Communication to the Editor

Removal of Chlorophenols from Wastewater by Immobilized Horseradish Peroxidase

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Immobilization of horseradish peroxidase on magnetite and removal of chlorophenols using immobilized enzyme were investigated. Immobilization by physical adsorption on magnetite was much more effective than that by the crosslinking method, and the enzyme was found to be immobilized at 100% of retained activity. In addition, it was discovered that horseradish peroxidase was selectively adsorbed on magnetite, and the immobilization resulted in a 20-fold purification rate for crude enzyme. When immobilized peroxidase was used to treat a solution containing various chlorophenols, *p*-chlorophenol, 2,4-dichlorophenol, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol, and pentachlorophenol, each chlorophenol was almost 100% removed, and also the removal of total organic carbon (TOC) and adsorbable organic halogen (AOX) reached more than 90%, respectively. However, in the case of soluble peroxidase, complete removal of each chlorophenol could not be attained, and in particular, the removal of 2,4,5-trichlorophenol was the lowest, with a removal rate of only 36%. © 1996 John Wiley & Sons, Inc.
Key words: chlorophenol • peroxidase • immobilization • magnetite • immobilized enzyme

INTRODUCTION

Horseradish peroxidase was shown to be able to remove a variety of phenols and aromatic amines from an aqueous solution[10,11] and to decolorize phenolic industrial effluents.[1] Elsewhere[17] it was shown that phenols are effectively removed by treatment with horseradish peroxidase in the presence of a coagulant. However, peroxidase quickly becomes inactivated during the reaction, and the coagulant prevents peroxidase inactivation and reduces the amount of peroxidase required for phenol removal. Arseguel and Baboulène[2] studied the removal of phenol using peroxidase in the presence of a mineral and showed that the mineral could prolong the catalytic action because of the adsorption of the reaction products.

Enzyme immobilization is excellent due its high storage stability and better control of the catalytic process.[18,19] In addition, immobilization allows for operational stability and no contamination of the solution treated by enzymes, because the immobilized enzymes can be easily separated from the solution.[18,19] Horseradish peroxidase was immobilized on controlled pore glass (CPG) and 300–400 units/g of immobilized peroxidase was obtained.[9] Siddique et al.[15] reported on the removal of *p*-chlorophenol from an aqueous solution by horseradish peroxidase immobilized on three different reactor matrices. Lignin peroxidase was immobilized on CNBr-Sepharose 4B at 80% of immobilization yield and 40% of retained activity.[3] Several immobilization methods (physical adsorption, covalent bonding, crosslinking, inclusion, and encapsulation) have been developed to immobilize enzymes. Physical adsorption is the most cost effective and simplest of all immobilization methods.[7] A variety of supports can be used to immobilize enzymes. The use of magnetic particles, as a support, has the advantage of being easily separable from the solution through the use of magnetic devices.

In the present work, the immobilization of horseradish peroxidase on magnetic particles and removal of chlorophenols in an aqueous solution by immobilized peroxidase was investigated.

MATERIALS AND METHODS

Chemicals

All chemicals are commercially available and of reagent grade. Phenol was obtained from Wako Chemicals (Tokyo, Japan); *p*-chlorophenol, 2,4-dichlorophenol, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol, and pentachlorophenol were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Pentachlorophenol was recrystallized twice from benzene. Magnetite, Fe_3O_4 (5 μm in average diameter) was obtained from Nacalai Tesque (Kyoto, Japan).

Enzymes

Purified horseradish peroxidase (EC 1.11.1.7) was purchased from Wako Chemicals (Tokyo, Japan) and had a specific activity of 100 units/mg. Peroxidase activity

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was determined from a change in the optical density (A_{405nm}) in a reaction mixture containing 2,2′-azinobis[3-ethyl-benzothiazoline-(6)-sulfonic acid] diammonium salt from Wako Chemicals.[13] The change was determined by using a spectrophotometer (Jasco Ubest-55, Japan Spectroscopic, Tokyo, Japan). One unit of peroxidase activity was defined from an increase in A_{405nm} per minute at pH 6.0 at 25°C in 2.4 mL reaction mixture.

