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Enzymatic oxidation of aqueous pentachlorophenol

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Abstract

The influence of the nonionic surfactant Tween 80 on pentachlorophenol (PCP) oxidation catalyzed by horseradish peroxidase was studied. The surfactant was tested at concentrations below and above its critical micelle concentration (CMC). Enhancement of PCP removal was observed at sub-CMCs. The presence of Tween 80 in the reaction mixture reduced enzyme inactivation which occurred through a combination of free radical attack and sorption by precipitated products. A simple first-order model was able to simulate time profiles for enzyme inactivation in the presence or absence of Tween 80. At supra-CMCs, the surfactant caused noticeable reductions in PCP removal, presumably through micelle partitioning of PCP which precluded the hydrophobic PCP molecule from interacting with the enzyme.

Key words: biocatalysis; horseradish peroxidase; pentachlorophenol; surfactant

Introduction

Pentachlorophenol (PCP) has been used extensively as a pesticide, herbicide, and wood-preserving agent at many wood treating sites. PCP is a probable human carcinogen and its presence in the environment is therefore of particular concern. In recent years many countries have banned the use of PCP. Unfortunately, past legal disposal practices coupled with the environmental stability of PCP have led to widespread contamination of soil, surface water, and groundwater aquifers.

Various methods are being developed to remove PCP from aqueous solution. Biodegradation of aqueous PCP by microorganisms has several advantages over chemical and physical methods, including mild operating conditions and better environmental compatibility. Several species of bacteria and fungi can biodegrade PCP. These organisms secrete a series of oxidative enzymes that are capable of catalyzing the oxidation of PCP. However, high concentrations of PCP can be inhibitory to the degrading organisms. As a result, direct application of isolated enzymes has been proposed as an alternative method of removing PCP from aqueous solution. Recent reviews on the *in vitro* use of oxidative enzymes to catalyze the oxidation of phenolic substances including PCP are available elsewhere (Durán and Esposito, 2000; Gianfreda *et al*., 2006; Torres *et al*., 2003). In the case of PCP oxidation, the enzymes that have been tested include horseradish peroxidase (Davidenko *et al*., 2004; Kim and Moon, 2005; Song *et al*., 2003; Tatsumi *et al*., 1996; Zhang and Nicell, 2000), laccase (Ricotta *et al*., 1996; Ullah *et al*., 2000), ligninase (Wang *et al*., 1999), and other extracellular peroxidases (Davila-Vazquez *et al*., 2005).

Because horseradish peroxidase (HRP) has been widely used to transform a wide range of phenolic contaminants, this communication focuses on the HRP-catalyzed oxidation of PCP in the presence of hydrogen peroxide (H_2O_2). As its name implies, HRP (EC 1.11.1.7) is isolated from the roots of horseradish (*Armoracia rusticana*). A comprehensive description of the structure, function, mechanism of action, and practical applications of HRP is available in the literature (Veitch, 2004). The HRP-catalyzed oxidation of PCP generates free aromatic radicals which combine to form polymers of low solubility that eventually precipitate from solution. The major product of the HRP-catalyzed oxidation of PCP over the pH range 4–7 is 2,3,4,5,6-pentachloro-4-pentachlorophenoxy-2,5-cyclohexadienone (PPCHD) (Kazunga *et al*., 1999), which is formed by the coupling of two pentachlorophenoxyl radicals, the expected products of one-electron oxidation reactions catalyzed by peroxidases.

Although the HRP-mediated oxidative coupling process has enormous potential for remediation of aqueous solution contaminated by PCP, its application is hampered by the low operational stability of HRP as a result of enzyme inactivation, which increases treatment costs owing to the high cost of HRP. This is considered a major drawback of the current enzymatic approach. To enhance the efficacy and cost-effectiveness of the enzymatic approach, various additives have been tested for their ability to

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minimize enzyme inactivation. These studies have shown that the oxidation of phenols catalyzed by peroxidases can be enhanced by adding additives such as surfactants, polyethylene glycol, chitosan, and gelatin (Sakurai *et al*., 2003; Tonegawa *et al*., 2003; Villalobos and Buchanan, 2002; Wu *et al*., 1997), but research relating to the influence of these additives on the peroxidase-catalyzed PCP oxidation is limited (Zhang and Nicell, 2000). The present study was therefore initiated to investigate the potential influence of a nonionic surfactant on the catalytic behavior of HRP in PCP transformation. The surfactant tested was Tween 80, a generally recognized safe ingredient for use in food processing, cosmetics, and household detergents.

1 Materials and methods

1.1 Materials

Horseradish peroxidase (type II), catalase, and hydrogen peroxide (30%, w/v) were purchased from Sigma Chemical Co. (St. Louis, USA). PCP was obtained from Janssen Chemical Co. (Geel, Belgium). Tween 80 was purchased from Shinyo Pure Chemical Co. (Osaka, Japan).

