

## Effect of Ionic Liquid on the Kinetics of Peroxidase Catalysis

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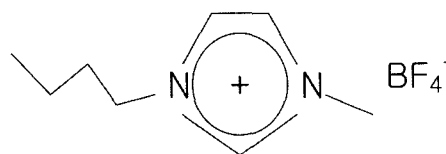
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**Abstract** The effect of a water-miscible ionic liquid, 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF<sub>4</sub>]), on the horseradish peroxidase (HRP)-catalyzed oxidation of 2-methoxyphenol (guaiacol) with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was investigated. HRP maintains its high activity in the aqueous mixtures containing various concentrations of the ionic liquid and even in 90% (v/v) ionic liquid. In order to minimize the effect of solution viscosity on the kinetic constants of HRP catalysis, the enzymatic reactions in the subsequent kinetic study were performed in water-ionic liquid mixtures containing 25% (v/v) ionic liquid at maximum. As the concentration of [BMIM][BF<sub>4</sub>] increased for the oxidation of guaiacol by HRP, the *K<sub>m</sub>* value increased with a slight decrease in the *k<sub>cat</sub>* value: The *K<sub>m</sub>* value increased from 2.8 mM in 100% (v/v) water to 22.5 mM in 25% (v/v) ionic liquid, indicating that ionic liquid significantly weakens the binding affinity of guaiacol to HRP.

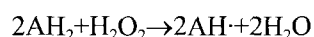
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Some ionic liquids maintain their liquidity at room temperatures lower than 100°C. These ionic liquids usually consist of bulky organic cations in association with inorganic anions. Unlike the common organic solvents, ionic liquids are nonvolatile, nonflammable, and highly stable, and thus, possessing great potential as environment-friendly green solvents. Applications of ionic liquids as reaction solvents for chemical, biochemical, and electrochemical processes have drawn much interest since the beginning of the 21<sup>st</sup> century [7, 18, 21]. Fig. 1 shows the structure of the ionic liquid, 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF<sub>4</sub>]), one of the few ionic liquids miscible with either water or polar organic solvents [18], which was used in this study.



**Fig. 1.** Structures of the ionic liquid, [BMIM][BF<sub>4</sub>], composed of a bulky organic cation in association with a small inorganic anion.

Peroxidases are a class of enzymes that catalyze the oxidation of a wide variety of aromatic compounds, including substituted phenols and aromatic amines, in the presence of peroxides. As described in the following equation, the overall catalytic reaction of a peroxidase is to oxidize two molecules of an aromatic compound (AH<sub>2</sub>) per molecule of a peroxide, such as H<sub>2</sub>O<sub>2</sub> producing the corresponding product radicals (AH·) and water. The product radicals further undergo rapid radical coupling and transfer reactions to form oligomers or polymers.



Applications of peroxidases have been exploited for environmental purposes such as the removal of recalcitrant phenolic pollutants [1, 9, 13, 14, 17] of higher concentrations than those for microbial treatments [2, 6, 20] and the development of benign alternative processes to produce phenolic polymers to replace phenol-formaldehyde resins [3, 13, 16]. Those processes using peroxidases have been carried out mostly in organic solvents with limited amount of added water, thereby entailing possible harmful effects on human health as well as on environments because of the vapors of the organic solvents.

Peroxidase catalysis in ionic liquids, therefore, holds great promise toward the development of green processes to solve environmental problems. Although many enzyme reactions have been tested in a variety of ionic liquids, most of the studies were concerned about the measurement of activity or stability of enzymes without focusing on the

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effects of ionic liquids on the kinetic behavior of the enzymatic catalysis [5, 8, 11, 12]. In this work, we investigated the effect of ionic liquids on the kinetics of HRP-catalyzed oxidation of guaiacol, a representative substrate of peroxidases, with  $H_2O_2$  as an oxidant. The kinetics of HRP's catalysis was studied systematically in order to elucidate the mechanism of interaction between the ionic liquid and the enzyme.

## MATERIALS AND METHODS

### Materials

The ionic liquid (<0.1% water, <0.01% chloride), [BMIM][BF<sub>4</sub>], was purchased from C-Tri Co. (Korea). HRP (Type II, RZ=2) and other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.) and used as received.

