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Letter

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Immobilized Horseradish Peroxidase (I-HRP) as Biocatalyst for Oxidative Polymerization of 2,6-Dimethylphenol

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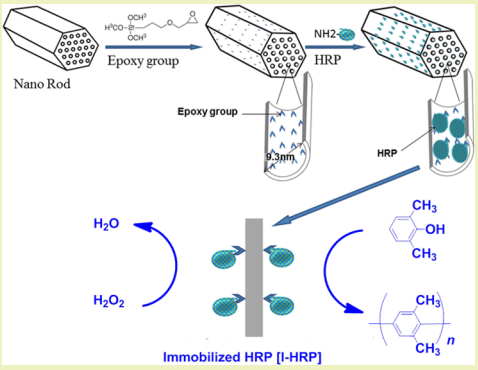
Supporting Information

ABSTRACT: An enzyme, horseradish peroxidase (HRP), was immobilized on silica nanorods and employed as a catalyst for the oxidative polymerization of 2,6-dimethylphenol. With this catalytic system, the polymer, poly(2,6-dimethyl-1,4-phenylene oxide), was successfully obtained from a water−acetone solvent system. The immobilized HRP exhibited substantially enhanced enzymatic activity toward oxidative polymerization as well as some degree of reusability compared with free HRP.



KEYWORDS: *Immobilized enzyme, Polyphenylene oxide, Oxidative polymerization, Water solvent, Horseradish peroxidase*

INTRODUCTION

Hay et al. discovered the oxidative polymerization of 2,6-dimethylphenol (DMP) in organic solvents in the presence of copper-amine complexes as catalysts can form poly(2,6-dimethyl-1,4-phenyleneoxide) (PPO).[1] PPO is an important engineering thermoplastic material with outstanding characteristics such as high melting point and good toughness over a wide temperature range and also with self-extinguishing and electrically insulating properties.[2] Many researchers have developed novel catalysts for PPO synthesis via oxidative polymerization.[3−11] The use of natural enzymes as catalysts is highly desirable and provides a green methodology for PPO synthesis.[12−20]

There has been much interest in enzyme-catalyzed polymerizations as these reactions can be performed under mild conditions (ambient temperature and pressure, physiological pH) in an environmentally acceptable solvent (water). In addition, the enzyme itself can usually be derived from renewable resources.[20]

Horseradish peroxidase (HRP) is the enzyme most commonly used in enzymatic oxidative polymerizations.[15−19] HRP is known to oxidize phenols and form radicals on phenols, and these radicals can react to form linear polymers via C−O coupling when only *o*-protected phenols such as 2,6-dimethylphenol are used. These radicals can also be used to decompose phenolic compounds when HRP is added to phenolic compounds.[21] HRP has an Fe-containing porphyrin-type structure and is known to catalyze oxidation of phenols and aniline derivatives using H_2O_2 as oxidizing agent. However, there are some drawbacks of HRP catalyzed polymerization such as its instability in harsh environments such as elevated temperature and organic solvents. Another drawback is the relatively short lifetime of HRP in organic solvents, which makes it difficult to recycle the enzyme. It is desirable to use less organic solvent and have reusable enzymes for practical applications of enzymatic oxidative polymerizations.

Enzyme immobilization is used in biocatalysis to improve the stability and recyclability of enzymes.[22−27] This immobilization process allows enzymes to be applied under extreme conditions, such as high temperature, high and low pH, and complex biological environments.[28] It has been suggested that different immobilization techniques such as noncovalent adsorption or desorption, encapsulation, covalent attachment, and cross-linking of enzymes may be employed to prepare suitable biocatalysts.[22] We have already reported the synthesis and characterization methods of immobilized HRP enzyme into silica nanorods.[25] Immobilization of HRP on mesoporous nanorods was carried out in the reaction of lysine residues of HRP with the epoxy functional groups on the mesoporous silica nanorods under basic conditions.[26,27,29]

In this letter, we describe the use of HRP immobilized silica nanorods (I-HRP) as a new type of enzyme catalyst for the oxidative polymerization of DMP (Scheme 1). In our experiments, the oxidative polymerization of DMP was carried out in the presence of I-HRP as a catalyst under different ratios of water−organic solvent mixture systems. We demonstrated

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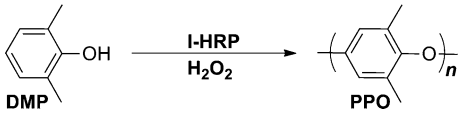
Scheme 1. Enzymatic Polymerization of DMP with Immobilized HRP



the enzymatic activity, stability, and reusability of I-HRP in the presence of small amounts of acetone.

