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Improved activity of horseradish peroxidase (HRP) in 'specifically designed' ionic liquid

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Abstract—The ionic liquid (IL), tetrakis (2-hydroxyethyl) ammonium triflouromethanesulfonate is rationally designed for horseradish peroxidase (HRP) on the basis of its stability and activity in the presence of an excipient, tris(hydroxymethyl)aminoethane (TRIS) in different ILs. The activity of HRP in this tailor made IL is at least 30–240-fold higher than that in conventional ILs. Also, the activity is more than 10 times greater than that in methanol, a common organic solvent used for HRP.

Research in the arena of ionic liquids (ILs)[1–3] has witnessed significant progress mainly due to their eco-friendly nature and designer flexibility. The application of these green solvents in the field of biocatalysis is also increasing rapidly. The merits of ILs in enzyme catalysis can be exploited if the enzyme exhibits improved efficiency within these green solvents. Significant enhancement in the stability and activity of enzymes in ILs could increase their versatility in biotransformations. Although preliminary reports on the hydrolytic activity of lipase and chymotrypsin in ILs are available,[4–14] literature on oxido-reductase enzymes has rapidly increased in recent years.[15–22] However, the efficiency of biocatalysts in conventional imidazolium based ILs is not substantial enough to substitute organic solvents. To this end, Walker and Bruce were the first to describe the design of an ionic liquid in which the cation contained a hydroxyalkyl group to stabilize the dissolved enzyme (alcohol dehydrogenase).[23,24] On the basis of this concept, herein, we report the design of ILs containing multiple hydroxyethyl groups for dissolving horseradish peroxidsase (HRP) while maintaining high activity by improving the compatibility of the enzyme in ILs. The stability and activity of HRP was investigated in different ILs (**1**–**6**, Fig. 1). To improve HRP activity further, the hydrophilic lyoprotectant tris(hydroxymethyl)aminoethane (TRIS) was applied as excipient[25] in the various ILs. The enhanced activity in the presence of TRIS led us to design novel ILs (**7**–**8**, Fig. 1), tailor made for the enzyme. The activity of HRP in **8** increased ~30–240-fold compared to that observed in other ILs. Stereoselective oxidation of thioanisole by HRP in the newly developed IL was also demonstrated.

The ILs ([bmim][Br], **2**–**4**) were prepared following microwave assisted synthesis and subsequent ion-exchange of the bromide ions by hydroxide ions which were neutralized by the respective acids for **2**–**4**. ILs **5** and **6** were synthesized directly from [bmim][Br] by stirring with ammonium tetrafluoroborate and hexafluorophosphoric acid, respectively. IL **7** was synthesized by quaternization of triethanolamine with 2-chloroethanol while **8** was prepared by subsequent replacement of the chloride anion of **7** with hydroxide which was neutralized by triflic acid. Detailed synthetic procedures with analytical data are given in the Supplementary data.

To improve the activity/stability of HRP in ILs, it was essential to study the dependence of HRP on the components of the ILs. Initially, the stability of HRP was investigated in three ILs, namely [bmim][Cl] (**1**), [bmim][CF_3CO_2] (**2**) and [bmim][alanine] (**3**). While **1** and **2** are conventional ILs, **3** is a relatively new IL, whose role in biocatalysis has not yet been probed. HRP loses half of its initial activity within 24 h at 5% water content in **1** and **2** (Fig. 2).[26a] Rapid deactivation

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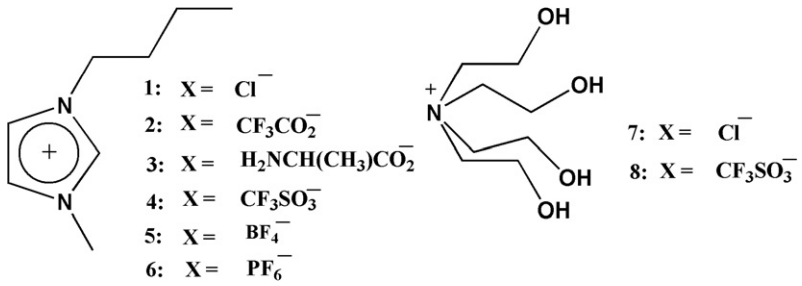

Figure 1. Structures of the ionic liquids.