### Assay of HRP Activity

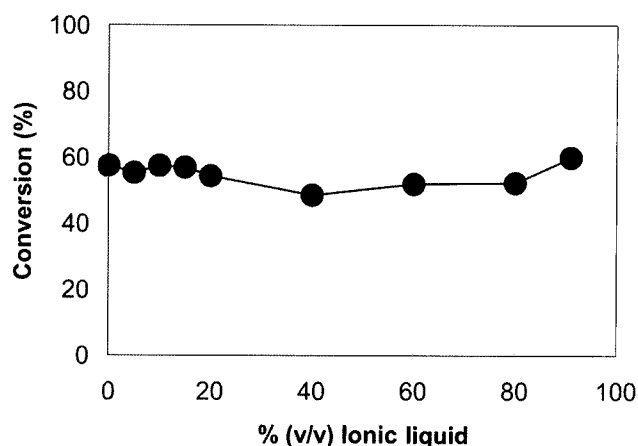
HRP-catalyzed oxidation of guaiacol with  $H_2O_2$  in the aqueous mixtures of the ionic liquid was performed in a 1-ml spectrophotometer cuvette at 25°C. The initial rate of HRP's catalytic reaction to oxidize guaiacol was measured spectrophotometrically by following the increase of absorbance at 436 nm of the reaction mixtures within the first 1 min after the addition of  $H_2O_2$  to a solution containing HRP and guaiacol [4]. A buffer solution (20 mM potassium phosphate, pH 7.0) was used as the aqueous component of the enzyme reaction solutions. The initial concentration of  $H_2O_2$  in all the reaction mixtures was 0.2 mM. For the kinetic experiment, the concentration of guaiacol was varied between 1 mM to 20 mM, and the concentration of HRP was approximately 6 nM, assuming the molecular weight of HRP as 42,000 [19]. In order to convert the spectrophotometric data into the amounts of guaiacol oxidized, some of the reaction samples were analyzed simultaneously by both a spectrophotometer and HPLC.

### HPLC Analysis

The concentration of guaiacol required to prepare the standard calibration curve was analyzed using a high-performance liquid chromatograph (HPLC) equipped with a Waters  $\mu$ Bondapak C<sub>18</sub> column (3.9×300 mm) and a UV-visible detector. Guaiacol was detected at 280 nm. The mobile phase was constituted of an acetonitrile-water mixture (50:50, v/v) at a flow rate of 1 ml/min.

### Viscosity Measurement

Viscosity of the aqueous mixtures of ionic liquid was measured with an Ostwald viscometer at 25°C. Water (0.89 cp), butyl alcohol (2.54 cp), 50% (v/v) glycerol (7.40 cp), sulfuric acid (24.2 cp), and cyclohexanol (57.5 cp) were used as standard materials [10] for the conversion of the data from the Ostwald viscometer into the corresponding viscosity values in centipoises (cp) for water-ionic liquid mixtures.

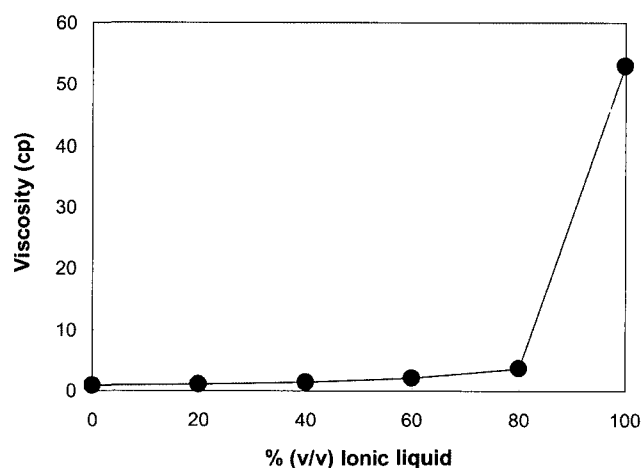


**Fig. 2.** Effect of ionic liquid, [BMIM][BF<sub>4</sub>], on the conversion of guaiacol with  $H_2O_2$  catalyzed by HRP for 4 h at 25°C. Initial concentrations of guaiacol,  $H_2O_2$ , and HRP were 0.4 mM, 0.2 mM, and 1.2  $\mu$ M, respectively. The content of the ionic liquid was varied from 0 to 90% (v/v).