1.2 Enzyme activity assay and PCP determination

HRP activity was determined by a colorimetric assay in which a 3-ml assay was prepared from 0.3 ml of 5.33% (w/v) pyrogallol, 0.2 ml of 0.12 mol/L H_2O_2, 0.1 ml of enzyme solution, and 2.4 ml of 0.1 mol/L sodium phosphate buffer (pH 6.5). The concentration of active HRP is proportional to the rate of purpurogallin formation at 420 nm with an extinction coefficient of 6.129 g/(L·cm). One unit of HRP activity is defined as the number of milligrams of purpurogallin formed in 20 s at pH 6.5 and 25°C. For the HRP stock used in this study, 1 mg of HRP corresponded to 200 units as measured by this assay. PCP concentrations were measured using an HP 8452A UV spectrophotometer at 320 nm.

1.3 PCP oxidation

PCP stock solutions were prepared by dissolving PCP in 0.5 mol/L NaOH. Batch reactions were initiated by adding a measured dose of HRP to 20 ml of reaction mixtures containing PCP, H_2O_2, and Tween 80 in the desired quantities. The reaction mixtures, held at pH 6.5 with 0.1 mol/L sodium phosphate buffer and 25°C, were continuously stirred. Reactions were halted by adding catalase which rapidly converts residual H_2O_2 to oxygen and water. Reaction samples were treated with 0.2 mmol/L alum to enhance coagulation of colloidal particles and centrifuged at 5500 g for 20 min to settle the precipitated products. Residual PCP concentration and HRP activity were measured as described above. In addition, several preliminary experiments were conducted in the absence of Tween 80 to examine the effects of solution pH and temperature on the extent of PCP oxidation.

2 Results and discussion

2.1 Effects of pH and temperature

The HRP-catalyzed oxidation of PCP by H_2O_2 generates free aromatic radicals which combine to form polymers of low solubility that eventually precipitate from solution. As with any enzymatic reaction, HRP performance in PCP oxidation is affected by pH and temperature. Preliminary experiments were carried out in the absence of surfactant to examine the effects of these two factors on PCP transformation by HRP. The effect of pH was examined in the pH range 6–8.8. The results, as shown in Fig.1a, have been obtained with PCP and H_2O_2 concentrations at 1 mmol/L, a temperature of 25°C, and a reaction time of 30 min. It can be seen that the extent of PCP removal was significantly affected by pH of the solution. PCP removal reached a maximum at pH 6.5 and declined sharply when the pH was moved either side of the optimum value. The extent of PCP removal ranged from a maximum of 81% at pH 6.5 to a minimum of 5% at pH 8.8. Variations in pH lead to changes in the ionic form of the active site of the enzyme and may also alter the conformation of the enzyme. These changes may adversely affect the activity and stability of the enzyme.

The effect of temperature on PCP removal was less pronounced within the temperature range examined. The following conditions were used to obtain the results for the temperature effect shown in Fig.1b: PCP concentration was 0.94 mmol/L; H_2O_2 concentration was 1 mmol/L; HRP concentration was 0.15 μmol/L; pH was 6.5; reaction time was 60 min; and temperature range was 6–38°C. The optimum temperature was found to be 25°C and PCP removal dropped from a maximum of 51% at 25°C to a minimum of 42% at 6°C. Based on these results, all further experiments were held at pH 6.5 and 25°C.

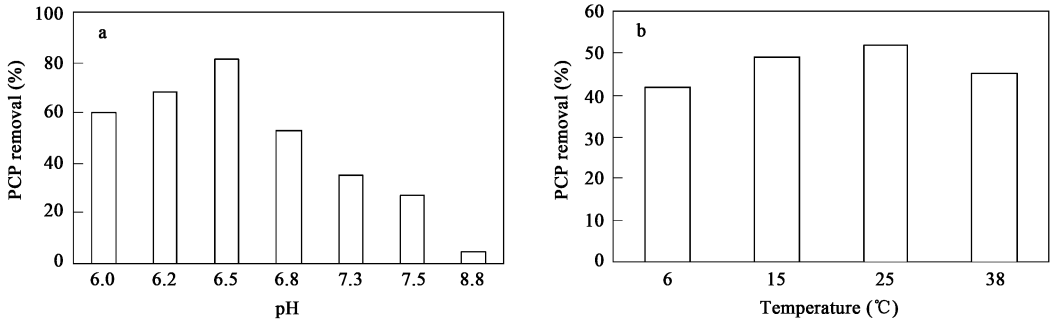

Fig. 1 Effect of pH (a) and temperature (b) on PCP oxidation.

