylphenols and chlorophenols from aqueous solutions. However, one of the major problems is that there is an optimum concentration range of each coagulant for maximum flocculation. When the coagulant dosage is less or more than the optimum concentration range, flocculation does not occur. In

addition, we have reported the usefulness of chitosan in the form of beads for tyrosinase-catalyzed removal of alkylphenols. A variety of alkylphenols can be effectively removed through the nonenzymatic adsorption of quinone derivatives on chitosan beads.[13,14] In this technique, quinone adsorption increases with an increase in the amount of chitosan beads dispersed in the reaction solutions, and a high removal efficiency can be obtained in shorter reaction times.

However, because tyrosinase dissolved in solutions was used in these investigations, the isolation of dissolved enzymes from the solutions after the enzymatic treatment of target pollutants was complicated, and it was difficult to obtain them without a considerable reduction of the activity. Therefore, enzyme immobilization has been brought up as a way to solve these problems.[22–24] The methods of enzyme immobilization to an insoluble carrier may be broadly classified into the following three groups: (1) covalent binding to a carrier, (2) ionic binding and physical adsorption to a carrier, and (3) entrapment in an insoluble crosslinked matrix.[25,26] Among them, the physical adsorption and entrapment methods are frequently used for the immobilization of enzymes because of the ease of the procedures. However, enzymes immobilized by these methods are more likely to be gradually released from the polymer matrix. On the other hand, the methods of binding immobilization of enzymes mainly include covalent binding or ionic binding. In this study, covalent binding was chosen because the binding between an enzyme and a carrier is rigid and leakage of the enzyme can be avoided to a great extent.[27–30]

We have to choose a support suitable for covalent immobilization of enzymes. In general, such a support should display the following properties: a hydrophilic nature, an appropriate concentration of reactive functional groups, resistance to biodegradation, and chemical and mechanical stability.[31–33] In addition, the covalent binding of enzymes on water-insoluble supports is required to be carried out under mild conditions to avoid considerable inactivation of the enzymes. Covalent immobilization involves the reaction between functional groups present in the polymeric supports and the functional ones in the enzymes.[34–38] Therefore, we focused our attention on the use of ion-exchange resins because they possess enough mechanical strength and hydrophilicity to immobilize enzymes and are not susceptible to microbial attack.

In this study, a weakly acidic cationic-exchange resin of poly(acrylic acid) (PAA) was chosen because carboxylic groups affixed to the PAA chains can react with amino groups in tyrosinase by the use of a water-soluble carbodiimide under mild conditions, resulting in stable peptide structures.[34,39–44] Process parameters such as the immobilization time, the amounts of tyrosinase-immobilized ion-exchange resins and chitosan beads packed in the column, and the temperature and pH value in removal experiments were systematically investigated to determine the optimum conditions for the removal of *p*-cresol from aqueous solutions with tyrosinase-immobilized resins and chitosan beads. In addition, to estimate the reusability of tyrosinase immobilized on ion-exchange resins, this procedure was applied to the removal of linear and branched alkylphenols suspected of weak endocrine-disrupting effects.

EXPERIMENTAL

Chemicals

Mushroom tyrosinase was purchased from Sigma Chemical (St. Louis, MO), and the specific activity of the tyrosinase was 2590 U/mg of solid (the activity was determined by the supplier). A PAA-type, weakly acidic cation-exchange resin (Daiaion WK10) from Mitsubishi Chemical Corp. (Tokyo, Japan; particle size = 300–1180 μm, ion-exchange capacity = 2.5 mequiv/cm^3) was used as a water-insoluble support for the immobilization of tyrosinase.[45] Sodium tripolyphosphate (TPP; Wako Pure Chemicals, Tokyo, Japan) was used for the protection of amino groups of chitosan in the preparation of chemically crosslinked chitosans. A water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride salt (EDC·HCl), was used as a coupling agent for the covalent immobilization and purchased from Tokyo Kasei Kogyo (Tokyo, Japan).[34,42,46] A porous chitosan bead (Chitopearl AL-01) from Fuji Spinning Co., Ltd. (Tokyo, Japan; particle size = 70–200 μm, specific surface area = 70–100 m^2/cm^3, water content = 92.5%), was used as an adsorbent and stored in a buffer of the same pH value as those of buffers used in the removal experiments described later. Chemically crosslinked chitosan beads were prepared from chitosan 500 (Wako Pure Chemicals) with epichlorohydrin (ECH) as a crosslinker.[47,48]