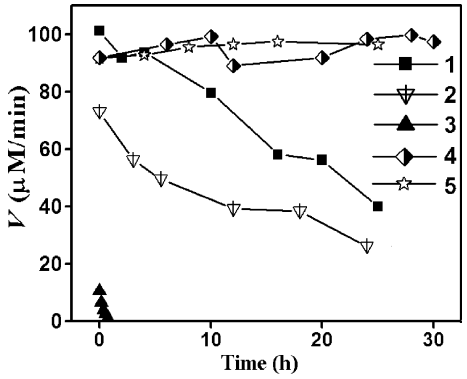

Figure 2. Stability of HRP in the ILs with 5% water. The concentration of HRP in the cuvette was 1 μg/mL. [Guaiacol] = 0.3 mM, $[H_2O_2]$ = 0.1 mM. The experimental error in these measurements was typically within ±8–12% in triplicate measurements.

of HRP in **1** and **2** led us to introduce **3**,[27] to determine whether the presence of an amino acid component in the IL would improve the activity and stability of the enzyme. However, IL **3** was found to be quite detrimental to the enzyme, causing irreversible deactivation. HRP becomes inactive within an hour of incubation in **3** (Fig. 3b) and also the stability was lower than that found in **1** (see Supplementary data) and **2** (Fig. 3a). The deactivation rate increases with decreasing the water content from 70% to 5% for all the three ILs (Fig. 3a and b for **2** and **3**, respectively, see Supplementary data for **1**) while the order of deactivation was **3** > **2** > **1**, particularly at lower water content. The high deactivation of HRP in **2** and **3** could be due to the inhibiting effect of the carboxylate anion, which binds to the sixth coordination position of Fe(III) in HRP, and thereby blocks further access to the substrate.[28,29] At high water (low IL) content, the enzyme active site becomes sufficiently hydrated to restore its natural conformation. Inhibition by the counterion is also reduced, resulting in greater stability of HRP. However, when the enzyme is exclusively dispersed in **3** at 0% water (lyophilized HRP), it takes 24 h for complete deactivation. In the presence of water, the active site of HRP is possibly more accessible to the inhibiting counterions of **2** and **3**, leading to its deactivation. To ascertain the influence of the anionic component of the IL, the stability of HRP was investigated (Fig. 3c) in [bmim][CF_3SO_3] (**4**), where $CF_3SO_3^-$ is an ineffective inhibitor of HRP. The results were encouraging as the enzyme was stable for up to 30 h irrespective of the water content in **4** and was stable for longer time even at 0% water.

In another common IL, [bmim][BF_4] (**5**), the stability of HRP was found to be comparable to that in **4** at high water content. Between 30% and 50% deactivation of HRP was observed in **5** at ⩽40% water content (Fig. 3d). In concurrence with earlier reports,[15,30] the stability of HRP was again hampered in the medium where **5** was the major component, presumably due to binding of fluoride anions released from tetrafluoroborate to the heme iron. The stability of HRP was also investigated in a hydrophobic IL, [bmim][PF_6] (**6**), where the lyophilized enzyme was quite stable for up to 24 h. However, further study in the presence of water could not be carried out due to the immiscibility of the enzyme solution with **6**. On the basis of the preceding

