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Removal of *p*-Alkylphenols from Aqueous Solutions by Combined Use of Mushroom Tyrosinase and Chitosan Beads

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Enzymatic removal of *p*-alkylphenols from aqueous solutions was investigated through the two-step approach, the quinone conversion of *p*-alkylphenols with mushroom tyrosinase (EC 1.14.18.1) and the subsequent adsorption of quinone derivatives enzymatically generated on chitosan beads at pH 7.0 and 45 °C as the optimum conditions. This technique is quite effective for removal of various *p*-alkylphenols from an aqueous solution. The % removal values of 97–100% were obtained for *p*-*n*-alkylphenols with carbon chain lengths of 5 to 9. In addition, removal of other *p*-alkylphenols was enhanced by increasing either the tyrosinase concentration or the amount of added chitosan beads, and their % removal values reached >93 except for 4-*tert*-pentylphenol. This technique was also applicable to remove 4-*n*-octylphenol (4NOP) and 4-*n*-nonylphenol (4NNP) as suspected endocrine disrupting chemicals. The reaction of quinone derivatives enzymatically generated with the chitosan's amino groups was confirmed by the appearance of peaks for UV–visible spectrum measurements of the chitosan films incubated in the *p*-alkylphenol and tyrosinase mixture solutions. In addition, 4-*tert*-pentylphenol underwent tyrosinase-catalyzed oxidation in the presence of hydrogen peroxide.

Key words: mushroom tyrosinase; alkylphenol; chitosan; quinone formation; adsorption

In recent years, there has been a growing concern that some chemicals, known as endocrine disrupting chemicals, may have adverse effects on humans and wildlife.[1,2] If endocrine disrupting chemicals are released into the environment, they may bioaccumulate and become biomagnified in the food chain. In addition, they have been confirmed to disturb sexual characteristics in wildlife populations such as fish and amphibians[3,4] and may be responsible for increases in cancer. Alkylphenols have received considerable attention due to high production and widespread usage. They are concentrated by organisms such as fish and birds, leading to contamination in their internal organs between 10 and several hundred times greater than in the surrounding environment.[5] In addition, when alkylphenol ethoxylates, which are widely used in manufacturing surfactants and antioxidants in the plastics industry, are discharged to wastewater treatment facilities or released into the environment, primary degradation of them generates alkylphenols as well as shorter-chain alkylphenol ethoxylates. Further transformation produces various intermediates such as alkylphenols, alkylphenoxy ethoxy acetic acid, and alkyl phenoxy acetic acid.[5,6] Alkylphenols have similar endocrine disrupting activities due to a phenyl ring in their chemical structures.[7,8] It has been reported that biodegradation of alkylphenol ethoxylates and alkylphenols stimulates production of biomarkers of estrogenic activity in male fish.[5,9,10]

Many researchers have a great interest in oxidoreductases such as tyrosinase,[11–15] laccase,[16–18] and peroxidase,[19–23] because their industrial potentials are recognized in degradation and detoxification of recalcitrant environmental pollutions. The potential advantages of the enzymatic treatment include operation at low concentrations over wide pH and temperature ranges and the easy control process. Tyrosinase is most used in treating wastewater containing phenolic contaminants and related compounds.[11–15] Tyrosinase is a copper-containing monooxygenase widely distributed in nature and is responsible for melanization in animals and browning in plants. It catalyzes two distinct reactions: the hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the oxidation of *o*-diphenols to *o*-quinones (catecholase activity), which undergo a non-enzymatic polymerization.[11]

In addition, many studies have been done on the enzymatic oxidation of phenol compounds by tyrosinase to the corresponding *o*-quinone derivatives in addition to the kinetic mechanism of action of tyrosinase,[24–26] but, little has been reported on the removal of phenol compounds. In addition, the successful use of cationic polymers such as chitosan and polyethylenimine to

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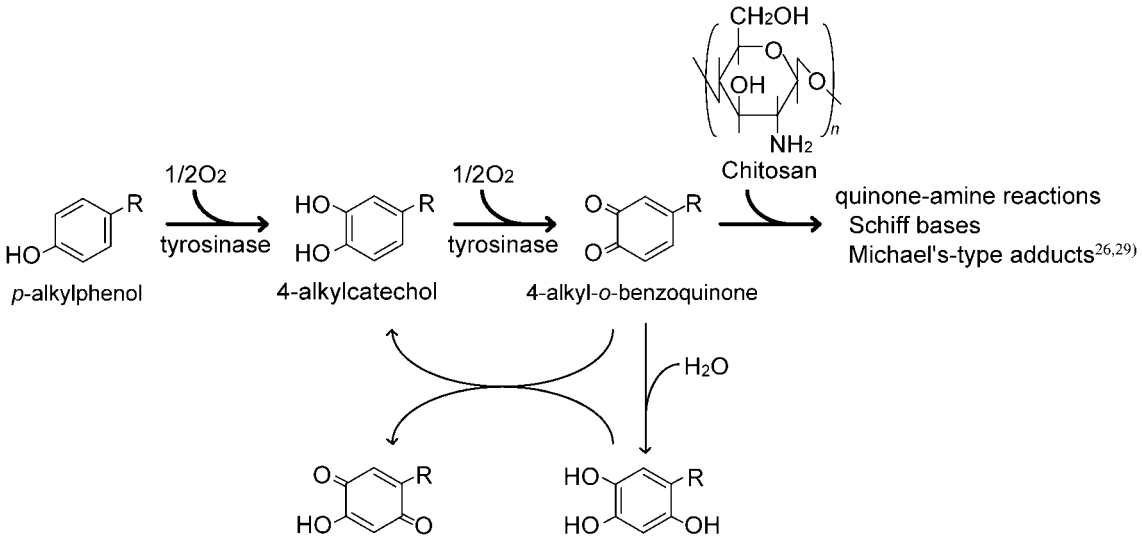

Scheme 1. Removal of *p*-Alkylphenols by the Quinone Conversion with Mushroom Tyrosinase and the Subsequent Adsorption of Quinone Derivatives on the Chitosan Beads.

remove tyrosinase-generated quinone derivatives has been reported by some researchers.[11,27,28] Their results indicate that the combination of tyrosinase and cationic polymer coagulants is an effective procedure in removing carcinogenic phenol compounds from aqueous solutions. But, one of the major problems in this procedure is that there is an optimum concentration range of the coagulant for maximum flocculation. When the dosage is less or more than the optimum concentration range, flocculation does not occur. We have reported on the enzymatic removal of phenol compounds by combined use of mushroom tyrosinase and chitosan in the form of beads.[12] Quinone derivatives enzymatically generated from *p*-alkylphenols can undergo either Schiff base or Michael's-type addition reaction with chitosan's amines as shown in Scheme 1. The quantities of phenol compounds of >90% were effectively removed by this procedure at pH 7.0 and 45 °C as the optimum conditions.