2.2 Effect of surfactant

Surfactants are frequently used in detergents and food products to alter the properties of solution interfaces. They are characterized by the presence of both hydrophilic and hydrophobic moieties. They typically have a hydrophilic group, known as a head, and a hydrophobic chain, known as a tail. The four general classifications of surfactants, based on charged groups in the hydrophilic moiety, are cationic, anionic, nonionic, and amphoteric. At concentrations above the critical micelle concentration (CMC), surfactant molecules cluster together and start forming dynamic aggregates known as micelles. Brief characteristics of the nonionic surfactant tested in this study, Tween 80, are given in Table 1. Because the CMC of a surfactant may be changed by pH, temperature, and ionic strength, the CMC of Tween 80 ranging from 13 to 45 mg/L have been reported in the literature (Luning Prak and Pritchard, 2002; Yeh *et al*., 1998; Zheng and Obbard, 2002). The CMC of Tween 80 listed in Table 1 is taken from the comprehensive study of Patist *et al*. (2000) which was conducted under well-defined conditions.

Table 1 Characteristics of Tween 80

Structure	Average MW	CMC (mg/L)
Sorbitan oleate ester (ethylene oxide$_{20}$)	1310	36.7

The effect of Tween 80 on the extent of PCP removal in batch tests for two different enzyme concentrations is shown in Fig.2. In the absence of Tween 80, Fig.2 shows that 36% of the initial concentration of PCP was removed from a reaction mixture containing 2.25 mmol/L PCP, 2.25 mmol/L H_2O_2, and 0.07 μmol/L HRP in 60 min. Under the same test conditions but with Tween 80 present at 10–40 mg/L, the extent of PCP removal increased considerably to 88%–92% (solid symbols). However, the surfactant appeared to be inhibitory at concentrations above 40 mg/L. A similar PCP removal trend can be seen in the tests containing 0.03 μmol/L HRP (open symbols).

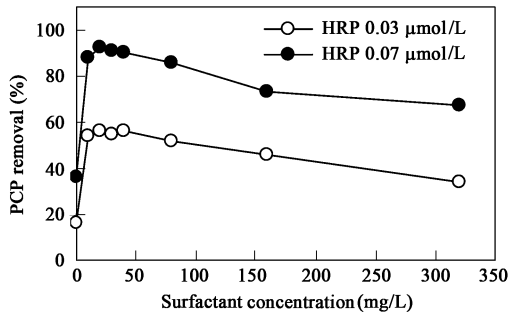

Fig. 2 Effect of Tween 80 on PCP oxidation for two different HRP concentrations. The reaction mixtures contained 2.25 mmol/L PCP and 2.25 mmol/L H_2O_2. The reaction time was 60 min.

At concentrations in excess of its CMC, Tween 80 monomers aggregate to form micelles that the hydrophobic PCP molecule can occupy. The reduction in PCP removal may thus be attributed to the reduced ability of HRP to gain access to micelle-partitioned PCP. These results suggest that enhancement of PCP removal by Tween 80 occurred at concentrations below the CMC. At concentrations above the CMC some of the PCP molecules were sequestered into the surfactant micelles and were thus precluded from interacting with the enzyme, resulting in lower PCP removal. These results agree with a previous study (Sakurai *et al*., 2003) that demonstrated the positive effect of other nonionic surfactants at sub-CMCs on phenol removal catalyzed by *Coprinus cinereus* peroxidase; however, supra-CMCs were not tested.

The positive effect of additives has been attributed to their ability to protect enzymes from inactivation. Fig.3 shows time profiles for enzyme inactivation in surfactant-free and surfactant-containing systems (symbols). Activity depletion curves calculated from a mathematical model are also included in Fig.3 (lines) and these are discussed later. It can be seen that inactivation of HRP occurred in the presence and absence of surfactant. For the surfactant-free system, an initial rapid rate of inactivation was observed to occur within the first 5 min of reaction, followed by a plateau of relatively slow inactivation. In the presence of Tween 80, HRP was inactivated during the reaction at much lower rates than in the surfactant-free system. HRP in the presence of 30 and 320 mg/L Tween 80 lost 67% and 49% of its initial activity, respectively, after a reaction time of 60 min. In contrast, the enzyme lost close to 95% of its initial activity in the absence of Tween 80 over the same reaction period.

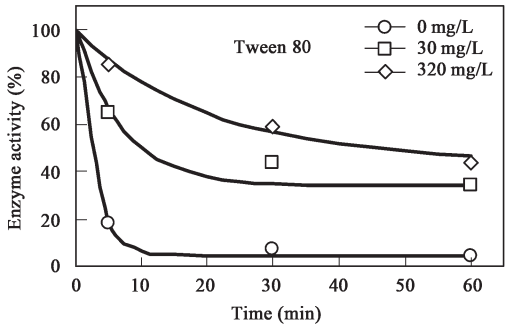

Fig. 3 Inactivation of HRP in the presence and absence of Tween 80. Solid curves are predictions calculated from Eq.(1).