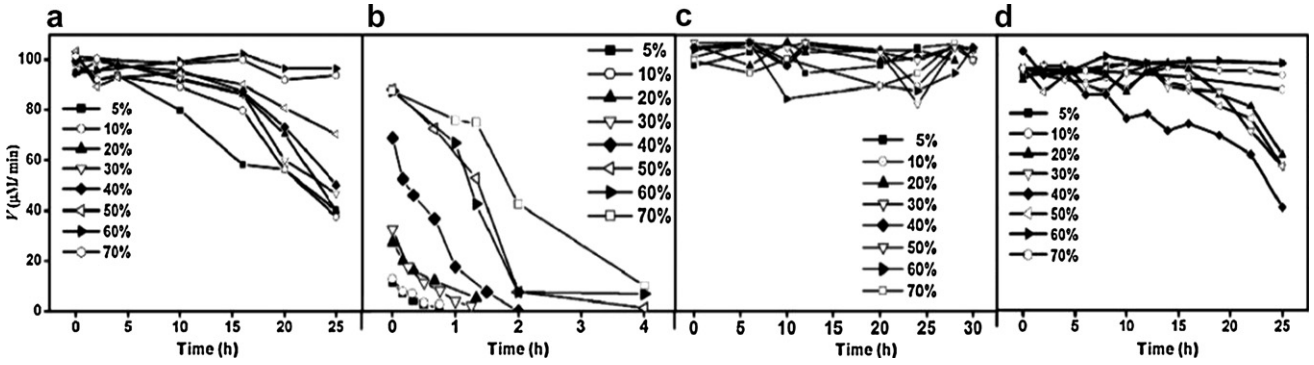

Figure 3. (a)–(d) Stability of HRP with varying water content in **2**–**5**, respectively. The concentration of HRP in the cuvette was 1 μg/mL. [Guaiacol] = 0.3 mM, $[H_2O_2]$ = 0.1 mM. The experimental error in these measurements was typically within ±8–12% in triplicate measurements.

observations, **4** was the best IL in terms of the stability of HRP.

Apart from the stability of HRP, it is important to determine its activity directly within these green solvents in order to be able to replace organic solvents.[26b] In concurrence with the stability study, **3** was found to be the most inferior medium for HRP as more than 92% water was necessary to show minimum visible activity (Fig. 4). HRP also showed poor efficiency in **2** where the presence of ~70–80% water was required for measurable activity. As discussed earlier, the presence of COO^- in both these ILs presumably deactivates peroxidase. In the case of **1**, the minimum amount of water essential for HRP activity was 20%. As expected (from the stability study), in all cases, the activity of HRP increased with water content and importantly, HRP needed only 5% water to remain active in **4** and **5**. Its efficiency was also markedly higher than in other ILs in comparable solution compositions.

To improve the activity of HRP in **2** and **3**, an excipient was added as an activity stimulator. In general, hydrophilic excipients maintain the native structure of the enzyme by holding water in proximity of the active site and thereby increase the efficiency of the biocatalyst.[25] Excipients have been previously used for enhancing enzyme activity in organic solvents.[25]Their use in ILs has however, not been reported previously. In this case, the hydrophilic lyoprotectant, TRIS was used as an excipient and succeeded in enhancing HRP activity in **2** and **3** up to an order of magnitude depending on the water content (Fig. 5).[26c] Moreover, in the presence of TRIS, the minimum amount of water necessary for HRP to be active was reduced by 10% in both these ILs. Thus, stability and activity studies in imidazolium based ILs have illustrated the crucial role of counterions on the efficiency of HRP.

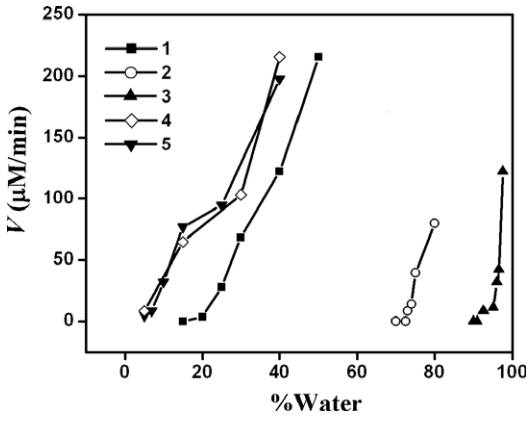

Figure 4. Initial rate of oxidation of guaiacol by HRP in different ILs with varying water content. [HRP] = 50 μg/mL. [Guaiacol] = 0.3 mM, $[H_2O_2]$ = 0.1 mM. The experimental error in these measurements was typically within ±8–12% in triplicate measurements.