The alkylphenols used in this study were *p*-cresol, 4-isopropylphenol (4IProP), 4-*sec*-butylphenol (4SBP), 4-*tert*-butylphenol (4TBP), 4-*tert*-pentylphenol (4TPenP), 4-*n*-octylphenol (4NOP), and 4-*n*-nonylphenol (4NNP), and they were purchased from Wako Pure Chemicals and Tokyo Kasei Kogyo. All alkylphenols except for *p*-cresol and 4IProP are said to have a weak estrogenic effect.[9]

Immobilization of tyrosinase

Phosphate buffers (ionic strength = 0.01*M*) were prepared over the pH values of 4–10 (HCl/KH_2PO_4

for pH 4 and $NaOH/KH_2PO_4$ for pHs 5–10). EDC·HCl (57.51 mg, 0.300 mmol) was dissolved in a pH 7.0 buffer (ionic strength = 0.01*M*, volume = 0.9 cm^3). Tyrosinase (1.930 mg, 5000 U) was dissolved in a pH 7.0 buffer (2.5 cm^3). A tyrosinase solution was added to ion-exchange resins (0.50 cm^3) stored in a buffer in advance, and then an EDC solution was added dropwise to the tyrosinase solution containing ion-exchange resins over 45 min.[45] Then, the mixture solutions were mildly stirred for prescribed times at 4°C to immobilize tyrosinase on the ion-exchange resins. After the reaction, the tyrosinase-immobilized ion-exchange resins were thoroughly washed with a pH 7.0 buffer to remove unreacted components and then stored in a buffer of the same pH value as that of the buffer used in the experiments studying the removal of alkylphenols at 4°C.

Determination of the amount of immobilized tyrosinase

An EDC solution (0.90 cm^3) at 0.333 mmol/cm^3 and a pH 7.0 buffer (0.366 cm^3) equivalent to the amount of water contained in equilibrium-swollen ion-exchange resins (0.50 cm^3) were added to tyrosinase solutions (2.5 cm^3, 250–2000 U/cm^3) prepared in a pH 7.0 buffer, and then the mixture solutions were mildly stirred for a prescribed time at 4°C. The amount of immobilized tyrosinase was determined with a bicinchoninic acid protein assay kit (BCA-1KT, Sigma Chemical). To tyrosinase solutions (0.5 cm^3) of different concentrations containing EDC at 0.333 mmol/cm^3, CuSO4(II) (0.4 cm^3) and bicinchoninic acid (4.0 cm^3) were added in test tubes. After color development by mild stirring of the solutions at 37°C for 30 min, the absorbance was measured at 562 nm.

After immobilization, each aliquot (0.5 cm^3) taken from the reaction solutions was color-developed in the same manner described previously. The concentration of tyrosinase was determined from a calibration curve relating the absorbance at 562 nm to the tyrosinase concentration prepared beforehand. The amount of tyrosinase immobilized on ion-exchange resins was determined from the concentration difference before and after immobilization.

Preparation of chemically crosslinked chitosan beads

A chitosan solution (1 wt/vol %) was prepared by the addition of 1.0 g of chitosan 500 flakes to 100 cm^3 of an aqueous acetic solution at 2.0 wt %. After most of the added chitosan flakes were dissolved, the solution was vacuum-filtered with a G3 glass filter to remove the insoluble parts. Chitosan beads were prepared by the dropwise addition of a chitosan solution (20 cm^3) to a TPP solution (1.0 wt %, 200 cm^3) with a burette.[49] Then, the prepared chitosan beads were dipped in an ECH solution (0.16*M*) in a 1.0*M* NaOH solution (100 cm^3). The crosslinking reaction was carried out by slow stirring of the reaction solution for 24 h at 75°C.[47,48] The chemically crosslinked chitosan beads were washed with HCl at pH 3.0 and with NaOH at pH 11.0 alternately to remove TPP and unreacted components and then stored in a pH 7.0 buffer at 4°C.