EXPERIMENTAL METHOD

I-HRP Catalyzed Polymerization. The typical polymerization procedure was undertaken as follows. Under air, 2,6-dimethylpenol (0.31 g, 2.5 mmol) and immobilized horseradish peroxidase (I-HRP) (100 mg of HRP immobilized particles) in a mixture of acetone (5.0 mL) and aqueous acetate buffer (20.0 mL, 0.1M, pH 5.0) with a total volume of 25 mL were placed in a flask. The mixture was stirred, and hydrogen peroxide (30% aqueous solution 28 μL, 0.25 mmol) was added every 15 min at room temperature until a total of 280 uL had been added. The stirring was continued for a total reaction time of 24 h when the reaction mixture was removed and centrifuged to separate I-HRP as a precipitate. The supernatant was extracted with chloroform (25 mL), and the chloroform layer was collected and evaporated under vacuum. All products were further dried under vacuum at room temperature, and the molecular weights of the samples were measured by GPC. Weight yield 40%. ^{1}H NMR (400 MHz, $CDCl_3$): δ_H(ppm) 2.06−2.21, (m, 6H, CH_3), 6.41−7.09 (m, 2H, ArH). ^{13}C NMR (400 MHz, $CDCl_3$): δ_C(ppm) 16.8, 114.45, 128.6, 132.5, 145.50, 154.7.

RESULTS AND DISCUSSION

The I-HRP catalyzed oxidative polymerization of DMP was performed at room temperature for 24 h under air. Hydrogen peroxide was employed as the oxidizing agent. Previous research based on free HRP catalyzed polymerization, reported that the highest yield of polymer was obtained in pH 5 buffer solutions of mixed water and acetone.[19] Therefore, 0.1 M acetate buffer at pH 5 was selected as the aqueous phase mixed with acetone. We aimed to decrease the amount of acetone and used two different proportions (5% and 20%) of acetone with 100 mg of I-HRP in the 25 mL-scale polymerization. The GPC analysis for the PPO polymerization was carried out on the reaction products (Figure 1). No monomer peak was evident in all the GPC results. These GPC results indicated that the monomer (DMP) was totally converted to polymer in both buffer−acetone solvent systems.

For comparison, polymerization of DMP using I-HRP in pure acetone was also carried out. The GPC chromatogram showed that monomer conversion to polymer in acetone was low (10%), and there was a clear monomer peak present at 18 min retention time (Figure 1). The activity of free HRP was reported to be affected by the composition of aqueous and organic solvents.[30−32] The low catalytic activity of I-HRP in acetone may be due to the active sites in enzymes being altered by the acetone, and also there might be some denaturation of the enzyme, even the immobilized HRP. These results further suggest that a water−acetone solvent system favors I-HRP catalytic activity compared with organic solvents (acetone) only.

Table 1 summarizes the highest molecular weight of the polymer obtained in the above three experiments and monomer conversion. On the basis of the GPC data, 5% v/v acetone in acetate buffer solution at pH 5 afforded the polymer of highest molecular weight (Table 1, entry 2 M_w = 1.9 × 10^3, M_w/M_n = 2.1). The amount of organic solvent had less effect

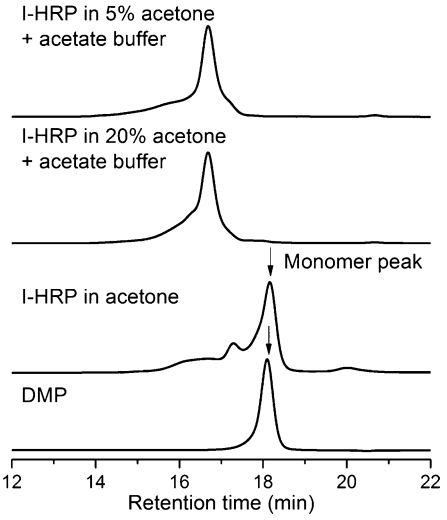


Figure 1. GPC chromatograms of monomer [2,6-dimethylphenol (DMP)] and after polymerization in different solvent systems; monomer peak indicated by arrow.