The goal of this study was to investigate the removal of various *p*-alkylphenols containing 4-*n*-octylphenol (4NOP) and 4-*n*-nonylphenol (4NNP) through their enzymatic oxidation and the subsequent adsorption of quinone derivatives formed on the chitosan beads. Specifically, we followed the effect of the addition of chitosan in the form of beads, and the capability of this two-step approach for removing various *p*-alkylphenols.

Materials and Methods

Chemicals. Mushroom tyrosinase (EC 1.14.18.1) of the specific activity of 2,590 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, 70–200 μm; specific surface area, 70–100 m^2/g; water content, 92.5%) was used as an adsorbent, and stored in a pH 7.0 phosphate buffer (ionic strength, 0.01 M). All *p*-alkylphenols used were purchased from Wako Pure Chemicals (Tokyo, Japan) and Tokyo Kasei Kogyo (Tokyo, Japan), and used without further purification. A chitosan solution (1 w/v%) was prepared using 1.0 g chitosan flakes (chitosan 1000, Wako Pure Chemicals).[12] The chitosan films were prepared by pipetting a chitosan solution of 4.0 g into a Petri dish of diameter 3.2 cm. The chitosan solution was allowed to dry in an oven at 60 °C. The chitosan films (average thickness, 31.4 μm) removed from the Petri dishes were thoroughly washed with 1 M NaOH and water to neutralize the amino groups, and then dried under reduced pressure.

Batch reactions. Enzymatic removal of *p*-alkylphenols was carried out in the batch systems. One of the *p*-alkylphenols used (0.5 mM) and tyrosinase (50 U/cm^3) were dissolved in a pH 7.0 phosphate buffer. A given amount of chitosan beads were added to the mixture solutions (20 cm^3) containing one of the *p*-alkylphenols used and tyrosinase at pH 7.0 and 45 °C.[12]

Quantitative assay of p-alkylphenols. The concentration of remaining *p*-alkylphenols was determined by Hitachi L-7000 high performance liquid chromatography combined with a spectrophotometer and an integrator (Hitachi L-7420) or a colorimetric assay with 4-aminoantipyrine (4-AAP).[14,29]

For the colorimetric assay, the reagents were added to an aliquot of 0.4 cm^3 taken from the reaction solutions in the following order: 3.0 cm^3 of 0.25 M sodium bicarbonate, 0.4 cm^3 of 20.8 mM 4-AAP, and 0.4 cm^3 of 83.4 mM potassium ferricyanide. After vigorous stirring for 5 min, the concentration of *p*-alkylphenols was determined from absorbance measured at wavelengths depending on the type of phenol compound.[24,30]

The concentration of remaining *p*-alkylphenols,

which had no color development by 4-AAP, was determined by the HPLC method. The reverse phase columns, Inertsil ODS-2 and C8-3 (5 μm, 4.6 mm i.d. × 15 cm), were used, and the ratio of the mobile phase with a flow rate of 1.0 cm^3/min consisting of acetonitrile and water depended on the carbon-chain lengths of *p*-alkylphenols used. After a prescribed time, 0.010 cm^3 of reaction solution was injected. The absorption spectra of the reaction solutions were determined by the spectrophotometer mentioned above.[12)]

Estimation of removal of p-alkylphenols. The % conversion values were calculated from the remaining *p*-alkylphenol concentration determined by the 4-AAP and HPLC methods, C_{rem}, and the initial concentration of the *p*-alkylphenols used, C_0, according to eq. 1:

$$\% \text{ conversion} = \frac{C_0 - C_{rem}}{C_0} \times 100 \qquad (1)$$

The % adsorption values were calculated from the absorbances at the wavelengths assigned to the generated quinone derivatives in the mixture solutions containing the chitosan beads, Abs_{chi}, and the maximum absorbance at the same wavelength in the absence of chitosan beads, Abs^{max}, according to eq. 2:

$$\% \text{ adsorption} = \frac{Abs^{max} - Abs_{chi}}{Abs^{max}} \times 100 \qquad (2)$$

In addition, the % removal values were obtained from eq. 3:[12)]

$$\% \text{ removal} = \frac{C_0 - C_{rem}}{C_0} \cdot \% \text{ adsorption} \qquad (3)$$

Results and Discussion

Removal of p-n-alkylphenols

For 4-n-pentylphenol

In our previous paper, removal of *p*-*n*-alkylphenols such as *p*-cresol, 4-ethylphenol, 4-*n*-propylphenol, and 4-*n*-butylphenol was investigated by the combined use of tyrosinase and chitosan beads, and the % removal values of 93–97% were obtained.[12)] Here, we began with enzymatic removal of other *p*-*n*-alkylphenols. The enzymatic quinone conversion of 4-*n*-pentylphenol (4NPenP) was carried out at pH 7.0 and 45 °C. Figure 1 shows the changes in the absorbance at 400 nm with the reaction time in the absence and presence of the chitosan beads for 4NPenP. The absorbance at 400 nm of a 4NPenP solution containing tyrosinase (50 U/cm^3) sharply increased in the absence of chitosan beads. According to Jiménez *et al.*, a gradual decrease in the absorbance indicates that some of the generated quinone derivatives undergo the water addition reaction.[26)] On the other hand, the absorbance of the 4NPenP + tyrosinase mixture solution with the chitosan beads of 0.025 cm^3/cm^3 sharply decreased 5 min after the enzymatic reaction started. This indicates that quinone