Removal of alkylphenols

Two glass columns, 5 mm in diameter and 12 cm long, were used for the experiments studying the removal of *p*-cresol with tyrosinase-immobilized ion-exchange resins and commercially available chitosan beads (Chitopearal AL-01). Tyrosinase-immobilized ion-exchange resins (0.5 cm^3) were packed in one column, and commercially available chitosan beads were packed in the other column. A pH 7.0 buffer was passed through the tandem arranged two columns. A *p*-cresol solution (0.5 m*M*) was prepared in a pH 7.0 buffer and flowed into the column packed with tyrosinase-immobilized resins. The eluate from the packed column flowed into the column packed with chitosan beads. Subsequently, the solutions were circulated through the two columns with tyrosinase-immobilized resins with a circulating pump. The circulation was carried out five times in all.

In addition, chemically crosslinked chitosan beads, in place of commercially available chitosan beads, were packed in a column 8 mm in diameter, and the removal experiments were carried out in the same manner described previously, as shown in Figure 1. After the optimum process parameters such as the pH value and temperature were determined for the removal of *p*-cresol as a model phenol compound, the optimum conditions determined were applied to the removal of different alkylphenols.

Quantitative assay of alkylphenols

The concentration of the remaining alkylphenols was determined with a Hitachi (Tokyo, Japan) L-7000 high-performance liquid chromatograph combined with a spectrophotometer and an integrator (L-7420, Hitachi). An aliquot (0.2 cm^3) taken from the eluate was filtered with a USY-1 positive-pressure-type ultrafilter unit (Advantec, Tokyo, Japan). A reverse-phase column (Inertsil ODS-3; 5 μm, 4.6-mm i.d. × 150 mm) was used. Aqueous acetonitrile solutions as the mobile phase were injected at a flow rate of 1.0 cm^3/min. The volume composition of the aqueous acetonitrile solutions and the wavelength of

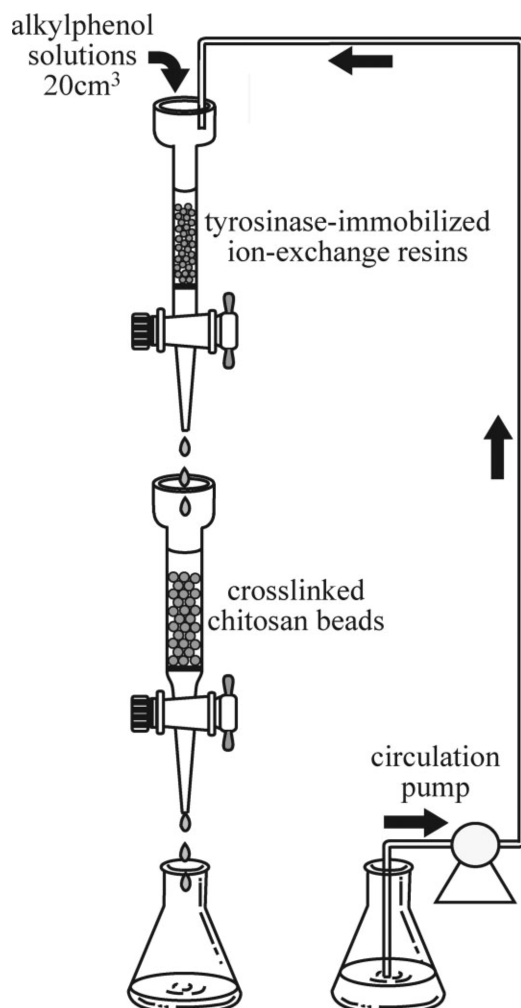

Figure 1 Schematic illustration of the experiments used to study the removal of alkylphenols with two columns packed with tyrosinase-immobilized ion-exchange resins and crosslinked chitosan beads.

the spectrophotometer depended on the types of alkylphenols used.

The conversion (%) values were calculated from the alkylphenol concentration remaining after circulation (C_n) and the initial concentration of alkylphenols used (C_0):

$$\text{Conversion}(\%) = \frac{C_0 - C_n}{C_0} \times 100 \quad (1)$$

where subscript n denotes the number of circulations of alkylphenol solutions through two columns packed with tyrosinase-immobilized ion-exchange resins and chitosan beads.