Interestingly, biocatalysis in ILs with structural variation of the cationic component is rarely reported,[23,24,31,32] and to our knowledge, no such work has been published on HRP. The activity study in the presence of excipient[33] motivated us to design an IL possessing four hydroxyethyl moieties (**7**, Fig. 1), whilst keeping a structural resemblance to TRIS. We expected that such a similarity in structure would help the IL (**7**) to act as an activator for the enzyme. HRP showed a 3-fold increase in activity in **7** compared to the best imidazolium based IL, **4** at 40% water content (Fig. 6, inset). However, the activity could not be measured below 40% water content in **7** due to solidification of the reaction medium. At this point, the Cl^- counterion of **7** was replaced by $CF_3SO_3^-$ to minimize the operational difficulty and also to have the most compatible counterion for HRP (as evidenced in the imidazolium based ILs). The resulting IL, **8** (tetrakis(2-hydroxyethyl)ammonium triflouromethanesulfonate) was notably better than its predecessor (**7**), as activity was observed even at 0.8% water content and **8** also showed ~6-fold higher activity compared to **4** at 40% water content.

In similar solution compositions (at 5% water), the activity of HRP in **8** was ~30 and 50-fold higher than that in the best performing imidazolium based ILs, **4** and **5**, respectively (Fig. 6). Also, the observed activity was ten times greater than that in methanol, a common

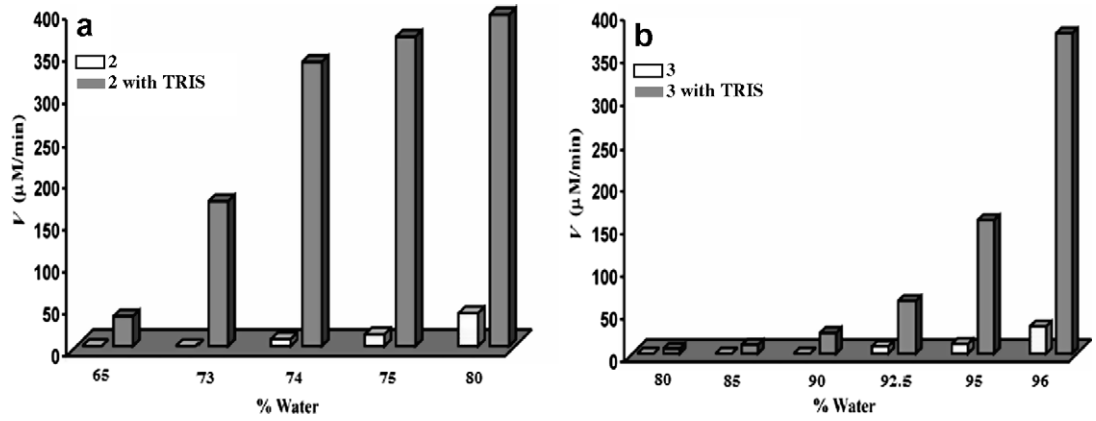

Figure 5. (a, b) Initial rate of oxidation of guaiacol by HRP in **2** (a) and **3** (b) in the presence and absence of TRIS (100 mM) with varying water content. [TRIS] = 0.1 M, [HRP] = 50 μg/mL [Guaiacol] = 0.3 mM, $[H_2O_2]$ = 0.1 mM.

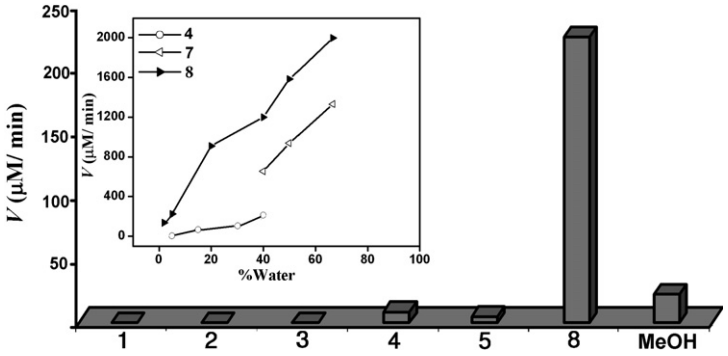

Figure 6. Initial rate of oxidation of guaiacol by HRP in different ILs and methanol at 5% water content. Inset shows the activity profiles of HRP in **4**, **7** and **8** at varying percentages of water. [HRP] = 50 μg/mL [Guaiacol] = 0.3 mM, [H_2O_2] = 0.1 mM. The experimental error in these measurements was typically within ±8–12% in triplicate measurements.

