Effects of Two Different Ionic Liquids, [BMPy][BF_4] and [BMIM][$MeSO_4$], on the Kinetics of Horseradish Peroxidase

Ensik Hong, Ik-Keun Yoo, and Keungarp Ryu

***Abstract*—The effect of two water-miscible ionic liquids, 1-butyl-3-methypyridinuim tetrafluoroborate ([BMPy][BF_4]) and 1-butyl-3-methylimidazolium methylsulfate ([BMIM][$MeSO_4$]), on the horseradish peroxidase-catalyzed oxidation of 2-methoxyphenol (guaiacol) with hydrogen peroxide (HRP) was investigated. In order to minimize the effect of solution viscosity on the kinetic constants of HRP catalysis, the enzymatic reactions for the kinetic study were performed in water-ionic liquid mixtures containing 25% (v/v) ionic liquid at maximum. As the concentration of [BMPy][BF_4] increased, the K_m value increased with a slight decrease in the k_{cat} value for the oxidation of guaiacol by HRP: The K_m value increased markedly from 2.8 mM in 100% water to 12.6 mM in 25% (v/v) ionic liquid indicating that ionic liquid significantly weakens the binding affinity of guaiacol to HRP. On the contrary, as the concentration of [BMIM][$MeSO_4$] increased, the K_m value decreased to 1.4 mM in 25% (v/v) ionic liquid. [BMIM][$MeSO_4$] also decreased k_{cat} from 13.8 s^{-1} in 100% water to 4.1 s^{-1} in 25% (v/v) ionic liquid. These results indicate that the ionic liquids interact with the enzyme at the molecular level as well as at a thermodynamic macroscopic scale.**

***Index Terms*—guaiacol, ionic liquid, kinetics, peroxidase**

I. INTRODUCTION

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, thus, possessing great potential as environmentally 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 21st century [1, 2]. 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_2) per molecule of a peroxide, such as H_2O_2 producing the corresponding product radicals ($AH\cdot$) and water. The product radicals further undergo rapid radical coupling and transfer reactions to form oligomers or polymers.

$$2AH_2 + H_2O_2 \rightarrow 2AH\cdot + 2H_2O$$

Applications of peroxidases have been exploited for the environmental purposes such as the removal of recalcitrant phenolic pollutants [3-5] of higher concentrations than those for microbial treatments [6-8] and the development of benign alternative processes to produce phenolic polymers to replace phenol-formaldehyde resins [5, 9, 10]. 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 due to the vapors of the organic solvents.

Peroxidase catalysis in ionic liquids, therefore, holds a 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 on the measurement of activity or stability of enzymes without focusing on the effects of ionic liquids on the kinetic behavior of the enzymatic catalysis [2, 11, 12]. In this work, we investigated the effect of two water-miscible ionic liquids, 1-butyl-3-methypyridinuim tetrafluoroborate ([BMPy][BF_4]) and 1-butyl-3-methylimidazolium methylsulfate ([BMIM][$MeSO_4$]), on the kinetics of HRP catalyzed oxidation of guaiacol, a representative substrate of peroxidases, with H_2O_2 as an oxidant. Kinetics of HRP's catalysis was studied systematically in order to elucidate the mechanism of interaction between the ionic liquids and the enzyme.

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Keungarp Ryu is with the School of Chemical Engineering and Bioengineering at the University of Ulsan, Ulsan 680-749, Korea (phone: 82-52-259-2822; fax: 82-52-259-1689; e-mail:kgryu@ mail.ulsan.ac.kr).

Eusik Hong was with the School of Chemical Engineering and Bioengineering at the University of Ulsan, Ulsan 680-749, Korea. He is now with the SK Chem. Co., Ulsan, Korea.

Ik-Keun Yoo is also with the School of Chemical Engineering and Bioengineering at the University of Ulsan, Ulsan 680-749, Korea (ikyoo@uou2.ulsan.ac.kr).

II. MATERIALS AND METHODS

Materials

The ionic liquids (< 0.1 % water, < 0.01% chloride), [BMPy][BF$_4$] and [BMIM][MeSO$_4$] were 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's 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℃. 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 [13]. 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 while the concentration of guaiacol was varied between 1 mM to 20 mM. The concentration of HRP was approximately 6 nM, assuming the molecular weight of HRP as 42,000 [14]. 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.

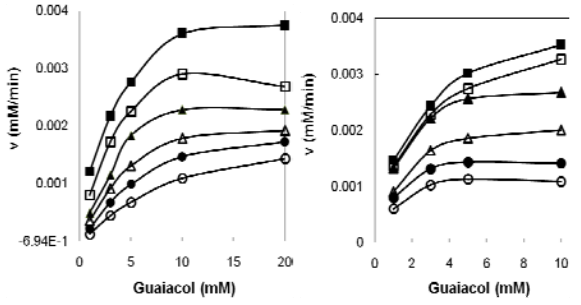
HPLC Analysis

The concentration of guaiacol required to prepare the standard calibration curve was analyzed using high performance liquid chromatograph (HPLC) equipped with a Waters μBondapak C$_{18}$ column (3.9x300 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.

III. RESULTS AND DISCUSSION

Effect of Ionic Liquid on the Activity of HRP

The effect of the two ionic liquids on the HRP's activity was studied with the initial presence of 0.4 mM guaiacol and 0.2 mM H_2O_2 at the stoichiometric ratio. HRP concentration was 1.2 μM assuming the molecular weight of HRP is 42,000. The reaction mixtures containing up to 90% (v/v) of the ionic liquids were incubated for 4 h at 25℃ with shaking at 150 rpm. More than half of the initial amount of guaiacol was oxidized by H_2O_2 in 4 h in all the aqueous mixtures of the ionic liquid (data not shown here). Although theoretical stoichiometric oxidation ratio of an aromatic donor to H_2O_2 is 2:1 in the peroxidase catalysis, 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 [12,15]. The molar ratios of guaiacol oxidized to mole of H_2O_2 initially present are over 1.1:1, which are almost 75% of the maximum molar oxidation ratio of aromatic donors to H_2O_2, which were experimentally obtainable. These results show that HRP maintains its high catalytic activity even in an aqueous solution containing 90% (v/v) ionic liquid.