Table 1. Measured GPC Values of I-HRP Catalyzed Polymerization of 2,6-Dimethyl Phenol Polymer formed in Different Solvent Systems[a]

entry	solvent	M_w
1	5% acetone + 0.1 M acetate buffer (pH 5.0)	1.7 × 10^3
2[b]	5% acetone + 0.1 M acetate buffer (pH 5.0)	1.9 × 10^3
3	20% acetone + 0.1 M acetate buffer (pH 5.0)	1.4 × 10^3
4	acetone only	9.0 × 10^2
5	80% acetone + 0.1 M acetate buffer (pH 5.0)	1.0 × 10^3
6	5% acetone + 0.1 M phosphate buffer (pH 7.4)	1.1 × 10^3

[a]All reactions except entry 2: H_2O_2 was added every 15 min up to a total of 280 μL. [b]H_2O_2 was added every 15 min up to a total of 560 μL

on the molecular weight. The structure of the polymer formed was analyzed by ^{1}H and ^{13}C NMR spectroscopy and confirmed the PPO structure (Supporting Information). On the basis of these results, we have selected 5% v/v acetone in acetate buffer solution at pH 5 as a condition for further polymerizations.

It is known that enzymes often have decreased activities after immobilization.[33] We first compared free HRP and I-HRP for their catalytic performance using the standard substrate 3,3′,5,5′-tetramethylbenzidine [TMB][34] in aqueous solution. The concentration of HRP immobilized on the nanorods after the immobilization reaction was estimated to be about 22.5 mg g^{-1} silica based on the mass balance as previously reported.[25] Indeed, there is significant decrease in activity observed for the TMB reaction in I-HRP.

The activity of 100 mg of I-HRP was only equivalent to 0.13 mg of free HRP for the reaction of TMB. Despite this significant decrease in activity for its standard substrate, the impact on enzymatic polymerization is less severe. Polymerization reactions were carried out with 2 and 10 mg of free HRP and 100 mg of I-HRP in 5% v/v acetone in acetate buffer solution (25 mL) at pH 5 to compare the polymerization with different enzyme quantities. The molecular weight of each reaction was analyzed by GPC (Figure 2). The GPC chromatograms showed that the polymerization undertaken using 10 mg of free HRP and 100 mg of HRP immobilized nanorods led to total conversion to polymer. However, the monomer conversion was less in the reaction undertaken with 2

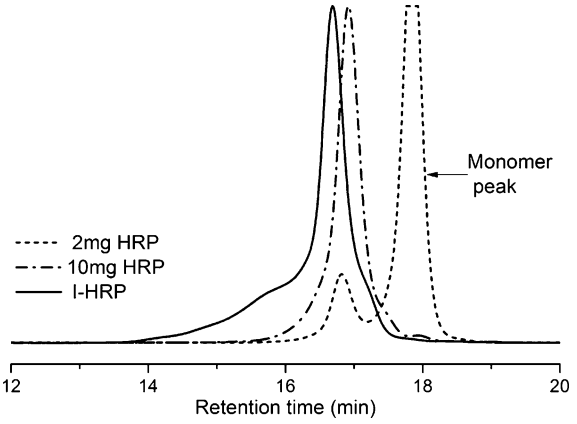


Figure 2. Comparison of GPC chromatograms showing polymerization of DMP using different amounts of HRP and 100 mg I-HRP.

mg of free HRP (Figure 2). The GPC chromatogram of the polymer obtained using 2 mg of free HRP showed that there is a clear monomer peak at 18 min retention time. The GPC results also indicated that the highest molecular weight of the polymer obtained using 100 mg of I-HRP ($M_w = 1.7 \times 10^3$) was higher than the reaction with 10 mg of free HRP ($M_w = 8.0 \times 10^2$). The molecular weight of polymer obtained from the polymerization using 100 mg of I-HRP (whose activity for TMB is equal to 0.13 mg of free HRP) was even higher than the one using 2 mg of free HRP. This result indicates that I-HRP is more catalytically active than free HRP for oxidative polymerization of DMP in buffer and solvent mixture.