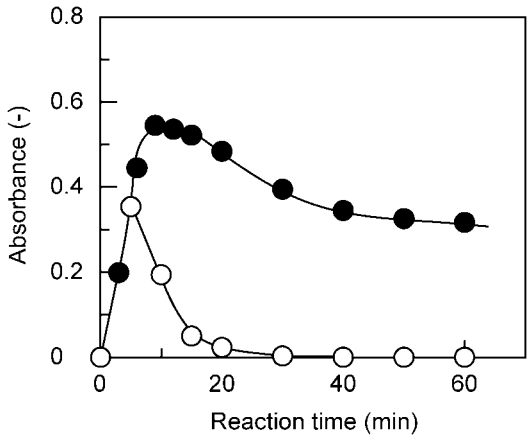

Fig. 1. Changes in the Absorbance at 400 nm of a 4-*n*-Pentylphenol (0.5 mM) Solution Containing Tyrosinase of 50 U/cm^3 with the Reaction Time in the Absence (●) and Presence (○) of Chitosan Beads of 0.025 cm^3/cm^3 at pH 7.0 and 45 °C.

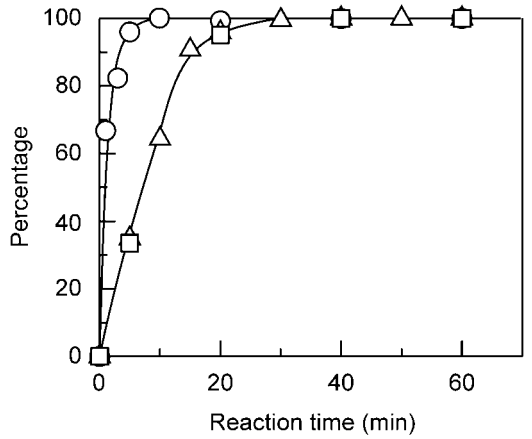

Fig. 2. Changes in % Conversion (○), % Adsorption (△), and % Removal (□) with the Reaction Time for 4-*n*-Pentylphenol (0.5 mM) at pH 7.0 and 45 °C in the Presence of Tyrosinase at 50 U/cm^3 and Chitosan Beads of 0.025 cm^3/cm^3.

derivatives enzymatically generated were adsorbed on the chitosan beads.[31–34)] Figure 2 shows the changes in the % conversion, % adsorption, and % removal values with the reaction time for 4NPenP. The quinone derivatives enzymatically generated from 4NPenP were effectively adsorbed on the chitosan beads through nonenzymatic chemical reaction of the quinone derivatives with the chitosan's amino groups. The % removal value rapidly increased and 4NPenP was completely removed at 40 min.

For 4-n-hexylphenol and 4-n-heptylphenol

The concentrations of 4NHexP and 4NHepP solutions prepared with a pH 7.0 buffer were 0.3 and 0.1 mM, respectively, due to their low solubility. Figure 3 shows the changes in the % conversion, % adsorption, and % removal values with the reaction time for 4NHexP and 4NHepP at 50 U/cm^3 in the presence of chitosan beads of 0.025 cm^3/cm^3. Both 4NHexP and 4NHepP were

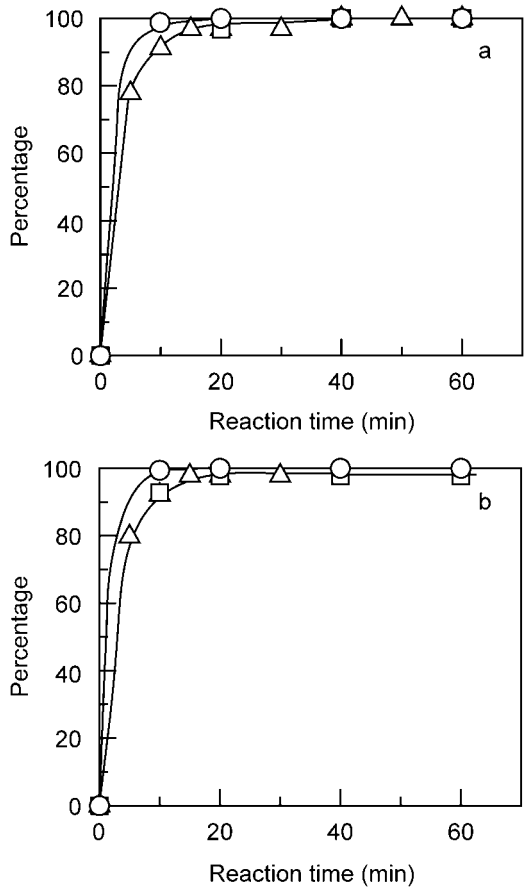

Fig. 3. Changes in % Conversion (○), % Adsorption (△), and % Removal (□) with the Reaction Time for (a) 4-*n*-Hexylphenol (0.3 mM) and (b) 4-*n*-Heptylphenol (0.1 mM) at pH 7.0 and 45 °C in the Presence of Tyrosinase at 50 U/cm^3 and Chitosan Beads of 0.025 cm^3/cm^3.

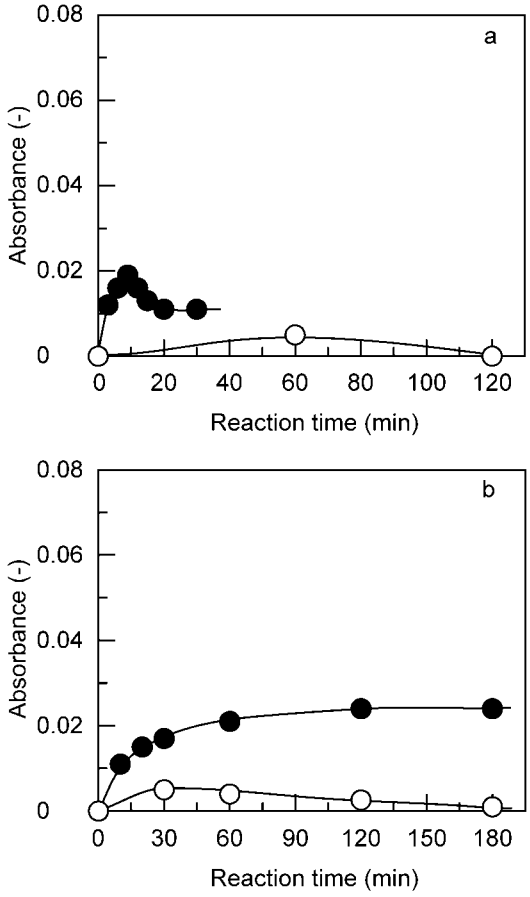

Fig. 4. Changes in the Absorbance at 400 nm of the (a) 4-*n*-Octylphenol (0.05 mM) and (b) 4-*n*-Nonylphenol (0.05 mM) Solutions Containing Tyrosinase at 50 U/cm^3 with the Reaction Time in the Absence (●) and Presence (○) of Chitosan Beads of 0.025 cm^3/cm^3 at pH 7.0 and 45 °C.

