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Application of hydrophilic ionic liquids as co-solvents in chloroperoxidase catalyzed oxidations

Cinzia Chiappe,* Lisa Neri and Daniela Pieraccini

Dipartimento di Chimica Bioorganica e Biofarmacia, Via Bonanno 33, 56126 Pisa, Italy

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Dedicated to the memory of Professor Giancarlo Berti (19 October, 2005)

Abstract—The effect of hydrophilic ionic liquids (ILs) on the activity of chloroperoxidase (CPO) was checked through kinetic and stereochemical studies. The possibility to employ this enzyme in synthesis has been demonstrated investigating the chemo- and stereoselectivity of oxidation of phenyl methylsulfide in several citrate buffer–IL mixtures.

The heme enzyme chloroperoxidase (CPO), produced by the marine fungus *Caldariomyces fumago*, is a versatile enzyme which exhibits a broad spectrum of chemical reactivities, including the reactions typical of peroxidases, the use of halide ions to halogenate a variety of organic molecules and the catalyses activity in the disproportionation of hydrogen peroxide.[1] Due to its versatility and selectivity CPO is recognized as the most promising enzyme for synthetic applications.[2] Despite the potentiality, actual synthetic applications of CPO are hampered by its limited stability due to inactivation by H_2O_2 and the low water solubility of many organic substrates of synthetic interest. Attempts to use CPO in aqueous buffer–organic solvent mixtures or in pure organic solvents have met with only moderate success, due to the decreased reaction rate and selectivity in these media.[3,4] At variance, adsorption on meso- or microporous solids,[5] covalent bound to silica gel[6] and micro-encapsulation in a microporous silica gel[7] have been shown to be alternative approaches able to ensure sufficient stability to CPO in the presence of hydrogen peroxide for carrying out the oxidation reaction with high activity and/or selectivity. On the other hand, a significant stabilization of CPO has been obtained also by the addition of poly(ethylene glycol)s to the reaction mixture.[8] Despite the widespread interest on this topic, only few papers have been published on peroxidase catalyzed reactions using ionic liquids as co-solvents.[9] During the last years, ionic liquids (ILs) have gained increased attention as new solvents for performing practically all types of reactions.[10,11] Several papers have been published[12] on the use of ILs as reaction media for biocatalyzed processes evidencing remarkable results with respect to the yield, enantioselectivity or enzyme stability.

In this letter, we report the kinetic and stereochemical studies carried out to determine the activity and selectivity of CPO in the presence of ionic liquids using both aqueous phosphate (pH 2.7) and citrate buffers (pH 5.0) as co-solvents. The possibility to employ this enzyme in synthesis has been demonstrated investigating the chemo- and stereoselectivity of oxidation of phenyl methylsulfide in several citrate buffer–IL mixtures.

Seven different hydrophilic ILs, 1,3-dimethylimidazolium methylsulfate [mmim][$MeSO_4$], **1**, 1,3-dimethylimidazolium dimethylphosphate [mmim][Me_2PO_4], **2**, 1-ethyl-3-methylimidazolium ethylsulfate [emim][$EtSO_4$], **3**, *N*,*N*-dimethylmorpholinium methylsulfate [Mor_{11}]-[$MeSO_4$], **4**, cholinium acetate [N_{1112OH}][OAc], **5**, cholinium phosphate [N_{1112OH}][H_2PO_4], **6**, and cholinium citrate [N_{1112OH}][Citr], **7**, were used as co-solvents to investigate the influence of ionic liquid structure on CPO activity and selectivity (Fig. 1). Cholinium based ILs,[13] which to the best of our knowledge have been applied here for the first time in biocatalyzed processes, have been chosen due to their low cost and extremely easy preparation procedure.

* Corresponding author. Tel.: +39 050 2219669; fax: +39 050 2219660; e-mail: cinziac@farm.unipi.it

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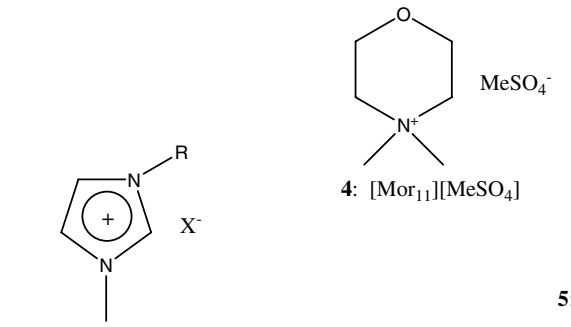
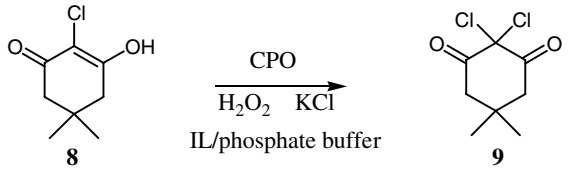


Figure 1.



