

Effects of Structural Difference of Ionic Liquids on the Catalysis of Horseradish Peroxidase

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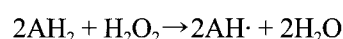
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The dependence of the catalytic properties of horseradish peroxidase on the structural changes of ionic liquids was investigated with two water-miscible ionic liquids, *N*-butyl-3-methylpyridinium tetrafluoroborate ([BMP₃][BF₄]) and 1-butyl-3-methylimidazolium methylsulfate ([BMIM][MeSO₄]), each of which shares an anion (BF₄⁻) or a cation (BMIM⁺) with 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF₄]), respectively. The oxidation of guaiacol (2-methoxyphenol) with H₂O₂ was used as a model reaction. In order to minimize the effect of solution viscosity on the kinetic constants of the enzymatic catalysis, the enzymatic reactions for the kinetic study were performed in water–ionic liquid mixtures containing 25% (v/v) ionic liquid at maximum. Similarly to the previously reported results for [BMIM][BF₄], as the concentration of [BMP₃][BF₄] increased, the K_m value increased with a decrease in the k_{cat} value: 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 the enzyme. On the contrary, [BMIM][MeSO₄] decreased the K_m value to 1.4 mM in 25% (v/v) ionic liquid. [BMIM][MeSO₄] also decreased k_{cat} more than 3-folds [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 macroscopic thermodynamic scale. Specifically, the anionic component of the ionic liquids influenced the catalysis of horseradish peroxidase in different ways.

Keywords: Anion, cation, ionic liquid, kinetics, peroxidase

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. Therefore, they possess 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 [8, 18, 21]. 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 (AH₂) per molecule of a peroxide, such as H₂O₂ 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, 10, 17] of higher concentrations than those for microbial treatments [2, 7, 20] and the development of benign alternative processes to produce phenolic polymers to replace phenol–formaldehyde resins [3, 14, 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, owing 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 about the measurement of activity or stability of enzymes without focusing on the effects of ionic liquids on the kinetic behavior of the enzymatic catalysis [5, 9, 12, 13].

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In our previous works, we explored the kinetics of horseradish peroxidase (HRP)-catalyzed oxidation of guaiacol, a representative substrate of peroxidases, with H_2O_2 as an oxidant in the aqueous mixtures of 1-butyl-3-methylimidazolium ([BMIM][BF₄]) in order to elucidate the mechanism of interactions between ionic liquids and the enzymes [6, 11]. We reported that [BMIM][BF₄] affects the catalysis of HRP in two ways. The ionic liquid exerts a macroscopic thermodynamic effect to stabilize the substrate in solution, thereby increasing the K_m values. It also has a microscopic molecular interaction with the enzyme, acting as a weak noncompetitive inhibitor for HRP to cause the decrease of the k_{cat} values [6].

In this work, we investigated the effects of two other water-miscible ionic liquids, *N*-butyl-3-methylpyridinium ([BMP_y][BF₄]) and 1-butyl-3-methylimidazolium ([BMIM][MeSO₄]), on the kinetics of HRP. As shown in Fig. 1, [BMP_y][BF₄] possesses an anion (BF₄⁻), and [BMIM][MeSO₄] a cation (BMIM⁺), in common with [BMIM][BF₄]. Our study revealed how structural features of ionic liquids influence enzymatic catalysis in a more general sense, both on the microscopic molecular and macroscopic thermodynamic levels.

MATERIALS AND METHODS

Materials

The ionic liquids (<0.1% water, <0.01% chloride) 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.