The absorbance of the eluate from the column packed with chitosan beads was measured at 400 nm after each circulation. The absorbance at 400 nm stood at 0.620 when a *p*-cresol solution (0.5 m*M*) underwent complete quinone oxidation by native tyrosinase at pH 7.0. Therefore, the adsorption of quinone derivatives generated by tyrosinase immobilized on chitosan beads was estimated for *p*-cresol:

$$\text{Adsorption}(\%) = \frac{0.620 \times 0.020 - \text{Abs}_t \times V^n_{\text{eluent}}}{0.620 \times 0.020} \times 100 \quad (2)$$

where $V^{\text{n}}_{\text{eluent}}$ and Abs_t denote the volume of eluate taken from the column packed with chitosan beads after each circulation and the absorbance of the eluate, respectively. The quantity of 0.020 was the volume (dm^3) of the *p*-cresol solution that flowed into the column packed with tyrosinase-immobilized ion-exchange resins.

In addition, the removal (%) values were obtained as follows:

$$\text{Removal}(\%) = \frac{\text{Conversion}(\%) \times \text{Adsorption}(\%)}{100} \quad (3)$$

RESULTS AND DISCUSSION

Immobilization of tyrosinase

The covalent immobilization of tyrosinase on ion-exchange resins was carried out with EDC at pH 7.0 as the optimum pH value for the native tyrosinase used.[13,14] As shown in Figure 2, the amount of immobilized tyrosinase increased in the range of immobilization times below 24 h and then leveled off with further increased immobilization times. Tyrosinase-immobilized ion-exchange resins (0.5 cm^3) prepared for different immobilization times and commercially available chitosan beads (4.0 cm^3) were packed in the two columns. Here, a *p*-cresol solution was circulated in the two columns three times for a single removal experiment. Figure 3

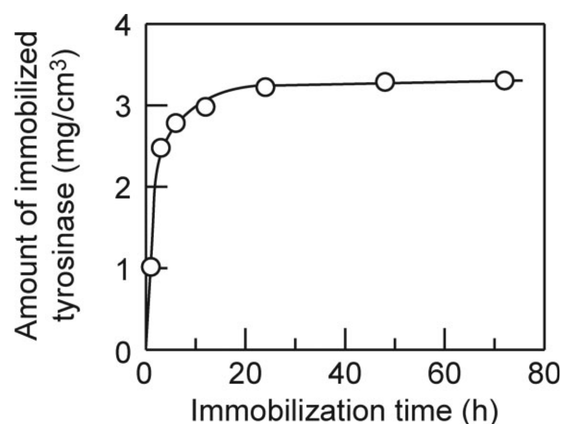

Figure 2 Change in the amount of tyrosinase immobilized on an ion-exchange resin (Daiaion WK10) with the immobilization time at 4°C.

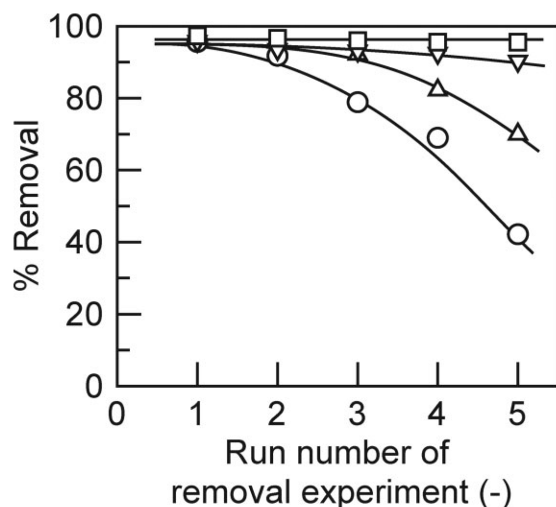

Figure 3 Effect of the immobilization time on the removal efficiency of *p*-cresol at pH 7.0 and 45°C. The immobilization times were (○) 1, (△) 6, (▽) 24, and (□) 72 h. The amount of packed commercially available chitosan beads was 4.0 cm^3.