Crude peroxidase was prepared from horseradish. Horseradish (1 kg) was grated and extracted with 1 L of water. The enzyme protein was precipitated by adding ethanol (5 L), and the precipitate was then dissolved in 50 mL of water. The crude enzyme was obtained by salting out using ammonium sulfate. The enzyme was dissolved in 35 mL of water, and then the solution was desalted and concentrated by ultrafiltration. Freeze drying produced 233 mg of crude enzyme powder. The yield of enzyme activity was 72% of the first water extract and the peroxidase activity was 2.5 units/mg.

Immobilization of Peroxidase

Peroxidase was chemically immobilized on magnetite as described previous.[19] Physical adsorption of peroxidase on magnetite was carried out in an aqueous solution containing peroxidase. The enzyme purchased or the crude enzyme was dissolved in pure water and stirred with magnetite (500 mg) at 25°C for 15 h. The immobilized protein was calculated from the difference between peroxidase concentration before and after immobilization. Protein concentration was determined by the Berden method.[4]

Incubation Conditions and Analysis

Reactions of each phenol with peroxidase in the presence of hydrogen peroxide were performed at 25°C in 7 mL of a 10 m*M* phosphate buffer (pH 7.0) as follows: A phenol solution was first added to a phosphate buffer solution containing immobilized peroxidase, followed by addition of hydrogen peroxide. Phenol solution was incubated under aerobic conditions using a stirrer (Tokyo Rika Kikai MDC-RT, Tokyo, Japan). A model wastewater containing various chlorophenols was prepared: *p*-chlorophenol (200 μM), 2,4-dichlorophenol (200 μM), 2,4,5-trichlorophenol (100 μM), 2,4,6-trichlorophenol (100 μM), 2,3,4,6-tetrachlorophenol (20 μM), and pentachlorophenol (10 μM) and treated by immobilized peroxidase in the presence of hydrogen peroxide. The model wastewater was incubated under the same conditions as that of phenol, except for the use of the phosphate buffer, pH 5.5. After a prescribed time, the sample was withdrawn and assayed for phenols, total organic carbon (TOC), and adsorbable organic halogen (AOX).

The determination of TOC was made using a Shimadzu TOC analyzer, model 500 (Kyoto, Japan), and AOX was determined with a Mitsubishi AOX analyzer, TOX-10Σ (Tokyo, Japan). The disappearance of each phenol was monitored by high-performance liquid chromatography using a Jasco PU-980 (Japan Spectroscopic) provided with an ultraviolet (UV) detector (Jasco UV-970) and an integrator (Jasco 807-IT). A reverse-phase column, Cosmosil C18-AR (5 μm, 4.6 mm i.d. × 25 cm, Nakarai tesque, Kyoto, Japan), was used, and the mobile phase (flow rate 1.0 mL/min) consisted of methanol and water containing 0.08% H_3PO_4 50:50 (v/v). During analysis of the model wastewater containing chlorophenols, the initial mobile phase consisted at 50:50 (v/v) methanol and 0.08% H_3PO_4, which was brought to 90:10 (v/v) after 20 min and held for another 20 min. After a specified time, 20 μL of reaction solution was injected. The formation of chloride ions was monitored by a Dionex ion chromatograph 4000i (Sunnyvale, CA) with a conductivity detector. The anion-exchange separation was achieved on a column (Dionex AS-4). The mobile phase was 1 m*M* $NaHCO_3$ and 2 m*M* Na_2CO_3 in deionized water. The absorbance at 400 nm of reaction solution was determined by the same spectrophotometer as above.

RESULTS AND DISCUSSION

Immobilization of Peroxidase

Peroxidase was crosslinked by glutaraldehyde on magnetite activated with 3-aminopropyltriethoxysilane (APTS) as described previously.[19] When 1 mg of peroxidase (100 units/mg) was stirred together with 500 mg of the magnetite in the presence of glutaraldehyde, 18% of the added enzyme was immobilized and the activity retained was only 52%. This indicates that the immobilized activity was 9% of that added, or 18 units/g. In the previous paper,[19] it was shown that 80% of tyrosinase was immobilized and 80% of the activity was retained under the same conditions, which means that the immobilized activity was 64% of that added. These facts indicate that immobilization of peroxidase using the crosslinking method was less effective than that of tyrosinase.