Scheme 1. CPO catalyzed chlorination of **8**.

First, CPO activity has been determined by examining the chlorination of monochlorodimedon, **8** (Scheme 1). This reaction, initially employed to characterize chloroperoxidase, nowadays represents a useful spectrometric assay to determine enzymatic activity in non-aqueous media.[4]

The ionic liquids used for this test were chosen such that their cut off point was less than 278 nm, the wavelength used for the assay. The chlorinating activity of CPO on **8** was initially measured in a phosphate buffer in the presence of increasing concentrations of IL. The concentration of **8**, chloride ion and hydrogen peroxide were as for the standard assay conditions.[4]

CPO was observed not to chlorinate **8** when the reactions were carried out in the presence of ILs **1**, **3**, **4** and **5**. No absorbance changes were detected for the period of the assay also adding very low amounts of these ILs (5%). At variance, CPO activity was observed in the presence of [mmim][Me_2PO_4], [N_{1112OH}][H_2PO_4] and [N_{1112OH}][Citr] (**2**, **6** and **7**). The results, expressed in terms of relative velocity, are reported in Table 1 and depicted in Figure 2. The relative velocity is the ratio of the initial rate in the presence of the ionic solvent, over the rate in pure aqueous buffer solution. In Figure 2, the data related to the activity of CPO in the presence of molecular solvents is also reported.[4] Experiments in

Table 1. Relative velocities (V_r) of CPO chlorination of **8** in the presence of ILs

Run	% (v/v) of co-solvent in potassium phosphate buffer		V_r	pH
1	5	[N_{1112OH}][Citr]	0.54	
2	10	[N_{1112OH}][Citr]	0.27	
3	20	[N_{1112OH}][Citr]	0.185	
4	30	[N_{1112OH}][Citr]	0.0409	
5	40	[N_{1112OH}][Citr]	—	3.74
6	5	[N_{1112OH}][H_2PO_4]/[H_3PO_4]	2.86	
7	10	[N_{1112OH}][H_2PO_4]/[H_3PO_4]	1.45	
8	15	[N_{1112OH}][H_2PO_4]/[H_3PO_4]	—	2.02
9	5	[N_{1112OH}][H_2PO_4]	—	3.53
10	5	[N_{1112OH}][OAc]/[HOAc]	—	4.64
11	5	[N_{1112OH}][OAc]	—	5.42
12	5	[mmim][Me_2PO_4]	0.770	
13	10	[mmim][Me_2PO_4]	0.58	
14	15	[mmim][Me_2PO_4]	0.455	
15	20	[mmim][Me_2PO_4]	0.347	
16	30	[mmim][Me_2PO_4]	0.292	3.24
17	30	[mmim][Me_2PO_4]	0.898	2.7[a]
18	40	[mmim][Me_2PO_4]	0.286	
19	50	[mmim][Me_2PO_4]	0.241	
20	90	[mmim][Me_2PO_4]	n.d.[b]	

[a] Reaction performed in a buffer–ionic liquid mixture (30% v/v), after correcting the pH value to 2.7 using a proper potassium phosphate buffer.
[b] The strong absorbance of the ionic liquids prevented the kinetic study.

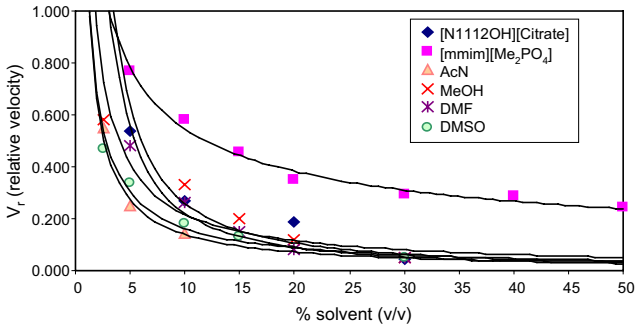


Figure 2. Relative velocities (V_r) of CPO chlorination of **8** in the presence of ILs and molecular solvents.

molecular solvents were carried out under identical conditions to those used in the present investigation.

As shown in Table 1, the enzyme activity varies significantly with the nature and concentration of the IL; as in conventional organic solvents, increasing amounts of IL progressively lowered the catalytic capability of the CPO. CPO shows, however, a higher tolerance towards ionic liquids than organic solvents: at least in the case of [mmim][Me_2PO_4], amounts of IL significantly higher than 20% v/v can be added before a complete lack of enzyme activity is recorded. In all examined molecular solvents (dimethyl sulfoxide, dimethyl formamide, methanol and acetonitrile) the relative velocity decreased[4] below 0.1 in the presence of 20% of organic solvent. Moreover, it is worth noting that in the presence of small amounts of [N_{1112OH}][H_2PO_4][H_3PO_4] (<5%) the enzyme is able to perform the chlorination of MCD with a rate superior to that observed in buffer solution.