shows the reusability of tyrosinase-immobilized ion-exchange resins prepared for different immobilization times in a pH 7.0 buffer at 4°C for *p*-cresol removal. When tyrosinase-immobilized ion-exchange resins prepared for the immobilization time of 72 h were used, *p*-cresol effectively underwent tyrosinase-catalyzed quinone oxidation, and the generated quinone was adsorbed onto the chitosan beads. As a result, *p*-cresol was almost completely removed from the aqueous solution. Here, the chitosan beads packed in the column were stained vermilion red because of quinone tanning. This indicated that quinone derivatives, which were generated by the enzymatic activity of immobilized tyrosinase, were chemically bound to the chitosan beads. Such a color development, called quinone tanning, has also been observed for the chemisorption of quinone derivatives generated from different phenol compounds in other studies.[3,40,41] A *p*-cresol solution (20 cm^3) passed through the two tandem arranged columns for 28–30 min, and it took 85–90 min to circulate a *p*-cresol solution three times in a single removal experiment. In addition, even when the removal experiments were repeated five times, removal values greater than 95% were obtained in each removal experiment. This indicates that the enzymatic activity of tyrosinase was iteratively used by covalent immobilization on ion-exchange resins.

On the other hand, when tyrosinase-immobilized ion-exchange resins prepared for shorter immobilization times were used, the removal (%) value gradually decreased against the number of the removal experiments. Immobilization for shorter times resulted in a considerable decrease in the removal (%) value, probably because of the thermal denaturation of immobilized tyrosinase or the allosteric effect by quinone derivatives enzymatically generated.

The prolongation of the immobilization time was effective in reducing deterioration in the removal efficiency for repeated use. The increase in the stability of immobilized tyrosinase was not considerably due to an increase in the immobilized amount because the amount of immobilized tyrosinase stayed almost constant in the range of immobilization times greater than 24 h, as shown in Figure 2. Therefore, we can conclude that the stability of tyrosinase was enhanced by multipoint attachment between tyrosinase molecules and PAA chains.[34,46]

Use of commercially available chitosan beads

In this section, a constant amount of tyrosinase-immobilized ion-exchange resins prepared for the immobilization time of 72 h (0.5 cm^3) was packed in one column, and the amount of commercially available chitosan beads packed in the other column was varied. Figure 4 shows the effect of the amount of packed chitosan beads on the removal (%) value for the consecutive removal of *p*-cresol at pH 7.0 and 45°C. The removal (%) value gradually decreased when the removal experiments were repeatedly carried out with 1.0 cm^3 of packed commercially available chitosan beads. In this case, because some of the quinone derivatives generated by immobilized tyrosinase remained unadsorbed on the chitosan beads, the solution containing quinone derivatives flowed into the column packed with tyrosinase-immobilized ion-exchange resins. Therefore, the

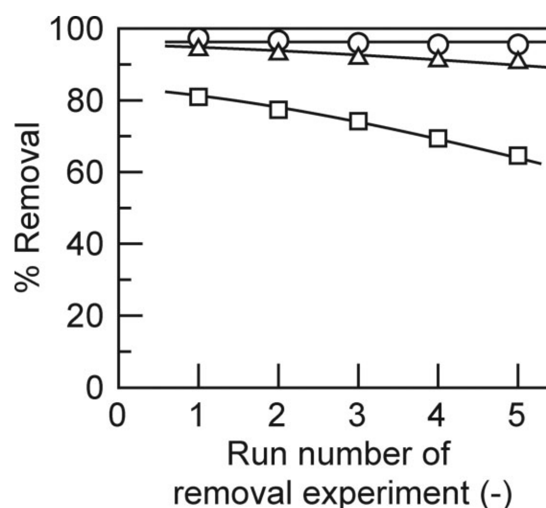

Figure 4 Effect of the amount of commercially available chitosan beads (AL-01) on the removal efficiency of *p*-cresol at pH 7.0 and 45°C. The amounts of packed commercially available chitosan beads were (□) 1.0, (△) 2.0, and (○) 4.0 cm^3. The concentration of immobilized tyrosinase was 3.13 mg/cm^3.

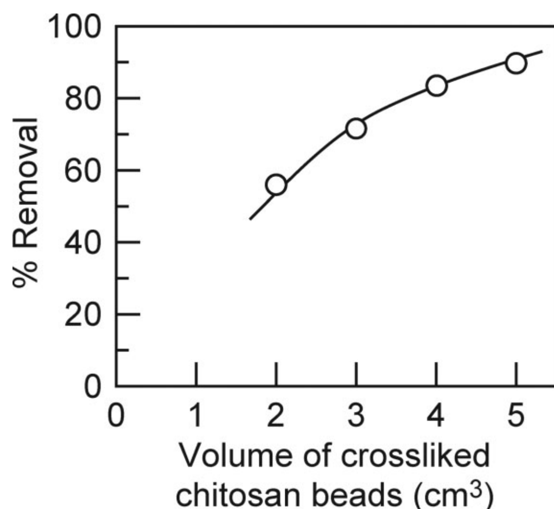

Figure 5 Effect of the amount of chemically crosslinked chitosan beads on the removal efficiency of *p*-cresol at pH 7.0 and 45°C. The average concentration of immobilized tyrosinase was 3.06 mg/cm^3.