quinone-converted within 10 min. The quinone adsorption successfully occurred in a short time, and the % removal values for 4NHexP and 4NHepP reached 100 and 98.7% at 60 min, respectively.

For 4-n-octylphenol and 4-n-nonyllpehnol

4NOP and 4NNP solutions of 0.05 mM were prepared with a pH 7.0 buffer. Figure 4 shows the changes in the absorbance at 400 nm with the reaction time at 50 U/cm^3 in the absence and presence of chitosan beads of 0.025 cm^3/cm^3 for 4NOP and 4NNP. 4NOP and 4NNP were enzymatically oxidized for 10 and 120 min, respectively, in the absence of chitosan beads. Quinone conversion was very fast, and the quinone derivatives generated from 4NOP were relatively unstable in the reaction solution. Therefore, the absorbance at 510 nm gradually increased concurrently with the decrease in the absorbance at 400 nm. The increase in the absorbance at 510 nm for 4NOP indicates that the water addition reaction occurs.[26] On the other hand, the absorbance at 400 nm gradually increased and then leveled off for 4NNP. It is thought that the quinone derivatives generated from 4NNP undergo the water addition reaction only under extreme conditions. Figure 5 shows the changes in the % conversion, % adsorption, and % removal values with the reaction time in the presence of chitosan beads of 0.025 cm^3/cm^3 for 4NOP and 4NNP. 4NOP was enzymatically oxidized for 40 min in the presence of chitosan beads. Although the reaction of quinone derivatives with chitosan was slower than that for other *p*-*n*-alkylphenols, the % removal value reached 100% at 120 min. Either nonenzymatic adsorption of quinone derivatives or enzymatic quinone conversion was slow for 4NNP, and the % removal value went up to 97.2% at 180 min. When the quinone derivatives generated from 4NPenP, 4NHexP, and 4NHepP were highly adsorbed on the chitosan beads, the chitosan beads were colored dark brown, but no distinct color development of the chitosan beads was observed for 4NNP and 4NOP. Therefore, the UV–visible spectra were measured of the chitosan films incubated in 4NOP and 4NNP solutions containing tyrosinase for 18 hr to confirm the reaction of quinone derivatives with chitosan's amino groups. The chitosan films turned light brown due to quinone tanning.[31–34] Figure 6 shows the UV–visible spectra of the chitosan films incubated in the 4NOP and 4NNP solutions

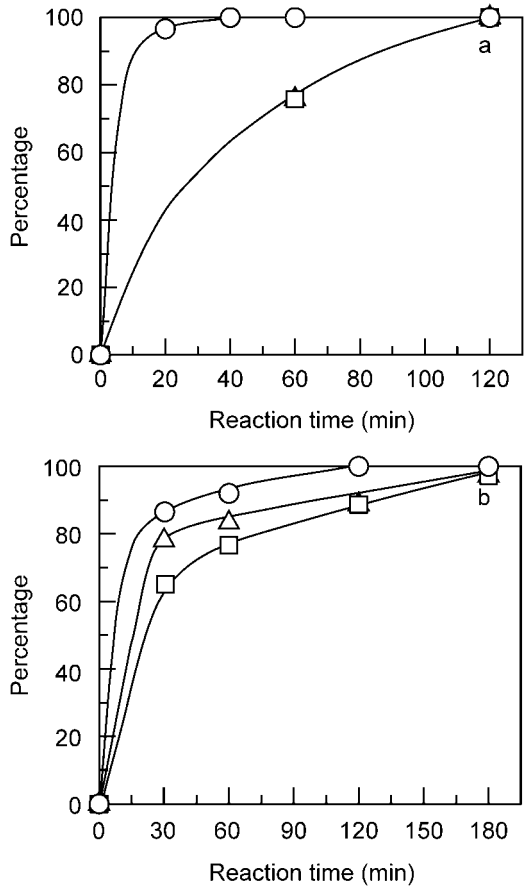

Fig. 5. Changes in % Conversion (○), % Adsorption (△), and % Removal (□) with the Reaction Time for (a) 4-*n*-Octylphenol (0.05 mM) and (b) 4-*n*-Nonylphenol (0.05 mM) at pH 7.0 and 45 °C in the Presence of Tyrosinase at 50 U/cm^3 and Chitosan Beads of 0.025 cm^3/cm^3.

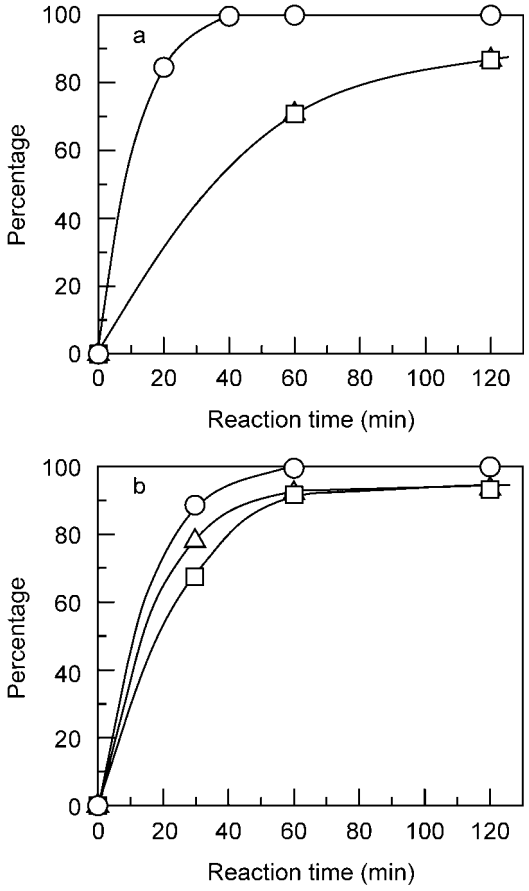

Fig. 7. Changes in % Conversion (○), % Adsorption (△), and % Removal (□) with the Reaction Time for 4-Isopropylphenol (0.5 mM) at pH 7.0 and 45 °C in the Presence of Tyrosinase at 50 U/cm^3 and Chitosan Beads.