The results in Fig.3 suggest that the susceptibility of HRP to inactivation can be reduced by some protection mechanisms associated with the surfactant. Similar protection of peroxidases from inactivation by various additives has been demonstrated in previous studies using phenols as the substrate (Sakurai *et al*., 2003; Tonegawa *et al*., 2003; Villalobos and Buchanan, 2002; Wu *et al*., 1997). Based on these studies, it has been proposed that enzyme inactivation occurs via three different mechanisms:

(1) Inactivation by the enzyme's own substrate, i.e., H_2O_2;

(2) Sorption by precipitated products; and

(3) Free radical attack.

Inactivation of HRP by H_2O_2 is only significant when no aromatic substrate is present (Nicell, 1994). This was not

the case for the three experiments reported in Fig.3 which are also displayed in Fig.2 (HRP concentration = 0.07 μmol/L). Fig.2 shows that considerable amounts of PCP remained in the reaction mixtures after 60 min of reaction. When the surfactant concentrations were 0, 30, and 320 mg/L, the amounts of residual PCP remaining at the end of reaction were 64%, 7%, and 33% of the initial PCP, respectively. Inactivation of HRP by H_2O_2 can therefore be ruled out under the experimental conditions of this study.

The second inactivation mechanism assumes that enzyme inactivation occurs when polymeric products adhere to HRP, hindering access of PCP to the enzyme's active site. It is postulated that the Tween surfactant can form complexes with HRP, thereby preventing the enzyme from associating with precipitated products. The third inactivation mechanism postulates that free radicals can bind to the active site of HRP, eliminating its catalytic ability. As mentioned earlier, the major product of the HRP-catalyzed PCP oxidation is PPCHD which is formed by the coupling of two pentachlorophenoxyl radicals (PPCHD chemical structure is shown in Fig.4). It may be inferred that Tween 80 is able to protect HRP primarily from inactivation by pentachlorophenoxyl radicals. The surfactant may associate with HRP in a way that the active site of HRP can be shielded from pentachlorophenoxyl radicals, effectively reducing the susceptibility of the enzyme to radical attack.

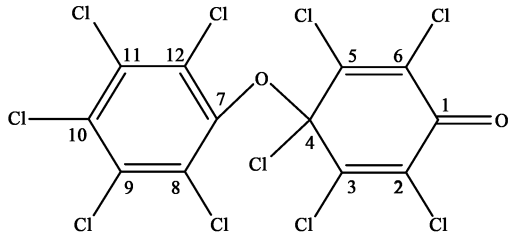

Fig. 4 Structure of 2,3,4,5,6-pentachloro-4-pentachlorophenoxy-2,5-cyclohexadienone (PPCHD).

2.3 Modeling of enzyme activity change

Mechanistic models for describing the effect of polyethylene glycol, a protective additive, on HRP activity in phenol removal are available in the literature (Buchanan and Nicell, 1999; Wu *et al*., 1998, 1999). These models in general consist of differential equations with several adjustable rate constants that require a numerical solution. We employ here a relatively simple first-order rate model in an effort to quantify the activity depletion trends reported in Fig.3:

$$E_A = \alpha \exp(-kt) + (1 - \alpha) \quad (1)$$

where, E_A is the residual enzyme activity (%) at time t (min), k is the first-order rate constant (min^{-1}), and α is the fraction of initial enzyme activity that is lost at the end of reaction. α values at t = 60 min for the three cases reported in Fig.3 are listed in Table 2. Eq. (1) was fitted to the data in Fig.3 to estimate the rate constant k using a nonlinear regression program based on a combination of the Gauss-Newton and Levenberg-Marquardt numerical algorithms. The best-fit k values are tabulated in Table 2. Activity depletion profiles calculated on the basis of this model are shown as solid lines in Fig.3, in comparison with the experimental results. It is evident that the three activity depletion profiles are well represented by the model. Given its empirical nature, the first-order model was found to be reasonably robust.

Table 2 Best-fit values of the first-order rate constant *k*

Surfactant concentration (mg/L)	α (%)	k (min^{-1})	R^2
0	95.5	0.39	0.999
30	66.0	0.14	0.967
320	56.0	0.05	0.989

3 Conclusions

The experimental results reported in this study indicate the potential of the nonionic surfactant Tween 80 to positively influence the HRP-catalyzed oxidation of PCP by H_2O_2. It was found that dosages of Tween 80 below its CMC enhanced PCP transformation and reduced HRP inactivation. These observations indicate that using nonionic surfactants in PCP removal could potentially increase enzyme performance and reduce treatment costs. The optimum pH and temperature were found to be pH 6.5 and 25°C, respectively.

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