enzymatic activity of immobilized tyrosinase went down because of the allosteric effect. In addition, a decrease in the activity of immobilized tyrosinase was suppressed by an increase in the amount of packed commercially available chitosan beads because quinone adsorption on the chitosan beads was enhanced. When the amount of packed chitosan beads was increased to 4.0 cm^3, *p*-cresol was almost completely removed by the circulation in the two columns. This result indicates that at least 4.0 cm^3 of chitosan beads was required to adsorb quinone derivatives generated by immobilized tyrosinase, by which the decrease in the enzymatic activity of immobilized tyrosinase was effectively depressed. However, a slow passage of a *p*-cresol solution through the column packed with commercially available chitosan beads would result in an increase in the contact of enzymatically generated quinone derivatives with tyrosinase-immobilized ion-exchange resins; consequently, the enzymatic activity of immobilized tyrosinase could be reduced. Therefore, chemically crosslinked chitosan beads larger than the commercially available chitosan beads were prepared to increase the flow rate of a *p*-cresol solution through the columns in the next section.

Use of chemically crosslinked chitosan beads

Amount of chemically crosslinked chitosan beads

Because quinone derivatives generated from phenol compounds react with amino groups through either a Schiff base or Michael-type addition reaction,[20,50–52] amino groups of chitosan were protected with TPP, and chitosan was chemically crosslinked with ECH between —CH_2OH or —OH groups. The chitosan beads prepared in this study had a diameter of 2.0–2.5 mm. Figure 5 shows the effect of the amount of crosslinked chitosan beads on the removal (%) value of *p*-cresol when a constant amount of tyrosinase-immobilized ion-exchange resins (0.5 cm^3) was packed. The removal efficiency increased with an increase in the amount of crosslinked chitosan beads packed in the column, and removal values greater than 90% were obtained with 5.0 cm^3 of crosslinked chitosan beads. Because the time for a *p*-cresol solution to pass through the two columns was shortened to approximately 16 min, it took 80 min for a single removal experiment consisting of five consecutive circulations.

pH and temperature dependence

The effects of the temperature and pH value on the removal of *p*-cresol were investigated with tyrosinase-immobilized ion-exchange resins and crosslinked chitosan beads. Figure 6(a) shows the effect of the temperature on the removal efficiency with 5.0 cm^3 of packed crosslinked chitosan beads and 0.5 cm^3 of tyrosinase-immobilized ion-exchange resins (immobilized amount = 2.81 mg/cm^3) in a pH 7.0 buffer. A high removal value was obtained in the temperature range below 45°C when the tyrosinase-immobilized ion-exchange resins were repetitively used at pH 7.0 as the optimum pH value for native tyrosinase. However, the removal (%) value gradually decreased for the fifth removal experiment at 50°C. In addition, at 60°C, the removal (%) value gradually decreased with the repetition of the removal experiments. This indicates that immobilized tyrosinase is susceptible to thermal denaturation at 60°C. On the other hand, as shown in Figure 6(b), immobilized tyrosinase was repeatedly used without a considerable decrease in the enzymatic activity in the pH range of 7–9 at 45°C (immobilized amount = 2.98 mg/cm^3). These results reveal that little leakage of tyrosinase covalently immobilized on ion-exchange resins occurred for the repeated-activity measurements and washings between the activity measurements. However, when the pH value deviated from this pH range, the removal efficiency decreased. The enzymatic activity of immobilized tyrosinase would be influenced by the ionization behavior of remaining negatively or positively chargeable functional groups of tyrosinase molecules. However, it was found from the aforementioned results that immobilized tyrosinase was reusable in the pH range of 7.0–9.0 below 50°C. The aforementioned results indicate that the thermostability and pH stability of tyrosinase is modified by covalent immobilization.