Many different methods can be used for immobilizing an enzyme. The immobilized enzyme must be manufactured by a cost-effective and technologically convenient method. Especially, it is important when an immobilized enzyme is used for wastewater treatment. Physical adsorption is the simplest immobilization method of all, but this method has the disadvantage that the enzyme is easily released into the solution. To further clarify the effects of horseradish peroxidase, we immobilized the enzyme on magnetite by physical adsorption and treated the aqueous solution containing chlorophenols.

Adsorption isotherms on magnetite of pure peroxidase purchased from Wako Chemicals and crude perox-

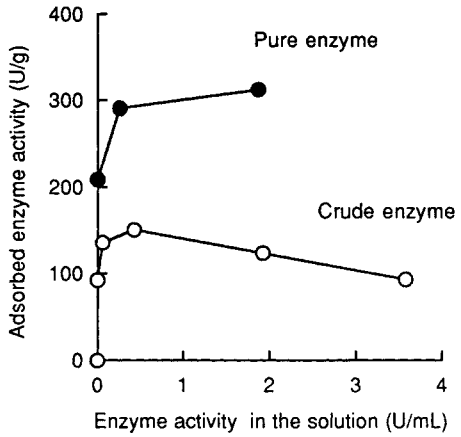

Figure 1. Relationship between adsorbed peroxidase activity on magnetite and remaining activity in the solution.

idase prepared from horseradish are shown in Figure 1. The amount of pure enzyme adsorbed was 300 units/g magnetite. During immobilization of the pure enzyme, no inactivation was observed. Since the activity of peroxidase used in this study was 100 units/mg, the adsorbed amount was 3 mg/g. Horisberger[8] investigated the adsorption amount of protein and polysaccharide on magnetite and showed that the amount of adsorbed trypsin was 5.5 mg/g.

The adsorption isotherm of crude enzyme showed that the maximum amount of adsorption was 150 units/g (Fig. 1). This was half of the amount of the pure enzyme. As described previously, the activity of the crude enzyme (2.5 units/mg) was one-fortieth of the pure one (100 units/mg). These facts indicate that peroxidase was adsorbed on magnetite more selectively than impurities in the crude enzyme, and immobilization resulted in purification of the enzyme (a 20-fold purification). As an example of the purification of the enzyme due to adsorption, the adsorptions of urease on kaolinite[16] and asparaginase on CM-cellulose[12] are known. Usually, an enzyme is purified from a crude one with column chromatography by using diethylaminoethyl (DEAE) cellulose and CM cellulose followed by freeze drying. The fact that we can produce an immobilized enzyme by simple adsorption from a crude enzyme without complicated purification is intriguing and suggests this method can be used to treat wastewater. The adsorption amount of crude enzyme reduced gradually more than 0.5 units/mL (Fig. 1). This is because adsorption of peroxidase was inhibited by impurities (probably other proteins) in the crude enzyme.

From these results, physical adsorption was found to be much more effective than the crosslinking method using glutaraldehyde in the immobilization of peroxidase. Since physical adsorption of an enzyme is a reversible reaction, it is difficult to ascertain that enzyme activity is due to a bound enzyme on a magnetite or due to a desorbed soluble enzyme. Therefore, it is necessary to take into account the desorption of peroxidase. To investigate the extent of desorption of immobilized peroxidase, peroxidase bound on magnetite was immersed into pure water overnight, and the concentration of peroxidase in water was measured. The results showed that no peroxidase was desorbed. Next, the effects of various ions and their concentrations on desorption of an enzyme were investigated. The results showed that the addition of Na_2SO_4, NaCl, and HCOONa hardly allowed the release of peroxidase from the support and 0.1 *M* $NaHCO_3$ released only 2% of peroxidase, even after over 20 h. However, in the phosphate solution, much more peroxidase was desorbed and 0.1 *M* Na_2HPO_4 released 44% of the immobilized enzyme. These results indicate that ionic strength is not responsible for the release of an enzyme from magnetite and that the type of ion is very important. Sabil et al.[14] reported the specific effects of phosphate ions; that is, phosphatase and cellulase adsorbed on a sediment of a lagoon were unaffected by chlorine and carbonate ions, but they were easily released by phosphate ions.

