Biotechnology and Bioprocess Engineering **2006**, 11: 462-465

Removal of Aqueous Pentachlorophenol by Horseradish Peroxidase in the Presence of Surfactants

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Abstract An important issue in the oxidation of pentachlorophenol (PCP) by the enzyme horseradish peroxidase (HRP) is enzyme inactivation during the reaction. This study was initiated to investigate the ability of two nonionic surfactants (Tween 20 and Tween 80) to mitigate HRP inactivation. The surfactants were tested at concentrations below and above their critical micelle concentrations (CMCs). Enhancement of PCP oxidation was observed at sub-CMCs, indicating effective protection of HRP by the two surfactants. Maximum levels of PCP removal were observed when the concentrations of Tween 20 and Tween 80 were 40 and 50% of the CMCs, respectively. At supra-CMCs, both surfactants caused a noticeable reduction in the extent of PCP removal.

Keywords: enzymatic catalysis, horseradish peroxidase, pentachlorophenol, surfactant

Pentachlorophenol (PCP) has been used extensively as a pesticide, herbicide, or wood-preserving agent. It is a probable human carcinogen and has been placed on the U.S. Environmental Protection Agency priority pollutant list. 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 treatment methods are being developed to remove PCP from contaminated environmental media. For example, enzyme-mediated oxidative reactions have been proposed as a promising method for treating aqueous PCP. Comprehensive reviews on the *in vitro* use of oxidative enzymes to catalyze the oxidation of phenolic substances including PCP are available in the literature [1,2]. In the case of enzyme-mediated PCP oxidation, the enzymes that have been tested include horseradish peroxidase (HRP) [3-7], laccase [8-10], ligninase [11], and other peroxidases [12,13].

The HRP-mediated PCP oxidation process was investigated in the current study. In the presence of hydrogen peroxide, PCP oxidation catalyzed by HRP generates free aromatic radicals that combine to form polymers of low solubility that eventually precipitate from solution. Although HRP has enormous potential for remediation of aqueous environments contaminated by PCP, enzyme inactivation under the reaction conditions that are generally encountered limits the extent of PCP removal. This shortcoming tends to compromise the economic viability of the enzymatic approach because enzymes are generally expensive.

Several approaches based on chemical modification, enzyme engineering, and immobilization have proven effective in mitigating enzyme inactivation, but the use of additives to preserve enzyme activity has attracted considerable attention due to its simplicity and cost-effectiveness. The positive effect of additives such as surfactants, polyethylene glycol, and gelatin on the oxidation of phenol catalyzed by peroxidases has been demonstrated in a number of earlier investigations [14-20]. However, research relating to the influence of these additives on HRP-mediated PCP oxidation is limited. Zhang and Nicell [5] investigated PCP transformation catalyzed by HRP in the presence of polyethylene glycol or chitosan. Their results showed that neither additive enhanced the extent of PCP removal. This observation suggests that the knowledge obtained from the widely studied phenol removal process is not enough to infer the roles of these additives in the PCP removal process.

The present study was initiated to investigate the potential influence of two nonionic surfactants on the catalytic behavior of HRP in PCP transformation. The two surfactants, Tween 20 (polyoxyethylene sorbitan monolaurate) and Tween 80 (polyoxyethylene sorbitan monooleate), were tested at concentrations below and above their critical micelle concentrations (CMCs).

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Table 1. Characteristics of Tween 20 and Tween 80

Surfactant	Structure	Average MW	CMC (mg/L)
Tween 20	Sorbitan laurate ester (ethylene $oxide_{20}$)	1228	51.6
Tween 80	Sorbitan oleate ester (ethylene $oxide_{20}$)	1310	36.7

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 the head, and a hydrophobic chain, known as the 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 CMC, surfactant molecules cluster together and start forming dynamic aggregates known as micelles. Brief characteristics of the two nonionic surfactants tested in this study, Tween 20 and Tween 80, are given in Table 1. The numeric values in the Tween nomenclature relate to the length of the carbon atom chain in the hydrophobic section of the molecule, *i.e.*, "20" represents C_{12} and "80" represents C_{18}. The CMCs of Tween 20 and Tween 80 listed in Table 1 are taken from Patist *et al.* [21]. The CMC of a surfactant may be changed by pH, temperature, and ionic strength.

Horseradish peroxidase (type II), catalase, and hydrogen peroxide (30% w/v) were purchased from Sigma (St. Louis, USA). PCP was obtained from Jassen Chemical Co. (Geel, Belgium). Tween 20 and Tween 80 were purchased from Shinyo Pure Chemical Co. (Osaka, Japan).

HRP activity was determined by a colorimetric assay in which a 3 mL solution was prepared from 0.3 mL of 5.33% w/v pyrogallol, 0.2 mL of 0.12 M H_2O_2, 0.1 mL of enzyme solution, and 2.4 mL of 0.1 M 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 sec 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 concentration was measured using an HP 8452A UV spectrophotometer at 320 nm.