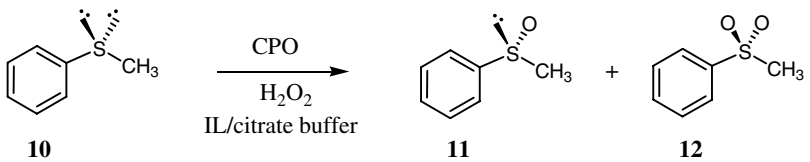
Since CPO activity is affected by the hydronium concentration we measured with a glass electrode the pH change due to the IL addition. Although these measurements reflect both bulk and surface effects,[14] it is evident that the presence of IL influences the measured pH and the enzyme activity. The addition of [mmim][Me_2PO_4] (30% v/v) to the potassium phosphate buffer solution (pH 2.7) enhances the pH of the medium to 3.74 determining a significant decrease in the CPO chlorination activity ($V_r = 0.292$). However, when the same amount of [mmim][Me_2PO_4] was added to a potassium phosphate buffer and the pH was adjusted to 2.7 after IL addition the V_r measured was not very far from unity ($V_r = 0.898$). The ability of the IL to affect the medium pH may be therefore considered one of the factors able to affect the activity of CPO in these non-conventional media.

Subsequently, we investigated the CPO catalyzed sulfoxidation reaction, an oxidative process often performed under completely different pH conditions (citrate buffer, pH 5.0).

CPO from *C. fumago* is known to be the heme peroxidase of first choice for sulfoxidation reactions owing to its high enantioselectivity.[2,15] Sulfoxidation of phenyl methylsulfide, thioanisole (**10**), by hydrogen peroxide in the presence of CPO was therefore investigated both in pure aqueous citrate buffer solution (0.1 M, pH 5.0) and in the presence of increasing amounts of ILs (Scheme 2). Generally, in CPO-catalyzed oxidations the oxidant is kept as low as possible by a slow addition to the reaction mixture, to avoid enzyme inactivation.[7,16] However, aiming to make CPO a more useful catalyst for synthetic applications, we have employed the procedure recently reported[8] by Savelli for CPO–oxidation in water–poly(ethylene glycol)s. H_2O_2 was used as oxidant: 1 equiv was added in a unique aliquot at the beginning of the reaction and 0.5 equiv after 2 h. The reaction was carried out at room temperature for 4 h. Sulfoxide (**11**) and sulfone (**12**) were extracted by diethyl ether and crude reaction mixtures were analyzed after addition of anisole as an internal standard by GC (on a chiral column) to determine the overall yield, the ratio between sulfoxide/sulfone and the enantiomeric excess of sulfoxide **11**.

The results are reported in Table 2. The chiral phenyl methyl sulfoxide obtained always had the *R* absolute configuration, as in pure buffer solution.[13]

As previously observed in the chlorination reaction, the CPO catalyzed oxidation of phenyl methylsulfide is affected by the nature of the IL. In the presence of [mmim][$MeSO_4$] [Mor_{11}][$MeSO_4$] and [N_{1112OH}][H_2PO_4]/[H_3PO_4] (**1**, **4** and **6**), CPO lost completely its activity and only racemic sulfoxide **11** was isolated. At variance, enantiomerically pure sulfoxide (*R*)-**11** was obtained performing the incubations in the presence of [mmim][Me_2PO_4], [N_{1112OH}][OAc] and [N_{1112OH}][Citr] (**2**, **5** and **7**). Using the corresponding aqueous buffer–IL mixtures, it is possible to obtain selectively (**11**:**12** ratio may rise the value of 98:2), in high yield (>74%, run 9) sulfoxide (*R*)-**11** characterized by an enantiomeric excess >99%. Generally, the higher conversions have been obtained in 1:1 IL/citrate buffer solutions although the enzyme maintained part of its activity also in the presence of higher amounts of IL (70%). The amount of IL in the presence of which it is possible to perform sulfide oxidation is therefore significantly higher than that found for chlorination activity (see above) and than that recently reported for indene oxidation (20% of



10 **11** **12**

Scheme 2. CPO catalyzed sulfoxidation of **10**.