Treatment of Phenol by Immobilized Peroxidase

Figure 2 shows the difference between the removal of phenol with peroxidase immobilized by crosslinking and physical adsorption and that of soluble peroxidase. Due to the immobilized peroxidase, inactivated in boiling water for 10 min, no reduction in phenol took place. In addition, a preliminary experiment showed that phenol was not adsorbed on free magnetite. The results clearly showed that the phenol reduction by immobilized peroxidase was caused only by an enzymatic reaction. The concentration of phenol decreased with time, and the

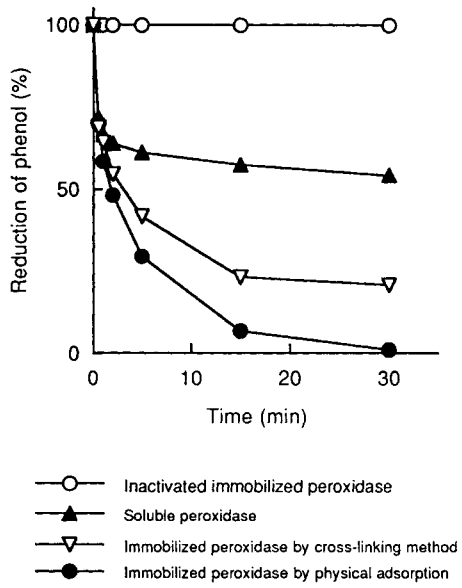

Figure 2. Reduction of phenol from aqueous solution by soluble and immobilized peroxidase in the presence of hydrogen peroxide.

immobilized enzyme was much more effective than the soluble enzyme. In the case of soluble peroxidase, almost no decrease was observed after 3 min. The reason immobilized peroxidase is much more effective might be due to the fact that the immobilized one was more stable than soluble peroxidase. We cannot completely describe the reason immobilized peroxidase was more stable for the enzymatic reaction. It is reported that tyrosinase, one of the phenoloxidases, is inactivated by the reaction of reactive products such as quinones or radicals with a free amino group in the enzyme.[20] Therefore, it can be considered that peroxidase was also inactivated by the reaction with the reactive products formed by the enzymatic reaction. It is assumed that immobilization decreases reactable free amino groups.

When we compared the two immobilizations—crosslinking and physical adsorption—peroxidase immobilized by physical adsorption was much more stable than that resulting from crosslinking. The phenol solutions were incubated by the same enzyme activity (0.2 units/mL). As previously described, the retained activities of the crosslinked enzyme and the physically adsorbed enzyme were 52 and 100%, respectively. Thus, in crosslinking immobilization, about twice the amount of enzyme protein adhered to magnetite compared with physical adsorption. However, the former enzyme could much easier be inactivated than the latter. This describes that the amount of protein was not responsible for prevention of enzyme inactivation. It can be therefore presumed that the product might react with an enzyme molecule before leaving the active center of the enzyme.

A phenol solution was colored dark brown by incubation with soluble peroxide. In the case of immobilized peroxidase, however, only a slight coloration was observed. This indicates that colored products were removed when phenol was treated by immobilized peroxidase. This is because the colored products were linked to the enzyme immobilized on magnetite by a chemical bond or by physical adsorption. The same results were observed for *p*-chlorophenol.

Treatment of Model Wastewater by Immobilized Peroxidase

Removal of each chlorophenol from the model wastewater containing six kinds of chlorophenols by peroxidase can be seen in Table I. We used the same enzyme activity (0.2 units/mL) for both these cases: soluble and immobilized enzyme. The results indicate, in agreement with the results described previously, that immobilized enzyme was more effective than the soluble one, and each chlorophenol was removed to almost 100%. For soluble peroxidase, 2,4,6-trichlorophenol was more reactive than 2,4,5,-trichlorophenol. Such an effect on the chlorinated position of phenol was not observed in immobilized peroxidase.

Table I. Removal of each chlorophenol from the model wastewater by soluble and immobilized peroxidase in the presence of hydrogen peroxide.

Chlorophenol	Initial concentration (μM)	Soluble peroxidase (%)	Immobilized peroxidase (%)
p-Chlorophenol	200	58	100
2,4-Dichlorophenol	200	82	100
2,4,5-Trichlorophenol	100	36	99
2,4,6-Trichlorophenol	100	97	98
2,3,4,6-Trichlorophenol	20	81	99
Pentachlorophenol	10	55	97

Note: Peroxidase activity: 0.2 units/mL; hydrogen peroxidase: 1m*M;* incubation time: 1.5 h.