Consequently, the optimum conditions were determined to be pH 7.0 and 45°C for the removal of *p*-cresol with tyrosinase-immobilized ion-exchange

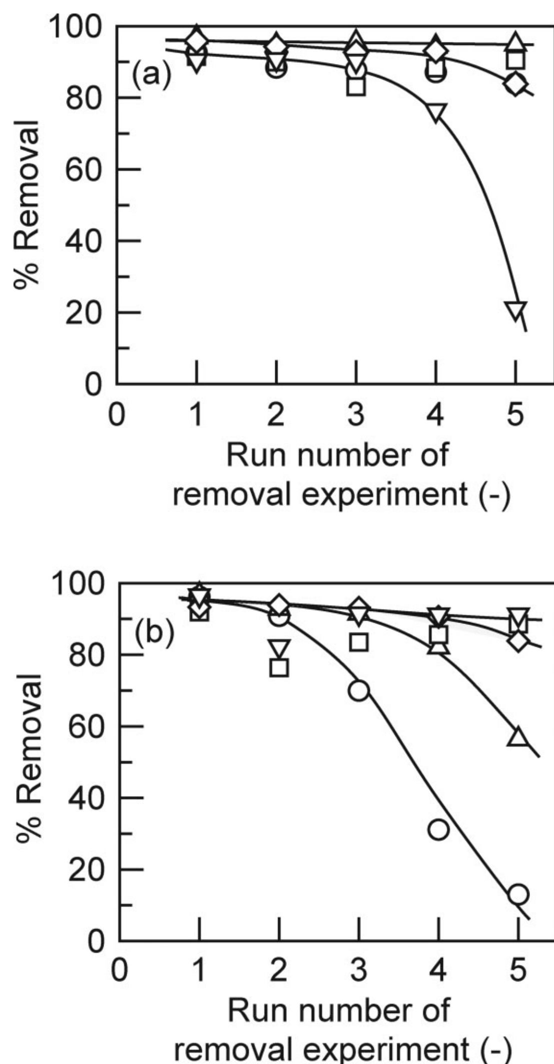

Figure 6 Effects of the (a) temperature at pH 7.0 and (b) pH value at 45°C on the removal efficiency of *p*-cresol. (a) The temperatures were (○) 20, (△) 40, (□) 45, (◇) 50, and (▽) 60°C. (b) The pH values were (○) 5.0, (△) 6.0, (□) 7.0, (◇) 8.0, and (▽) 9.0. The average concentrations of immobilized tyrosinase were (a) 2.81 and (b) 2.98 mg/cm^3.

resins and crosslinked chitosan beads. Quinone oxidation by immobilized tyrosinase was estimated under the optimum conditions. It was found from the high-performance liquid chromatography measurements that when a *p*-cresol solution at 0.5 m*M* flowed into the column packed with 0.5 cm^3 of tyrosinase-immobilized ion-exchange resins (immobilized amount = 3.44 mg/cm^3), the *p*-cresol concentration in the elute from the tyrosinase-immobilized ion-exchange resins decreased to 0.35 m*M*. Subsequently, when the eluate flowed into the column packed with tyrosinase-immobilized ion-exchange resins, the *p*-cresol concentration went down to 0.20 m*M*. The *p*-cresol concentration was gradually reduced by the repetition of circulations. During the circulation, the flow rate was kept almost constant at 1.0–1.2 cm^3/min. For the experiments of *p*-cresol removal, the absorbance at 400 nm of the eluate was 0.022 after the fifth consecutive circulation in the two columns. This indicates that most of the quinone derivatives generated by immobilized tyrosinase were removed by adsorption on crosslinked chitosan beads. Therefore, it is thought that the adsorption of enzymatically generated quinone derivatives is an indispensable procedure in depressing the decline of the enzymatic activity of immobilized tyrosinase.