Table 2. Oxidation of phenyl methylsulfide **10** with H_2O_2 and CPO at room temperature in IL/citrate buffer

Run	% (v/v) of co-solvent in citrate buffer[a]		pH	CPO U	Conv. (%)	**11**:**12**	(*R*)-**11**[b] ee (%)
1	0		5.0	67.4	35	89:11	97
2	0		5.0	0	8	67:33	0
3	30	$[N_{1112OH}][Citr]$	3.84	67.4	38	90:10	>99
4	30	$[N_{1112OH}][Citr]$	3.84	0	11	86:14	0
5	50	$[N_{1112OH}][Citr]$		67.4	48	95:5	>99
6	70	$[N_{1112OH}][Citr]$		67.4	17	90:10	>99
7	30	$[mmim][Me_2PO_4]$	4.93	67.4	37	98:2	95
8	30	$[mmim][Me_2PO_4]$	4.93	0	8	70:30	0
9	50	$[mmim][Me_2PO_4]$		67.4	76	98:2	>99
10	70	$[mmim][Me_2PO_4]$		67.4	19	70:30	>99
11	30	$[N_{1112OH}][OAc]$	6.08	67.4	42	100:0	>99
12	30	$[N_{1112OH}][OAc]/[HOAc]$	4.80	67.4	25	90:10	50
13	30	$[N_{1112OH}][OAc]/[HOAc]$		0	11	79:21	0
14	50	$[N_{1112OH}][OAc]/[HOAc]$		67.4	24	90:10	38
15	70	$[N_{1112OH}][OAc]/[HOAc]$		67.4	10	86:14	38

[a] Citrate buffer 0.1 M, pH 5.0.

[b] Determined by GC on a chiral 30 m Chiradex G-TA (ASTEC) column (helium flow 50 kPa, with evaporator and detector set at 200 °C, column temperature 90 °C per 1 min, 8 °C/min, 170 °C).

$[mmim][MeSO_4]$ in citrate buffer).[9a] Although more factors are responsible for the activity of enzymes in ILs, probably in this case the pH of the medium is an important factor able to affect enzyme activity. Racemic sulfoxide **11**, arising exclusively from the chemical oxidation, was formed in the presence of ILs able to shift the pH of the medium at values higher than 6.0 or lower than 2.7 ($[N_{1112OH}][H_2PO_4]/[H_3PO_4]$, $[Mor_{11}]$-$[MeSO_4]$ and $[mmim][MeSO_4]$). Even for this oxidation process, therefore, the best ILs to use as co-solvents are $[mmim][Me_2PO_4]$, cholinium citrate and cholinium acetate.

In conclusion, results reported here are clearly evident about the potential to use ILs as co-solvents for CPO catalyzed reactions. As compared to the behavior observed in conventional organic solvents, CPO in ILs presents enhanced activity, stability and selectivity. Moreover, the presence of IL increases substrate solubility in the reaction medium. However, to take full advantage of these perceived benefits, it is necessary to choose the ionic liquid accurately. The ability of the IL anion to modify the medium pH is probably the first parameter to consider in the selection of the IL to use in CPO catalyzed reactions.

Acknowledgements

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13. *Synthesis of cholinium-based ionic liquids*: a choline hydroxide methanol solution was added dropwise to an equimolar (for neutral ILs) or a two fold excess (for the acidic ILs) solution of the organic or inorganic acid. The mixture was stirred under cooling for 12 h, then methanol was evaporated under reduced pressure. Cholinium citrate $[N_{1112OH}][Citr]$: ^1H NMR (MeOH-d_6) δ (ppm): 3.79

(m, 2H, CH_2OH); 3.26 (m, 2H, CH_2N); 2.94 (s, 9H, 3 CH_3N); 2.64–2.50 (AA'BB'system, 4H). ^{13}C NMR (MeOH-d_6) δ (ppm): 177.93; 174.1; 74.15; 68.95; 57.09; 54.73; 44.28. ESI-MS (MeOH): positive ion, 104.12 $[N_{1112}OH]^+$; negative ion, 191.01 [citrate]$^-$. Cholinium phosphate $[N_{1112}OH][H_2PO_4]$: ^1H NMR (D_2O) δ (ppm): 3.76 (m, 2H, CH_2OH); 3.24 (m, 2H, CH_2N); 2.91 (s, 9H, 3 CH_3N). ^{13}C NMR (D_2O) δ (ppm): 68.18; 56.36; 54.64; 44.33. Cholinium acetate $[N_{1112OH}][OAc]$: ^1H NMR (DMSO-d_6) δ (ppm): 4.63 (m, 2H, CH_2OH); 4.23 (m, 2H, CH_2N); 3.92 (s, 9H, 3 CH_3N); 2.41 (s, 3H, CH_3COO^-). ^{13}C NMR (DMSO-d_6) δ (ppm): 173.78; 67.30; 54.94; 53.10; 25,45. ESI-MS (MeOH): positive ion, 104.12 $[N_{1112}OH]^+$; negative ion, 59 $[CH_3COO]^-$.

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