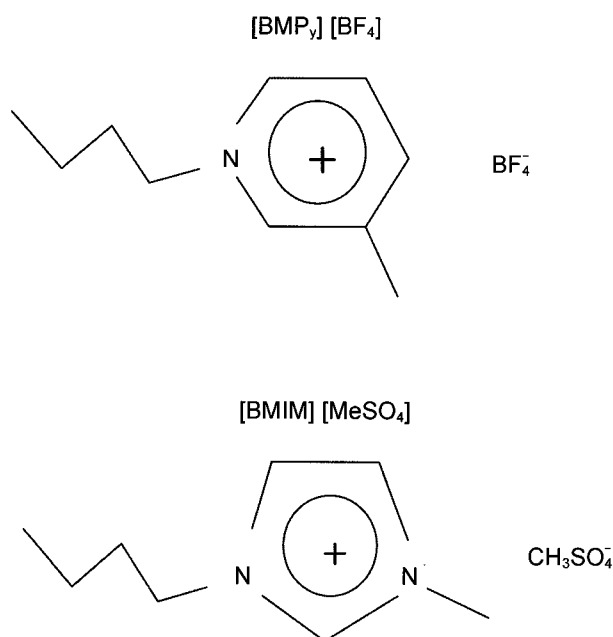


Fig. 1. Structures of the two ionic liquids, [BMP_y][BF₄] and [BMIM][MeSO₄], which share either a cation, (BMIM⁺), or an anion, (BF₄⁻), with [BMIM][BF₄].

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. Initial rates of the HRP catalytic reaction to oxidize guaiacol were 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 and 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 calibration line was analyzed using high performance liquid chromatography (HPLC) equipped with a Waters μ Bondapak C₁₈ column (3.9×300 mm) and a UV-visible detector. Guaiacol was detected at 280 nm. The mobile phase consisted of an acetonitrile–water mixture (50:50, v/v) at a flow rate of 1 ml/min.

RESULTS AND DISCUSSION

Effect of Ionic Liquids on the Activity of HRP

The effect of the two ionic liquids on the HRP activity was studied 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 M, assuming the molecular weight of HRP to be 42,000. The reaction mixtures containing up to 40% (v/v) [BMIM][MeSO₄] and 90% (v/v) [BMP_y][BF₄] were incubated for 4 h at 25°C with shaking at 150 rpm. Owing to the formation of precipitates above 40% (v/v) [BMIM][MeSO₄], the content of this ionic liquid was maintained below 40% (v/v). Fig. 2 shows that 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 liquids. The theoretical stoichiometric oxidation ratio of an aromatic donor to H_2O_2 is 2:1 in the peroxidase catalysis. However, 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, 14]. The molar ratios of guaiacol oxidized to mole of H_2O_2 initially present were over 1:1, implying that HRP maintained its high catalytic activity, even in an aqueous solution containing 90% (v/v) [BMP_y][BF₄].

Effect of Ionic Liquid on the Kinetics of HRP Catalysis

To elucidate the mechanisms of the effects of ionic liquids on HRP catalysis, the variation of two enzymatic kinetic constants of HRP, turnover number (k_{cat}) and Michaelis

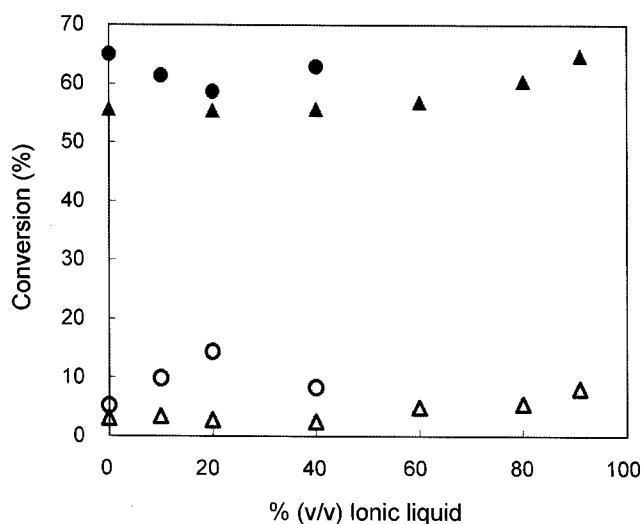


Fig. 2. Effect of [BMP_y][BF₄] (▲, △) and [BMIM][MeSO₄] (●, ○) on the conversion of guaiacol with H₂O₂ catalyzed by HRP for 4 h at 25°C.