Removal of alkylphenols

The removal experiments with linear and branched alkylphenols suspected of weak endocrine-disrupting chemicals were carried out at pH 7.0 and 45°C as the optimum conditions determined for the removal of *p*-cresol. The removal of different linear and branched alkylphenols is summarized in Table I. The enzymatic quinone oxidation of 4IProP and 4SBP was slower than that of *p*-cresol. We have reported previously[16,17] that the activity of native tyrosinase toward branched alkylphenols is lower than that toward linear alkylphenols. Therefore, in this study, the quinone conversion of 4IProp and 4SBP was enhanced by the amount of packed tyrosinase-immobilized ion-exchange resins being increased to 1.0 cm^3, and removal values of 95–97% were obtained. 4TBP and 4TPenP underwent no tyrosinase-catalyzed quinone oxidation.[53] Therefore, when the 4TBP and 4TPenP solutions containing H_2O_2 (0.5 m*M*) flowed into the column packed with tyrosinase-immobilized ion-exchange resins, 4TBP and 4TPenP were enzymatically converted into the corresponding quinones. Six kinds of linear and branched alkylphenols (shown in Table I) were effectively removed by circulation of the columns packed with tyrosinase-immobilized ion-exchange resins and crosslinked chitosan beads. 4NOP and 4NNP were also removed by this procedure. In addition, Table I shows that immobilized tyrosinase was repeatedly usable in removing these alkylphenols.

CONCLUSIONS

In this study, process parameters such as the immobilization time, the amount of tyrosinase-immobilized ion-exchange resins packed in the column, the pH value and temperature of the removal experiments, and the amount of chitosan beads packed in the column were systematically investigated for removing *p*-cresol as a model phenol compound from aqueous solutions, and the optimum conditions determined for *p*-cresol were applied to various linear and branched alkylphenols suspected of endocrine-disrupting chemicals.

TABLE I
Removal Efficiency of Different Alkylphenols Through the Combined Use of Tyrosinase Immobilized on Ion-Exchange Resins and Chemically Crosslinked Chitosan Beads at pH 7.0 and 45°C

Alkylphenol	Concentration of alkylphenol (m*M*)	Concentration of H_2O_2 (m*M*)	Amount of immobilized tyrosinase (mg/cm^3)	Volume of tyrosinase-immobilized ion-exchange resin (cm^3)	Circulation	Conversion (%)	Removal (%)				
							1st	2nd	3rd	4th	5th
p-Cresol	0.5		3.44	0.5	5	100	97.6	97.9	96.0	97.1	97.6
4IProP	0.5		3.46	1.0	5	97.1	97.0	96.5	95.4	95.2	95.2
4SBP	0.5		3.32	1.0	5	95.7	96.0	96.7	95.1	96.9	95.8
4TBP	0.5	0.5	3.37	1.0	5	96.0	93.7	95.5	90.7	91.3	95.9
4TPenP	0.5	0.5	3.28	1.0	5	98.3	97.8	97.5	97.5	97.9	96.3
4NOP	0.05		3.60	0.5	2	100	99.2	99.4	99.4	100	100
4NNP	0.05		3.64	0.5	2	100	99.4	100	100	100	100

Mushroom tyrosinase was covalently immobilized onto a PAA-type, weakly acidic cation-exchange resin (Daiaion WK10) with EDC·HCl as a water-soluble carbodiimide. In addition, chitosan beads were prepared via crosslinking with ECH as an alternative to commercially available chitosan beads. Ion-exchange resins immobilized with tyrosinase were packed in one column, and chemically cross-linked chitosan beads prepared with ECH were packed in another column. The enzymatic activity of tyrosinase was modified by the covalent immobilization, and immobilized tyrosinase had a high activity in the temperature range of 30–45°C and the pH range of 7–9. When a *p*-cresol solution was circulated through the two columns packed with tyrosinase-immobilized ion-exchange resins and crosslinked chitosan beads at 45°C and pH 7.0, *p*-cresol was effectively removed by quinone oxidation with immobilized tyrosinase and subsequent quinone adsorption on chitosan beads. The use of chemically crosslinked chitosan beads in place of commercially available chitosan beads was effective in removing alkylphenols from the aqueous solutions because the circulation time was shortened. The removal efficiency increased with an increase in the amount of crosslinked chitosan beads packed in the column because the rate of quinone adsorption became higher than the rate of enzymatic quinone generation. The activity of tyrosinase was iteratively used by covalent immobilization on ion-exchange resins. In addition, linear and branched alkylphenols suspected of weak endocrine-disrupting effects were removed by this procedure. Among them, 4TBP and 4TPenP were also effectively removed in the presence of H_2O_2. One of the most important findings obtained in this study is the fact that the tyrosinase-immobilized ion-exchange resins were repeatedly used for quinone oxidation of various alkylphenols, and linear and branched alkylphenols suspected of weak endocrine-disrupting effects were effectively removed by this procedure.

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