Amount of chitosan beads (cm^3/cm^3): (a) 0.025, (b) 0.100.

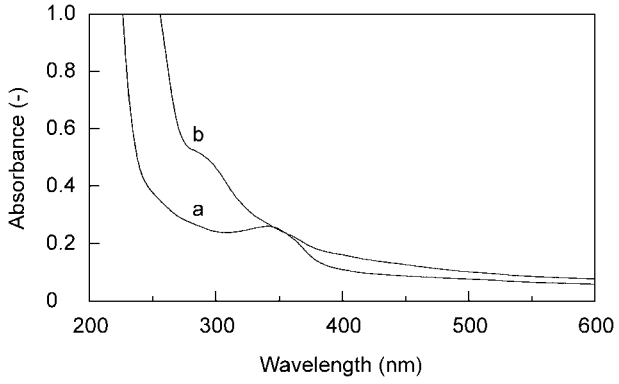

Fig. 6. UV–Visible Spectra of the Chitosan Films Incubated in (a) 4-*n*-Octylphenol and (b) 4-*n*-Nonylphenol Solutions Containing Tyrosinase at 50 U/cm^3 for 18 h at pH 7.0 and 45 °C.

containing tyrosinase of 50 U/cm^3. A broad peak emerged at 350 nm for 4NOP. A small peak at 300 nm was observed for 4NNP. No browning and increase in UV–visible adsorption were observed for the chitosan films immersed in the 4NOP or 4NNP solution without tyrosinase. These results indicate that quinone derivatives enzymatically generated from 4NOP and 4NNP react with chitosan's amino groups.

Removal of other p-alkylphenols

Since *p-n*-alkylphenols were removed as described above, we tried to remove other *p*-alkylphenols enzymatically with a nonlinear carbon chain such as 4-isopropylphenol (4IProP), 4-*sec*-butylphenol (4SBP), and 4-*tert*-pentylphenol (4TPenP). Of these, 4SBP and 4TPenP were suspected to be the endocrine disrupting chemical. 4IProP and 4SBP were oxidized at pH 7.0 and 45 °C with tyrosinase. The optimum conditions were determined for enzymatic removal of these three kinds of *p*-alkylphenols.

Removal of 4-isopropylphenol

Figure 7(a) shows the changes in the % conversion, % adsorption, and % removal values with the reaction time for 4IProP (0.5 mM) at pH 7.0 and 45 °C in the presence of chitosan beads of 0.025 cm^3/cm^3. 4IProP was completely converted into the corresponding quinone derivative at 40 min, but, the increase in the % adsorption value was more gradual than the increase in the % conversion value because some of the quinone

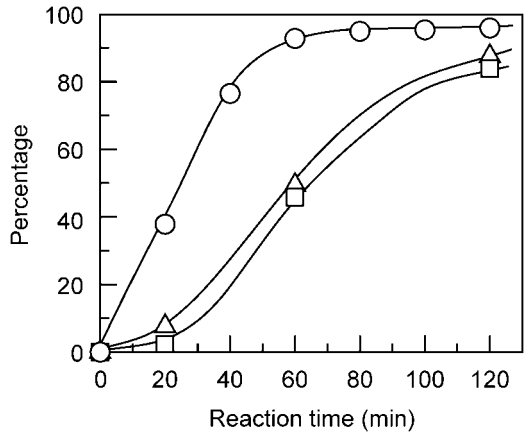

Fig. 8. Changes in % Conversion (○), % Adsorption (△), and % Removal (□) with the Reaction Time for 4-*sec*-Butylphenol (0.5 mM) at pH 7.0 and 45 °C in the Presence of Tyrosinase at 50 U/cm^3 and Chitosan Beads of 0.025 cm^3/cm^3.

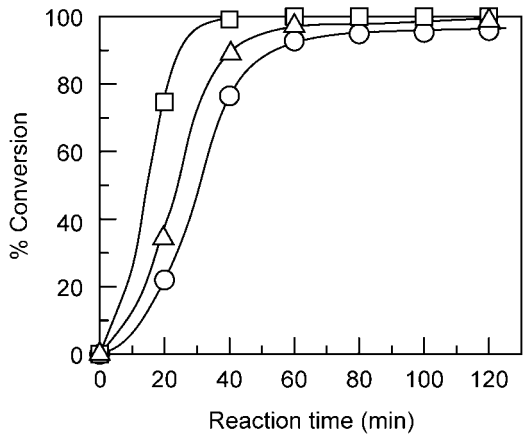

Fig. 9. Changes in % Conversion with the Reaction Time for 4-*sec*-Butylphenol (0.5 mM) at pH 7.0 and 45 °C in the Presence of Tyrosinase at 50 (○), 75 (△), and 100 (□) U/cm^3 and Chitosan Beads of 0.025 cm^3/cm^3.

derivatives generated from 4IProP underwent the water addition reaction,[26] and the % removal value was limited to 86.7% at 120 min. Therefore, the amount of added chitosan beads was further increased, while the tyrosinase concentration was kept unchanged. The % removal values went up to 93.3% as shown in Fig. 7(b), since the increase in the amount of chitosan beads added to the 4IProP + tyrosinase mixture solution led to the increase in the reaction of the quinone derivatives with chitosan's amino groups.