The TOC and AOX of the model wastewater were 43 and 45 ppm, respectively. In the soluble peroxidase of 0.2 units/mL, TOC and AOX could not be removed, due to a lack of precipitation. Phenol removal by enzyme-catalyzed polymerization has been investigated. However, there are almost no reports on contamination due to the remaining soluble enzyme and nonprecipitated products in the aquatic solution after the peroxidase treatment. From the results of the previous study,[17] the amount of enzyme is found to be directly connected to whether the reaction products were precipitated or not. Thus, a large amount of enzyme was required to remove the products as a precipitate. The evaluation of TOC and AOX removal provides very important information on using enzymes for wastewater treatment.

Removal of TOC and AOX from model wastewater containing various chlorophenols by immobilized peroxidase can be seen in Figure 3. As described previously, no release of enzymes occurred from the immobilized peroxidase. In 10 min, about 90% of TOC and AOX was found to be removed. When magnetite was added to the solution treated by soluble enzyme, no more

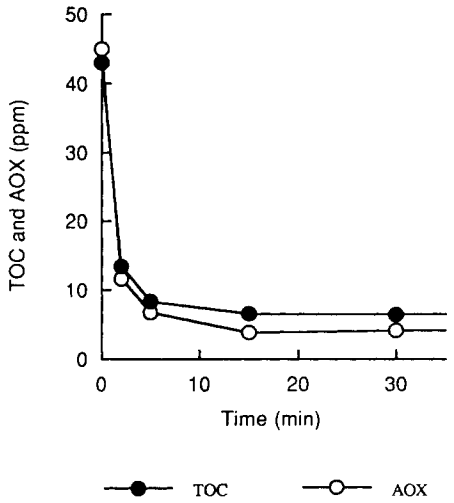

Figure 3. Removal of TOC and AOX from model wastewater containing various chlorophenols by immobilized peroxidase.

reduction of TOC and AOX was observed. These results indicate that the products formed from each chlorophenol were not adsorbed to magnetite and linked to enzymes immobilized on magnetite. Elswhere[17] we showed that the colored products were effectively removed by a combination treatment with peroxidase and a coagulant. This study shows that when you use immobilized peroxidase, chlorophenols can be removed without any coagulant.

Chloride ions are known to be released during the enzymatic coupling processes of chlorophenols.[6] Figure 4 shows the concentration of chloride ions released from chlorophenols during enzymatic treatment. The concentration of released chloride ions was 12 ppm, and it was found to constitute about 25% of the total chlorine initially associated with chlorophenols. An increase in chloride ions took place at almost the same time as the decrease in TOC and AOX. Dec and Bollag[5] showed that the processes of dechlorination coincide almost exactly with the course of the 2,4-dichlorophenol disappearance. They also showed that the maximum amount of chloride ions released was 20% of the total chlorine initially associated with 2,4-dichlorophenol.

In conclusion, peroxidase is seen as being very easily immobilized on magnetite by physical adsorption. Peroxidase was immobilized preferentially from crude horseradish peroxidase, and the enzyme was purified. The immobilized peroxidase can effectively remove phenols because of the binding of colored reaction products to the immobilized enzyme. In the treatment of model wastewater containing chlorophenols, about 90% of TOC and AOX was found to be removed by immobilized peroxidase.

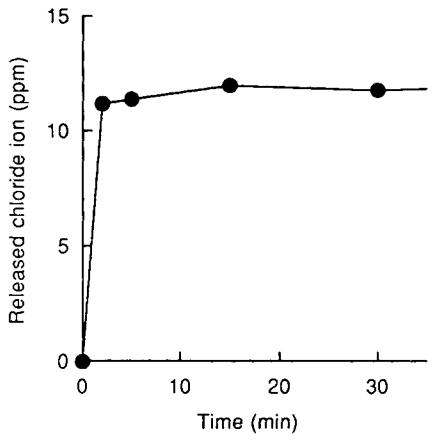

Figure 4. Release of chloride ions from chlorophenols in model wastewater by immobilized peroxidase.

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