A PCP stock solution was prepared by dissolving PCP in 0.5 N NaOH. Batch reactions were initiated by adding a measured dose of HRP to 20 mL of a reaction mixture containing PCP, H_2O_2, and either Tween 20 or Tween 80 in the desired quantities. The reaction mixtures, held at 25°C and pH 6.5 with 0.1 M sodium phosphate buffer, were stirred continuously. Reactions were halted by adding catalase, which rapidly converts residual H_2O_2 to oxygen and water. Reaction samples were treated with 0.2 mM alum to enhance coagulation of colloidal particles and centrifuged at 5,500 g for 20 min to settle the precipitated products. Residual PCP concentration and HRP activity were measured as described above.

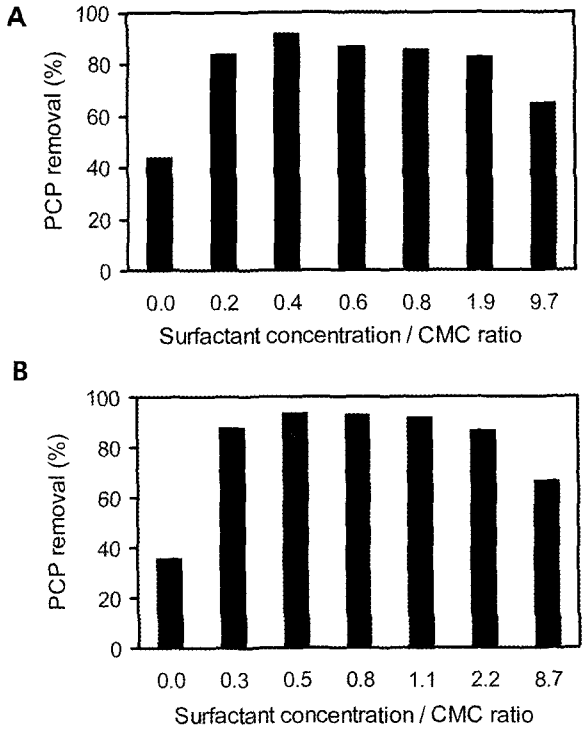

Fig. 1. (A) Effect of Tween 20 on HRP-mediated PCP oxidation. The reaction mixtures contained 2.25 mM PCP, 2.25 mM H_2O_2, 0.08 μM HRP, and varying amounts of Tween 20. The reaction time was 60 min. (B) Effect of Tween 80 on HRP-mediated PCP oxidation. The reaction mixtures contained 2.25 mM PCP, 2.25 mM H_2O_2, 0.07 μM HRP and varying amounts of Tween 80. The reaction time was 60 min.

The effects of the two surfactants on the extent of PCP removal in aqueous batch tests are shown in Fig. 1. The results are presented as percent PCP removed as a function of normalized surfactant concentration (ratio of actual surfactant concentration to the CMC listed in Table 1). Various normalized surfactant concentrations ranging from 0 to 9.7 and from 0 to 8.7 were tested with Tween 20 concentrations ranging from 0 to 500 mg/L and Tween 80 concentrations ranging from 0 to 320 mg/L, respectively.

In the absence of Tween 20, Fig. 1A shows that 44% of the initial concentration of PCP was removed from a reaction mixture containing 2.25 mM PCP, 2.25 mM H_2O_2, and 0.08 μM HRP in 60 min. Under the same test conditions but with Tween 20 present at a normalized surfactant concentration of 0.2, the extent of PCP removal increased considerably to 84%. However, increasing the normalized surfactant concentration to 0.4 resulted in only a small incremental increase in PCP removal. Adding Tween 20 up to twice its CMC did not improve the extent of PCP removal. The surfactant appeared to be slightly inhibitory at a normalized surfactant concentration of about 10, resulting in an overall PCP removal of 60%.

Similar PCP removal trends can be seen in the tests with Tween 80 as an additive. Fig. 1B shows that a Tween

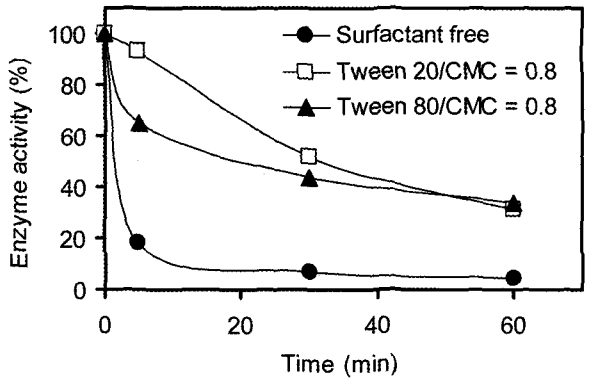

Fig. 2. HRP inactivation in the presence and absence of surfactant.