Initial concentrations of guaiacol, H₂O₂, and HRP were 0.4 mM, 0.2 mM, and 1.2 μM, respectively. The content of the ionic liquid was varied from 0 to 90% (v/v). Empty symbols represent the blank reactions in the corresponding water–ionic liquid mixtures in the absence of the enzyme.

constant (K_m), in the aqueous mixtures containing increasing concentration of the ionic liquids was determined. The results were also compared with our previous experimental findings with [BMIM][BF₄], which shares the anion BF₄⁻ with [BMP_y][BF₄] and the cation BMIM⁺ with [BMIM][MeSO₄], to estimate the magnitude of the effects an anion or a cation exerts on the catalysis of HRP. Since fast enzymatic reactions were known to be affected by solvent viscosity [15], the changes in solvent viscosity were maintained negligible by adding the ionic liquids up to 25% (v/v) at a maximum, as in our previous reports [6, 11]. Fig. 3 shows that the catalytic reaction of HRP followed typical Michaelis–Menten kinetics, with saturation at high guaiacol concentrations in all the water–ionic liquid mixtures.

The two kinetic constants, turnover number (k_{cat}) and Michaelis constant (K_m), were determined by fitting the initial reaction rate data in Fig. 3 to the Michaelis–Menten equation using a nonlinear least squares method. Figs. 4 and 5 show the changes in the values of k_{cat} and K_m as the content of the ionic liquids increased from 0% to 25% (v/v) in the reaction mixtures.

For the aqueous mixtures of [BMP_y][BF₄], the K_m value increased almost 5-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). In contrast, the k_{cat} value in 25% (v/v) [BMP_y][BF₄] decreased to almost 50% of the value in 0% ionic liquid. These results are similar to the result reported previously with [BMIM][BF₄] and infer that the binding affinity of guaiacol to HRP significantly weakens because of the presence

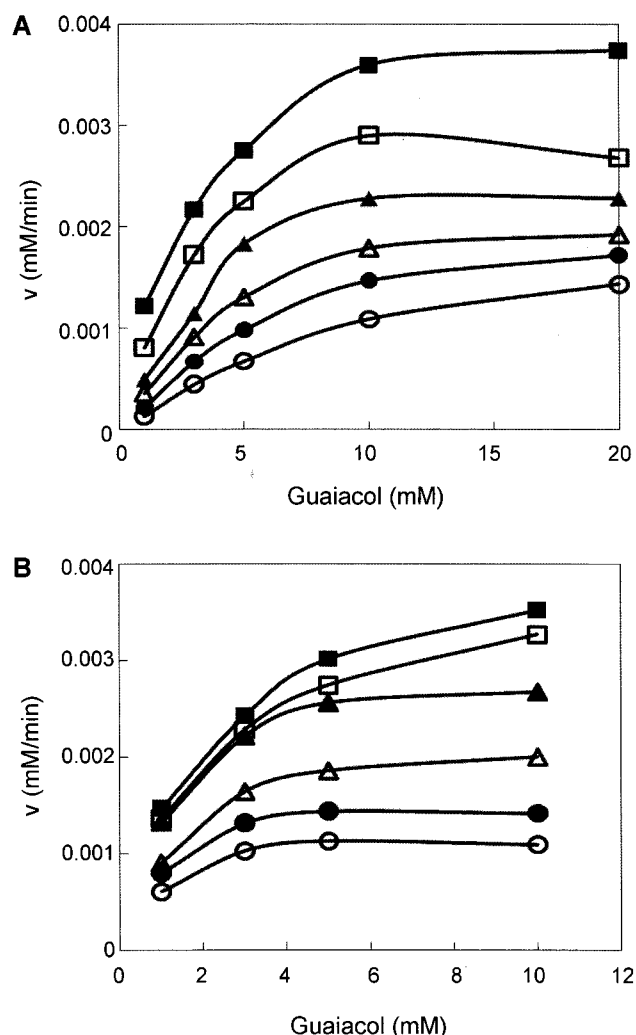


Fig. 3. Michaelis–Menten type reaction of HRP for the initial oxidation rate (v) of guaiacol with H₂O₂ at 25°C in the reaction mixtures containing 0% (■), 5% (□), 10% (▲), 15% (△), 20% (●), and 25% (○) (v/v) [BMP_y][BF₄] (A) and [BMIM][MeSO₄] (B). Initial concentrations of H₂O₂ and HRP were 0.2 mM and 6 nM, respectively.