Removal of 4-sec-butylphenol

Enzymatic removal of 4SBP was carried out at pH 7.0 and 45 °C. As Fig. 8 shows, quinone conversion sharply increased against the reaction time in the initial stage of the enzymatic reaction, and the % conversion value leveled off at 80 min in the presence of chitosan beads of 0.025 cm^3/cm^3. Quinone adsorption gradually increased with the reaction time. However, since the quantities of 4SBP of 4.1% remained in the reaction solution and the % removal value was limited to 83.9% at 120 min, both tyrosinase concentration and the amount of added chitosan beads were further increased for removal of 4SBP. Figure 9 shows the changes in the % conversion value with the reaction time at different tyrosinase concentrations. Quinone conversion more sharply increased at shorter reaction times at higher tyrosinase concentrations. 4SBP was completely converted to the quinone derivative at 75 and 100 U/cm^3. Since the time required to convert 4SBP enzymatically was shorter at 100 U/cm^3, a further increased amount of chitosan beads were added to the 4SBP solution containing tyrosinase of 100 U/cm^3. Figure 10 shows the effect of the amount of added chitosan beads on 4SBP removal. The absorbance at 400 nm gradually decreased 60 min after the enzymatic reaction started, and decreased with an increase in the amount of added chitosan beads. Quinone adsorption on the chitosan beads takes place in competition with water addition reaction of the quinone

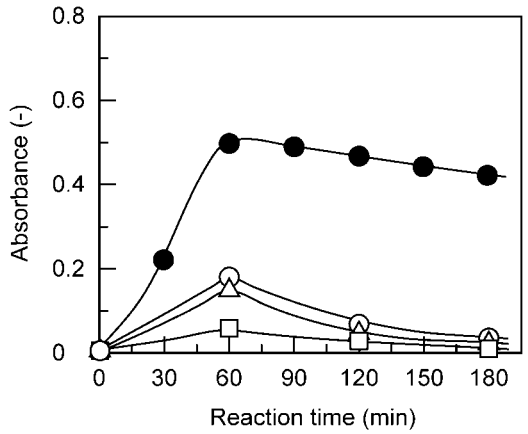

Fig. 10. Changes in the Absorbance at 400 nm of 4-*sec*-Butylphenol (0.5 mM) Solutions Containing Tyrosinase at 50 U/cm^3 with the Reaction Time in the Absence (●) and Presence of Chitosan Beads of 0.025 (○), 0.050 (△), and 0.100 (□) cm^3/cm^3 at pH 7.0 and 45 °C.

derivatives generated.[26] Since an intermediate generated from the quinone derivatives through water addition reaction is not considered to react with chitosan, water addition reaction more preferentially occurs at lower amounts of chitosan beads. This reaction results in the decrease in the % adsorption value. The results of 4SBP removal at 100 U/cm^3 are shown in Fig. 11. In the case where chitosan beads of 100 cm^3/cm^3 were added to a 4SBP solution containing tyrosinase at 100 U/cm^3, 4SBP was completely converted to quinone derivatives at 60 min. The % removal value gradually increased with an increase in the reaction time, and went up to 95.2% at 120 min.

For 4-tert-pentylphenol

No enzymatic oxidation of 4TPenP occurred at pH 7.0 in the absence of hydrogen peroxide (H_2O_2). In the presence of H_2O_2 of 0.5 mM at pH 6.0 as the optimum

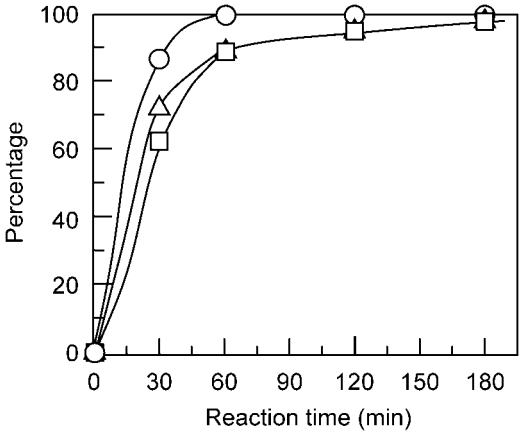

Fig. 11. Changes in % Conversion (○), % Adsorption (△), and % Removal (□) with the Reaction Time for 4-*sec*-Butylphenol (0.5 mM) at pH 7.0 and 45 °C in the Presence of Tyrosinase at 100 U/cm^3 and Chitosan Beads of 0.100 cm^3/cm^3.

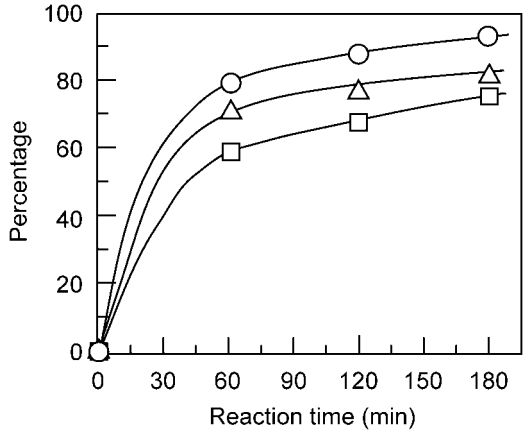

Fig. 12. Changes in % Conversion (○), % Adsorption (△), and % Removal (□) with the Reaction Time for 4-*tert*-Pentylphenol (0.5 mM) at pH 6.0 and 45 °C in the Presence of Tyrosinase at 50 U/cm^3, H_2O_2 (0.5 mM), and Chitosan Beads of 0.025 cm^3/cm^3.

conditions for 4-*tert*-butylphenol (4TBP) as determined in our previous study,[12] the % conversion value of 4TPenP gradually increased. Since some tyrosinase molecules are transformed from met into oxy forms by the addition of H_2O_2, enzymatic oxidation proceeds under the above-mentioned conditions.[26] Oxy-form tyrosinase is complexed with 4TPenP, leading to the corresponding quinone derivative. In the absence of H_2O_2, the portion of oxy form tyrosinase is so small that the efficiency of the enzymatic oxidation is very slow, and oxy-form tyrosinase is recovered in the turnover. In addition, since the peak position of the product enzymatically generated from 4TPenP was in fair agreement with one of 4-*tert*-butyl-*o*-benzoquinone generated from 4TBP under the same condition,[12,26] a product from 4TPenP in our system is considered to be 4-*tert*-pentylbenzoquinone (4TPenBQ).