organic solvent for HRP. Under varying water content, HRP showed ~60–240 times higher activity in **8** as compared to that found in **1** and was beyond comparison with respect to **2** and **3**. Although the activity of HRP was significantly improved in **8** compared to other ILs and methanol, it was 15–20 times lower than that in water. Spectroscopic studies are in progress to understand the effects of the components of the IL on the enzyme.

Improving the stereoselectivity of an enzyme towards a given substrate is also a very challenging objective in the field of biocatalysis. The prospect of ionic liquids as reaction media in this field has been studied recently.[19,21] At this time, we questioned whether the present ILs could also boost the stereoselectivity of HRP in asymmetric sulfoxidation. Accordingly, we investigated three ILs **3**, **4** and **8**, with varying percents of water as reaction media for carrying out the sulfoxidation of thioanisole (0.5 mM) by HRP (15 mg/mL) using 1 mM H_2O_2 as the oxidant. It was found that no sulfoxide was formed using **3** and **4** in the presence of varying percents of water (Table 1); whereas in **8** with 10%, 30% and 50% v/v content of water, HRP showed >99% ee with conversions ranging from 16% to 40% on reaction for 2 h (details of the experimental procedure are given in the Supplementary data). In concurrence with the observed trend in guaiacol oxidation by HRP, here also peroxidase showed improved activity as well as stereoselectivity in designed IL, **8**.

In conclusion, the superior efficiency of HRP in the oxidation of guaiacol as well as the asymmetric sulfoxidation of thioanisole was observed in IL **8**, which was developed on the basis of enzyme stability and activity in different ILs. Hence, the present approach provides a prelude to the development of novel green solvents which are tailor made for enzyme catalysis.

Table 1. Asymmetric oxidation of phenyl methylsulfide by HRP at room temperature in ILs[a]

Ionic liquid	Water added to IL (%)	Conversion (%)	(*R*) ee (%)
3	50	0	—
4	50	0	—
8	10	15.8	>99
	30	23	>99
	50	40.34	>99

[a] Concentrations in the reaction mixtures: [H_2O_2] = 1 mM, [HRP] = 15 mg/mL, [phenyl methylsulfide] = 0.5 mM.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2007.06.022.

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26. (a) For 0% water, HRP (lyophilized from 10 mM phosphate buffer, pH 7) was dispersed in IL at a concentration of 2 mg/mL; while in the presence of water the same concentration of enzyme was achieved by adding the required volume of aqueous enzyme stock (40 mg/mL) in the IL. Water content was varied by addition of the desired volume of buffer. This was followed by brief sonication, and stirring for a specific period. The solution was diluted (100–500 μL) with buffer and an aliquot (3.75 μL) was added to a cuvette (1.5 mL) to monitor the initial rate of oxidation (V) of guaiacol spectrophotometrically at room temperature ([HRP] = 1 μg/mL in cuvette); (b) IL was directly taken into the cuvette containing the required % water (buffer). Subsequently, the enzyme, guaiacol and H_2O_2 were added to monitor the rate of oxidation of guaiacol. [HRP] = 50 μg/mL; (c) Aqueous HRP stock solution (10 mg/mL) in phosphate buffer containing 100 mM TRIS at pH = 7 was used. The addition of TRIS increased the pH to ≈9. Hydrochloric acid was added to decrease the pH to 7. Subsequently, the enzyme, guaiacol and H_2O_2 were added. [HRP] = 50 μg/mL. In all cases, [guaiacol] = 0.3 mM; [H_2O_2] = 0.1 mM. The extinction coefficient for the guaiacol dehydrogenation product (GDHP) at 436 nm was 6400 M^{-1} cm^{-1}. The experimental error was typically within ±8–12% in triplicate measurements.
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