of the ionic liquid in the reaction solution as a result of the strong substrate stabilizing effects of the ionic liquid [6].

For the aqueous mixtures of [BMIM][MeSO₄], on the contrary, the 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 [BMP_y][BF₄]. However, the k_{cat} value in 25% (v/v) [BMP_y][BF₄] decreased three times of the value in 100% water.

The above results imply that alteration of the anion of ionic liquids may affect the enzymatic catalysis quite distinctively, whereas changing the cation may not significantly alter the way ionic liquids effect enzyme catalysis. Clearly,

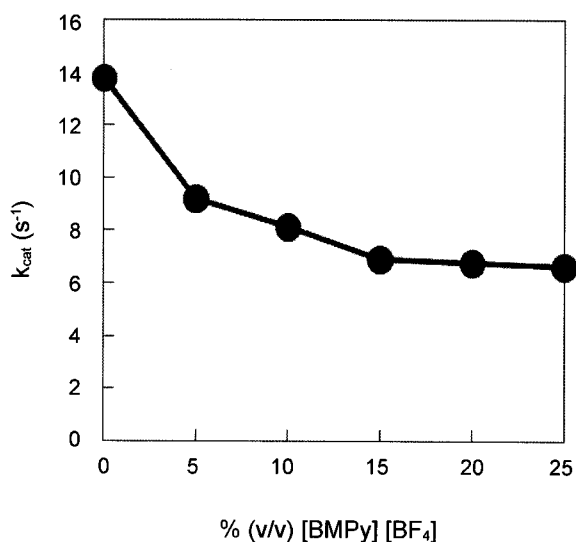


Fig. 4. Effect of [BMPy][BF₄] on the values of k_{cat} and K_m in the HRP-catalyzed oxidation of guaiacol with H₂O₂ at 25°C.

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 similar macroscopic properties, such as polarity, dielectric constant, etc., the two ionic liquids may exert common effects 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 HRP 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. In

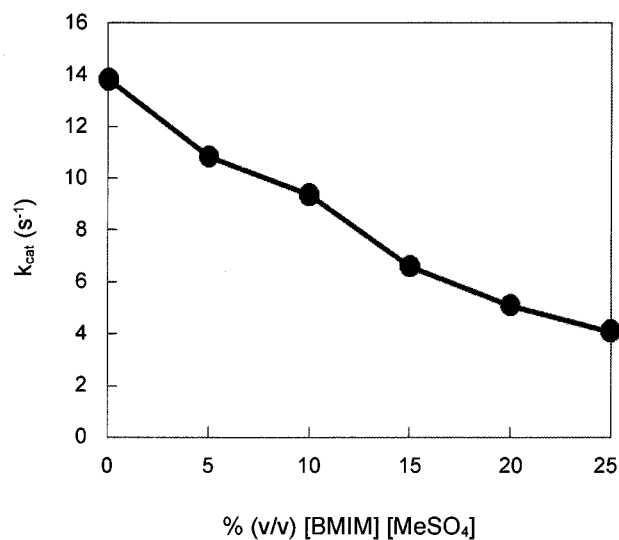


Fig. 5. Effect of [BMIM][MeSO₄] on the values of k_{cat} and K_m in the HRP-catalyzed oxidation of guaiacol with H₂O₂ at 25°C.

addition, an anionic component plays a more dominant role than the cationic counterpart in the enzymatic catalysis.

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