When H_2O_2 was added to the 4TPenP + tyrosinase (50 U/cm^3) mixture solutions, the enzymatic oxidation of 4TPenP was enhanced and reached the maximum at an H_2O_2 concentration of 0.5 mM, but the % conversion and % adsorption values were 40 and 38%, respectively, at 120 min. Therefore, the % removal value was limited to 16%. It was found from these results that enzymatic quinone conversion and the subsequent quinone adsorption were very slow under the above-mentioned conditions, and that removal of 4TPenBQ was carried out at 150 U/cm^3 in the presence of chitosan beads of 0.100 cm^3/cm^3.

Figure 12 shows the changes in the % conversion, % adsorption, and % removal values with the reaction time at an H_2O_2 concentration of 0.5 mM and a tyrosinase concentration of 150 U/cm^3 in the presence of chitosan beads of 0.100 cm^3/cm^3 at pH 6.0. The % conversion value increased to 92.3% at 180 min. The increase in chitosan beads led to the increase in the reaction of generated 4TPenBQ with chitosan. The % removal value went up to 71.3%. The increase in the tyrosinase concentration and the amount of added chitosan beads had a favorable influence on removal of 4TPenP like 4TBP.

The removal of *p*-alkylphenols by the combined use of tyrosinase and chitosan beads is summarized in Table 1. This procedure is quite useful for removal of various *p*-alkylphenols from aqueous solutions. The % removal values of 97–100% were obtained for *p*-*n*-alkylphenols. In addition, the % removal value increased by adjusting either the tyrosinase concentration or the amount of added chitosan beads for some of the *p*-alkylphenols shown in Table 1. In conclusion, removal of *p*-alkylphenols by this procedure is much more effective than other methods repeated previously. This method is an easy-to-operate procedure, and the chitosan beads are readily separable from the reaction solutions after removal of *p*-alkylphenols. It should be emphasized that this procedure can also be applied to remove 4NOP and 4NNP suspected endocrine disrupting chemicals. But, one of the problems that must be solved is low chemical resistance to organic solvents, since tyrosinase was deactivated by the use of ethanol. In addition, the repeated use of tyrosinase is a significant challenge in applying this procedure for practical purposes. One way to solve the problem is to immobilize tyrosinase on a water-insoluble support. Retention of the activity of immobilized tyrosinase is required. On this point, the covalent immobilization of tyrosinase on hydrophilic polymer supports with a water-soluble carbodiimide is an effective procedure, and this is our goal for the future.

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Table 1. Removal of *p*-Alkylphenols by the Combined Use of Tyrosinase and Chitosan Beads at 45 °C

Phenol compound	Phenol concentration (mM)	Tyrosinase concentration (U/cm^3)	Chitosan beads (cm^3/cm^3)	Reaction time (min)	H_2O_2 (mM)	pH	% Conversion	% Adsorption	% Removal
4-*n*-pentylphenol	0.5	50	0.025	40		7.0	100	100	100
4-*n*-hexylphenol	0.3	50	0.025	40		7.0	100	100	100
4-*n*-heptylphenol	0.1	50	0.025	40		7.0	100	98.7	98.7
4-*n*-octylphenol	0.05	50	0.025	120		7.0	100	100	100
4-*n*-nonylphenol	0.05	50	0.025	180		7.0	100	97.2	97.2
4-isopropylphenol	0.5	50	0.025	120		7.0	99.9	86.7	86.6
	0.5	50	0.025	120		7.0	99.9	93.3	93.2
4-*sec*-butylphenol	0.5	50	0.025	120		7.0	95.9	87.5	83.9
	0.5	100	0.100	180		7.0	100	98.2	98.2
4-*tert*-pentylphenol	0.5	50	0.025	120	0.5	6.0	43.0	40.7	17.5
	0.5	150	0.100	180	0.5	6.0	92.3	77.2	71.3