## RESULTS AND DISCUSSION

### Effect of Ionic Liquid on the Activity of HRP

Preliminary study on the effect of the ionic liquid, [BMIM][BF<sub>4</sub>], on HRP activity was performed with the initial presence of 0.4 mM guaiacol and 0.2 mM  $H_2O_2$  at the stoichiometric ratio. The HRP concentration was 1.2  $\mu$ M, assuming the molecular weight of HRP as 42,000. The reaction mixtures were incubated for 4 h at 25°C with shaking at 150 rpm. Fig. 2 shows that the conversion (%) of guaiacol by  $H_2O_2$  catalyzed by HRP changed little in the aqueous mixtures containing various concentrations of the ionic liquid from 0% to 90% (v/v).

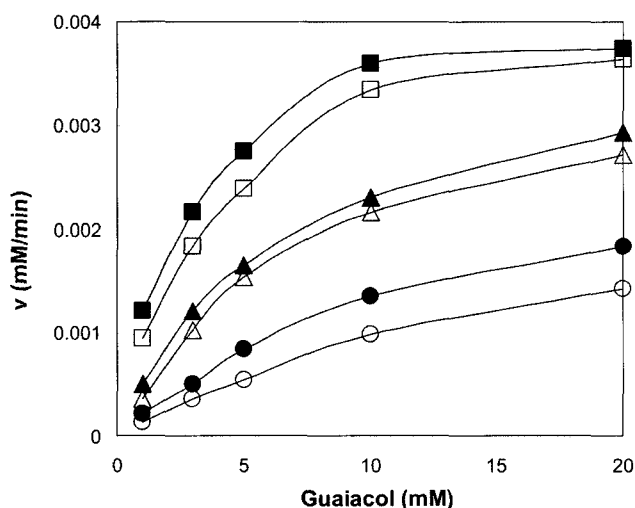


**Fig. 3.** Effect of ionic liquid, [BMIM][BF<sub>4</sub>], on the solution viscosity at 25°C. Water (0.89 cp), butyl alcohol (2.54 cp), 50% (v/v) glycerol (7.40 cp), sulfuric acid (24.2 cp), and cyclohexanol (57.5 cp) were used as standard materials [10].

More than half of the initial amount of guaiacol was oxidized by  $\text{H}_2\text{O}_2$  in 4 h in all the aqueous mixtures of the ionic liquid. Although the theoretical stoichiometric oxidation ratio of an aromatic donor to  $\text{H}_2\text{O}_2$  is 2:1 in the peroxidase catalysis, the experimentally determined maximum values of the moles of an aromatic compound oxidized per mole of a peroxide were close to 1.5:1, as evidenced in many studies [4, 13]. The molar ratios of guaiacol oxidized to mole of  $\text{H}_2\text{O}_2$  calculated from the data in Fig. 2 was over 1.1:1, reaching almost 75% of the maximum molar oxidation ratio of aromatic donors to  $\text{H}_2\text{O}_2$ , which can be experimentally obtainable. Therefore, it can be concluded that HRP maintains its high catalytic activity even in an aqueous solution containing 90% (v/v) ionic liquid.