Despite the decrease in activity, the solid immobilized catalyst can provide the advantages of stability and reusability of the catalyst. In order to examine the reusability of this I-HRP, the same immobilized enzyme sample was used consecutively in three polymerization reactions. After each polymerization reaction, I-HRP was separated using centrifugation and collected and used again for the next polymerization without drying the enzyme. GPC results showed that the first polymerization gave the highest monomer conversion (100%) with $M_w = 1.7 \times 10^3$ (Figure 3). In the second run, the enzyme activity of the I-HRP still remained and gave a similar molecular weight polymer to the first run ($M_w = 1.7 \times 10^3$); however, monomer conversion was reduced to 45%. The third run again gave a similar molecular weight polymer with low monomer conversion (about 10%). Monomer conversion dropped from 100% to 10% after the third run. These results suggested that the activity of I-HRP decreased after the reuse; however, some amount of catalytic activity in I-HRP still remained even after the third recycle, which indicated that the immobilization of HRP onto the silica nanorods increased the stability of the HRP as free HRP cannot be reused. The decrease in catalytic activity of the enzyme after the second cycle may be due to the loss of immobilized particles during recovery of nanoparticles or denaturing of the enzyme after a few cycles of polymerization. The IR spectrum of I-HRP after the centrifugation from each cycle showed bands that corresponded to PPO such as a single band at 1188 cm^{-1} for C−O−C stretching (Supporting Information). This result suggests that the PPO adsorptions to the surface of the I-HRP after centrifugation can be the reason for the decrease in catalytic activity during consecutive polymerization runs. In addition, there was no HRP leaching from the support during the reaction (Supporting Information). At this stage, the exact reason for the decrease in the enzyme catalytic activity is unknown. More studies are required to optimize the immobilization conditions and the nature of solid supports that might be used. For example, the use of magnetic particles can be used to improve the recovery and reusability of immobilized HRP to obtain 100% recovery of the immobilized enzymes.

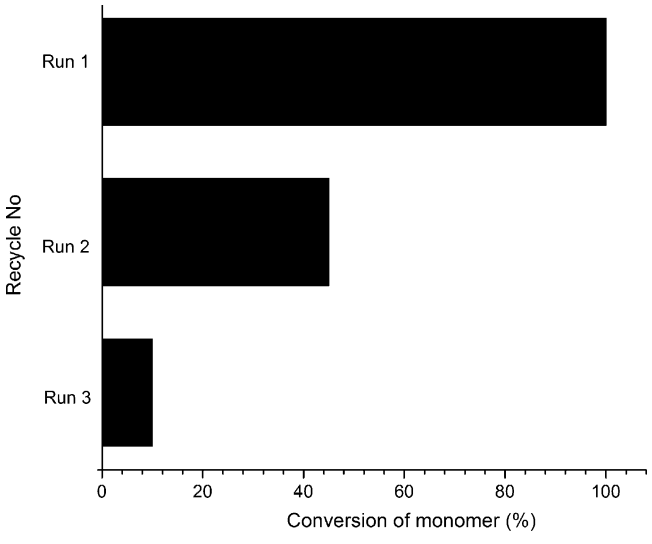


Figure 3. Recycling of I-HRP in polymerization of PPO.

CONCLUSIONS

In summary, we have demonstrated I-HRP can be used as a new biocatalyst for the oxidative polymerization of DMP in a water−acetone solvent system. I-HRP exhibited substantially enhanced enzymatic activity as well as some degree of reusability compared with the free HRP.

ASSOCIATED CONTENT

Supporting Information

Methods: I-HRP preparation, enzyme catalytic activity by TMB, I-HRP reusability in PPO polymerization, and control reaction with silica nanorods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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