References

1) Guillette, L. J., and Gunderson, M. P., Alteration in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. *Reproduction*, **122**, 857–864 (2001).
2) Gunderson, M. P., LeBlanc, G. A., and Guillette, L. J., Alterations in sexually dimorphic biotransformation of testosterone in juvenile American alligators (*Alligator mississippiensis*) from contaminated lakes. *Environ. Health Perspect.*, **109**, 1257–1264 (2001).
3) Gimeno, S., Komen, H., Gerritsen, A. G. M., and Bowmer, T., Feminisation of young males of the common carp, Cyprinus Carpio, exposed to 4-*tert*-pentylphenol during sexual differentiation. *Aquatic Toxicology*, **43**, 77–92 (1998).
4) Hayes, T. B., Collins, A., Lee, M., Mendoza, M., Noriega, N., Stuart, A. A., and Vonk, A., Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proc. Natl. Acad. Sci. USA*, **99**, 5476–5480 (2002).
5) Ying, G. G., Williams, B., and Kookana, R., Environmental fate of alkylphenols and alkylphenol ethoxylates: a review. *Environ. Int.*, **28**, 215–226 (2002).
6) Blackburn, M. A., and Waldock, M. J., Concentrations of alkylphenols in rivers and estuaries in England and Wales. *Water Res.*, **29**, 1623–1629 (1995).
7) Kwack, S. J., Kwon, O., Kim, H. S., Kim, S. S., Kim, S. H., Sohn, K. H., Lee, R. D., Park, C. H., Jeung, E. B., An, B. S., and Park, K. L., Comparative evaluation of alkylphenolic compounds on estrogenic activity *in vitro* and *in vivo*. *J. Toxicol. Environ. Health A*, **65**, 419–431 (2002).
8) Chikae, M., Ikeda, R., Hasan, Q., Morita, Y., and Tamiya, E., Effect of alkylphenols on adult male medaka: plasma vitellogenin goes to the level of estrous female. *Environ. Toxicol. Pharmacol.*, **15**, 33–36 (2003).
9) Rankouhi, T. R., Sanderson, J. T., van Holsteijn, I., van Leeuwen, C., Vethaak, A. D., and van den Berg, M., Effects of natural and synthetic estrogens and various environmental contaminants on vitellogenesis in fish primary hepatocytes: comparison of bream (*Abramis brama*) and carp (*Cyprinus carpio*). *Toxicol. Sci.*, **81**, 90–102 (2004).
10) Tsutsumi, Y., Haneda, T., and Nishida, T., Removal of estrogenic activities of bisphenol A and nonylphenol by oxidative enzymes from lignin-degrading basidiomycetes. *Chemosphere*, **42**, 271–276 (2001).
11) Sun, W, Q., Payne, F. G., Moas, M., Chu, J. H., and Wallace, K. K., Tyrosinase reaction/chitosan adsorption for removing phenols from wastewater. *Biotechnol. Prog.*, **8**, 179–186 (1992).
12) Yamada, K., Akiba, Y., Shibuya, T., Kashiwada, A., Matsuda, K., and Hirata, M., Water purification through bioconversion of phenol compounds by tyrosinase and chemical adsorption by chitosan beads. *Biotechnol. Prog.*, **21**, 823–829 (2005).
13) Wada, S., Ichikawa, H., and Tatsumi, K., Removal of phenols and aromatic amines from wastewater by a combination treatment with tyrosinase and a coagulant. *Biotechnol. Bioeng.*, **45**, 304–309 (1995).
14) Payne, G. F., Sun, W. Q., and Sohrabi, A., Tyrosinase reaction/chitosan adsorption for selectively removing phenols from aqueous mixtures. *Biotechnol. Bioeng.*, **40**, 1011–1018 (1992).
15) Ikehata, K., and Nicell, J. A., Characterization of tyrosinase for the treatment of aqueous phenols. *Bioresource Technol.*, **74**, 191–199 (2000).
16) Fukuda, T., Uchida, H., Takashima, Y., Uwajima, T., Kawabata, T., and Suzuki, M., Degradation of bisphenol A by purified laccase from *trametes villosa*. *Biochem. Biophy. Res. Commun.*, **284**, 704–706 (2001).
17) Okazaki, S., Michizoe, J., Goto, M., Furusaki, S., Wariishi, H., and Tanaka, H., Oxidation of bisphenol A catalyzed by laccase hosted in reversed micelles in organic media. *Enzyme Microb. Technol.*, **31**, 227–232 (2002).
18) Michizoe, J., Ichinose, H., Kamiya, N., Maruyama, T., and Goto, M., Biodegradation of phenolic environmental pollutants by a surfactant-laccase complex in organic media. *J. Biosci. Bioeng.*, **99**, 642–647 (2005).
19) Nicell, J. A., Bewtra, J. K., Taylor, K. E., Biswas, N., and St. Pierre, C., Enzyme catalyzed polymerization and precipitation of aromatic compounds from wastewater. *Water Sci. Technol.*, **25**, 157–164 (1992).
20) Reactor development for redoxidase catalyzed polymerization and precipitation of phenols from wastewater. *Water Res.*, **27**, 1629–1639 (1993).
21) Caza, N., Bewtra, J. K., Biswas, N., and Taylor, K. E., Removal of phenolic compounds from synthetic waste-

water using soybean peroxidase. *Water Res.*, **33**, 3012–3018 (1999).
22) Wagner, M., and Nicell, J. A., Detoxification of phenolic solutions with horseradish peroxidase and hydrogen peroxide. *Water Res.*, **36**, 4041–4052 (2002).
23) Sakurai, A., Masuda, M., and Sakakibara, M., Effects of surfactants on phenol removal by the methods of polymerization and precipitation catalysed by *Coprinus cinereus* peroxidase. *J. Chem. Technol. Biotechnol.*, **78**, 952–958 (2003).
24) Ikehara, K., and Nicell, J. A., Color and toxicity removal following tyrosinase-catalyzed oxidation of phenols. *Biotechnol. Prog.*, **16**, 533–540 (2000).
25) Fenoll, L. G., Rodríguez-López, J. N., García-Sevilla, F., Tudela, J., García-Ruiz, P. A., Varón, R., and García-Cánovas, F., Oxidation by mushroom tyrosinase of monophenols generating slightly unstable *o*-quinones. *Eur. J. Biochem.*, **267**, 5865–5878 (2000).
26) Jiménez, M., and Carcía-Carmona, F., Hydrogen peroxide-dependent 4-*t*-butylphenol hydroxylation by tyrosinase: a new catalytic activity. *Biochim. Biophys. Acta*, **1297**, 33–39 (1996).
27) Wada, S., Ichikawa, H., and Tatsumi, K., Removal of phenol from wastewater by soluble and immobilized tyrosinase. *Biotechnol. Bioeng.*, **42**, 854–858 (1993).
28) Sun, W. Q., and Payne, G. F., Tyrosinase-containing chitosan gels: a combined catalyst and sorbent for selective phenol removal. *Biotechnol. Bioeng.*, **51**, 79–86 (1996).
29) Endo, Y., Zuan, Y. J., and Fujimoto, K., The oxidation of *p*-octylphenol by mushroom tyrosinase. *Nippon Nōgeikagaku Kaishi* (in Japanese), **74**, 1337–1341 (2000).
30) Ettinger, M. B., Ruchhort, C. C., and Lishka, R. J., Sensitive 4-aminoantipyrine method for phenolic compounds. *Anal. Chem.*, **23**, 1783–1788 (1951).
31) Kumar, G., Smith, P. J., and Payne, G. F., Enzymatic grafting of a natural product onto chitosan to confer water solubility under basic conditions. *Biotechnol. Bioeng.*, **63**, 154–165 (1999).
32) Payne, G. F., and Sun, W. Q., Tyrosinase reaction and subsequent chitosan adsorption for selective removal of contaminant from a fermentation recycle stream. *Appl. Environ. Microbiol.*, **60**, 397–401 (1994).
33) Yamada, K., Chen, K., Kumar, G., Vesnovsky, O., Topoleski, L. D. K., and Payne, F. G., Chitosan based water-resistant adhesive: analogy to mussel glue. *Biomacromolecules*, **1**, 252–258 (2000).
34) Chen, T. H., Kumar, G., Harris, M. T., Smith, P. J., and Payne, G. P., Enzymatic grafting of hydroxylation onto chitosan to alter surface and rheological properties. *Biotechnol. Bioeng.*, **70**, 33–39 (2000).