#### Effect of Ionic Liquid on the Kinetics of HRP Catalysis

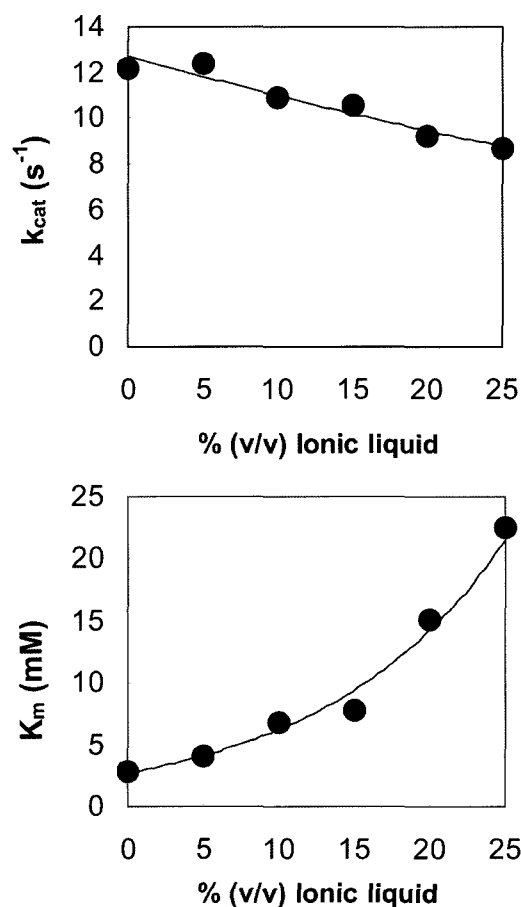
To elucidate the mechanisms of the effect of ionic liquid on HRP catalysis, the preliminary experiment described in the previous section was not enough to give any clues at a microscopic level. Therefore, the following experiments were carried out to determine the variation of two enzymatic kinetic constants of HRP, turnover number ( $k_{\text{cat}}$ ) and Michaelis constant ( $K_{\text{m}}$ ), in the aqueous mixtures containing increasing concentrations of the ionic liquid. Since fast enzymatic reactions are influenced by solvent viscosity [15], changes in solvent viscosity due to the addition of the ionic liquid with a very high viscosity need to be minimized in order to obtain the kinetic constants unbiased by the changes in solvent viscosity. Fig. 3 shows that the viscosity of the aqueous mixtures of the ionic liquid increased markedly when more than 50% (v/v) of the ionic liquid was added to the aqueous buffer. Another potential bias in the kinetic



**Fig. 4.** Michaelis-Menten-type reaction of HRP for the initial oxidation rate ( $v$ ) of guaiacol with  $\text{H}_2\text{O}_2$  at  $25^\circ\text{C}$  in the reaction mixtures containing 0% (■), 5% (□), 10% (▲), 15% (△), 20% (●), and 25% (○) (v/v) ionic liquid. Initial concentrations of  $\text{H}_2\text{O}_2$  and HRP were 0.2 mM and 6 nM, respectively.

study of enzyme catalysis is the effect of high concentrations of ionic liquids on an enzyme's structural integrity, entailing changes in enzyme stability within the time frame of the reaction rate measurement. In order to exclude those two potential biases in determining kinetic constants for HRP catalysis, we conducted a kinetic study with the aqueous mixtures containing the ionic liquid up to 25% (v/v) at maximum. Fig. 4 shows that the catalytic reaction of HRP to oxidize guaiacol with  $\text{H}_2\text{O}_2$  followed typical Michaelis-Menten kinetics with saturation at high guaiacol concentration in all the water-ionic liquid mixtures.

The two kinetic constants, turnover number ( $k_{\text{cat}}$ ) and Michaelis constant ( $K_{\text{m}}$ ), were determined by fitting the initial reaction rate data in Fig. 4 to the Michaelis-Menten equation using a nonlinear least-squares method. Fig. 5 shows the changes in the values of  $k_{\text{cat}}$  and  $K_{\text{m}}$  as the concentration of the ionic liquid in the reaction mixtures increased from 0% to 25% (v/v). The notable effect of the ionic liquid on the HRP catalysis was that the  $K_{\text{m}}$  value increased almost nine-fold as the concentration of the ionic liquid increased from 0% to 25% (v/v). This result infers



**Fig. 5.** Effects of ionic liquid, [BMIM][ $\text{BF}_4$ ], on the values of  $k_{\text{cat}}$  and  $K_{\text{m}}$ , in the HRP-catalyzed oxidation of guaiacol with  $\text{H}_2\text{O}_2$  at  $25^\circ\text{C}$ .

that the binding affinity of guaiacol to HRP significantly weakens owing to the presence of the ionic liquid in the reaction solution. In contrast, the  $k_{cat}$  value in 25% (v/v) ionic liquid decreased slightly to 70% of the value in 0% ionic liquid. These results imply that the ionic liquid may act as an inhibitor. However, the exact mechanism of the role played by the ionic liquid in the HRP catalysis can be elucidated only after the effects of the ionic liquid on the thermodynamic states of the reaction components have been deciphered.

## Acknowledgment

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