(A) (B)

Fig. 1. Michaelis-Menten type reaction of HRP for the initial oxidation rate (v) of guaiacol with H_2O_2 at 25℃ in the reaction mixtures containing 0% (■), 5% (□), 10% (▲), 15% (△), 20% (●), and 25% (○) (v/v) [BMPy][BF$_4$] (A) and [BMIM][MeSO$_4$] (B). Initial concentrations of H_2O_2 and HRP were 0.2 mM and 6 nM, respectively.

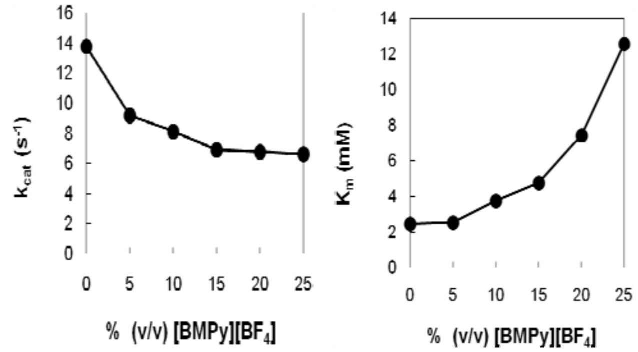


Fig. 2. Effects of ionic liquid, [BMPy][BF$_4$], on the values of k_{cat} and K_m, in the HRP-catalyzed oxidation of guaiacol with H_2O_2 at 25℃.

Effect of Ionic Liquid on the Kinetics of HRP Catalysis

To elucidate the mechanisms of the effect of ionic liquids on HRP's catalysis, the variation of two enzymatic kinetic constants of HRP, turnover number (k_{cat}) and Michaelis constant (K_m), in the aqueous mixtures containing increasing concentration of the ionic liquid were determined. Since fast enzymatic reactions were known to be influenced by solvent viscosity [16], the changes in solvent viscosity were maintained negligible by adding the ionic liquids up to 25% (v/v) at maximum. Fig. 1 shows that the catalytic reaction of HRP to oxidize guaiacol with H_2O_2 followed the typical Michaelis-Menten kinetics with saturation at high guaiacol concentration in all the water-ionic liquid mixtures.

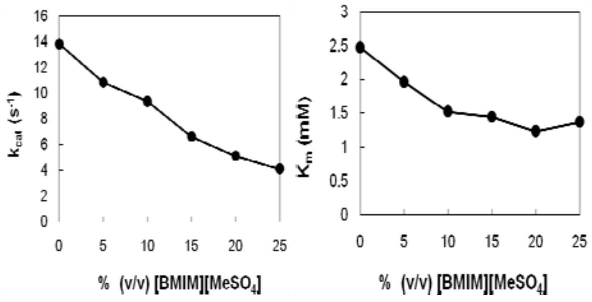


Fig. 3. Effects of ionic liquid, [BMIM][MeSO$_4$], on the values of k_{cat} and K_m, in the HRP-catalyzed oxidation of guaiacol with H_2O_2 at 25℃.

The two kinetic constants, turnover number (k_{cat}) and Michaelis constant (K_m), were determined by fitting the initial reaction rate data in Fig. 1 to the Michaelis-Menten equation using a non-linear least squares method. Fig. 2 and Fig. 3 show the changes in the values of k_{cat} and K_m as the content of the ionic liquids in the reaction mixtures increased from 0% to 25% (v/v).

For the aqueous mixtures of [BMPy][BF$_4$], K_m value increased almost five-fold as the concentration of the ionic liquid increased from 0% to 25% (v/v) (from 2.8 mM in 100% water to 12.6 mM in 25% (v/v) ionic liquid). This result infers that the binding affinity of guaiacol to HRP significantly weakens due to the presence of the ionic liquid in the reaction solution. In contrast, k_{cat} value in 25% (v/v) [BMPy][BF$_4$] decreased to almost 50% of the value in 0% ionic liquid.

For the aqueous mixtures of [BMIM][MeSO$_4$], K_m value decreased as the concentration of the ionic liquid increased from 0% to 25% (v/v) (from 2.8 mM in 100% water to 1.4 mM in 25% (v/v) ionic liquid). This result is the opposite to that in the aqueous mixtures of [BMPy][BF$_4$]. However, k_{cat} value in 25% (v/v) [BMPy][BF$_4$] decreased three times of the value in 100% water.

The above results imply that each of the ionic liquids may affect the catalysis of HRP in different ways. Clearly, the ionic liquids will affect the macroscopic thermodynamic state of the substrate causing the alteration of the solubility of the substrate in solution. Considering the fact that the two ionic liquids possess the similar macroscopic properties such as polarity, dielectric constant, etc., the two ionic liquids may exert common effect on the thermodynamic property of the substrate in solution. This will lead to the changes in K_m value in a similar direction by the two ionic liquids. Our results, however, implicate that the ionic liquids have additional effects on the HPR's catalysis. The decrease in k_{cat} values upon the addition of the two ionic liquids showed that these ionic liquids also act as inhibitors of HRP, the mechanism of which should be deciphered in further studies. In conclusion, our study elucidated that the ionic liquids affect the catalysis of HRP at the molecular levels as well as at a macroscopic thermodynamic state.

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