80-free test containing 2.25 mM PCP, 2.25 mM H_2O_2, and 0.07 μM HRP resulted in 36% PCP removal in 60 min. A normalized Tween 80 concentration of 0.3 improved the PCP removal to 88%. Addition of Tween 80 up to twice its CMC did not provide additional benefit. At a much higher surfactant concentration (8.7 times the CMC), the extent of PCP removal was only 31% higher than that of the surfactant-free test (67% vs 36%).

The surfactant concentrations at which noticeable reductions of PCP removal occurred were well above the CMCs of Tween 20 and Tween 80. Micelle formation occurs at surfactant concentrations above the CMC where the transition from a monomeric solution to a solution containing both monomers and micelles takes place. Further increase in surfactant concentration leads to an increase in the number of micellar aggregates, which are roughly spherical and have hydrophobic interiors and hydrophilic exteriors. The results in Fig. 1 suggest that the onset of micelle formation did not directly affect PCP removal until the surfactant concentration was about 9~10 times the CMC. It is postulated that at these surfactant concentration levels some of the hydrophobic PCP molecules became entrapped in the hydrophobic interior of the micelles. The reduction of PCP removal may thus be attributed to the reduced ability of HRP to gain access to these PCP molecules that have been partitioned into the micellar phase of the Tween surfactants.

Fig. 1 clearly shows that PCP removal levels observed in tests with the surfactants were higher than those of their surfactant-free counterparts. The positive effect of additives has been attributed to their ability to protect enzymes from inactivation. Fig. 2 shows time profiles for enzyme inactivation in surfactant-free and surfactant-containing systems. 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 occurred within the first 5 min of reaction, followed by a plateau of relatively slow inactivation. In the presence of Tween 20 or Tween 80 at a normalized concentration of 0.8, HRP was inactivated at much lower rates than in the surfactant-free system. The enzyme lost 69 and 66% of its initial activity in the presence of Tween 20 and Tween 80 after a reaction time of 60 min. In contrast, HRP lost close to 95% of its initial activity in the absence of surfactant over the same reaction period.

HRP inactivation can occur via three mechanisms: (1) inactivation by the enzyme's own substrate, *i.e.*, H_2O_2, (2) sorption by precipitated products, and (3) free radical attack. Guidelines are available in the literature for avoiding HRP inactivation by H_2O_2 [4]. According to these guidelines, inactivation of HRP by H_2O_2 can be ruled out under the experimental conditions of this study. The progressive loss of enzyme activity can therefore be attributed largely to the second and third inactivation mechanisms.

The results in Fig. 2 suggest that the susceptibility of HRP to inactivation by means of the second and third mechanisms can be reduced by some protection mechanism associated with the two Tween surfactants. The second inactivation mechanism is based on the assumption that HRP becomes entrapped and its active site occluded when large amounts of polymeric products are formed. It is postulated that the Tween surfactants can form complexes with HRP, thereby preventing the enzyme from associating itself with precipitated products.

The third inactivation mechanism postulates that free radicals can bind to the active site of HRP, eliminating its catalytic ability. Kazunga *et al.* [22] found that the major product of HRP-catalyzed PCP oxidation over the pH range 4~7 was 2,3,4,5,6-pentachloro-4-pentachlorophenoxy-2,5-cyclohexadienone (PPCHD). PPCHD is formed by the coupling of two pentachlorophenoxyl radicals, the expected products of one-electron oxidation reactions catalyzed by HRP and other peroxidases. It may be inferred that the Tween surfactants are able to interact with HRP in such a way that the active site of HRP can be shielded from pentachlorophenoxyl radicals, effectively reducing the susceptibility of the enzyme to free radical attack. There is clear evidence indicating that Tween 80 has the ability to form weak hydrophobic interactions with enzymes [23].

It should be mentioned that surfactant binding to enzyme may activate the enzyme, resulting in enhanced substrate conversion. Activation of enzymes by nonionic surfactants has been widely documented. For example, Gong *et al.* [24] reported that the apparent activity of the enzyme epoxide hydrolase increased by 1.8-fold in the presence of 0.5% w/v Tween 80. The greater extent of PCP removal observed in the presence of the two Tween surfactants is therefore attributable in part to mitigation of HRP inactivation, which is believed to occur through binding with free radicals and/or precipitated products, and also to increased interaction between activated HRP and PCP.

In summary, the experimental results reported in this study indicate the potential of the nonionic surfactants Tween 20 and Tween 80 to positively influence the HRP-mediated PCP oxidation process. Dosages of the two surfactants below their CMCs were found to enhance PCP transformation, probably by a combination of enzyme activation and mitigation of enzyme inactivation. These observations indicate that using the Tween surfactants in PCP removal could potentially increase enzyme perform-

ance and reduce treatment costs. The positive results suggest both surfactants are valuable additives for enhancing PCP removal and that further study on the roles of other nonionic surfactants in enzyme-mediated PCP removal is warranted.

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[Received May 2, 2006; accepted October 10, 2006]