Effect of ionic liquids as additives in the catalytic properties of different immobilized preparations of *Rhizomucor miehei* lipase in the hydrolysis of peracetylated lactal†

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The addition of small amount of different ionic liquids modified the activity and regioselectivity of different immobilized preparations of *R. miehei* lipase catalyzing the hydrolysis of hexa-*O*-acetyl lactal in aqueous media. ILs with [emim] as cation and different anions were first evaluated affecting in a different manner depending on the immobilized preparation used. The enzymatic activity of RML immobilized on octyl-agarose or CNBr-agarose decreased in the following order: NO_3^- ≈ BF_4^- > $MeOSO_3^-$ > PF_6^-, whereas when RML was immobilized on Q-Sepharose, the enzymatic activity decreased in a different order: $MeOSO_3^-$ > PF_6^- > BF_4^- > NO_3^-. Using [bdmim], the activity of octyl-RML and CNBr-RML preparations resulted higher in the presence of PF_6^- than BF_4^-, 6-fold for octyl-RML and 2-fold for CNBr-RML if compared with the enzyme activity without additive. In both preparations the enzyme was completely regioselective in the presence of the IL hydrolyzing at *C*-3 position in 99% yield. The modification of the cation in the IL did not affect to the activity of Q-RML with BF_4^- or decreased the activity with PF_6^-, although affected to the regioselectivity producing another undesired product, a bihydrolized product in 20–25% yield. In this case, the addition of [emim][$MeOSO_3$] caused the best increment in the activity for this RML biocatalyst, 2-fold with only 8% of bihydrolized production.

Introduction

Ionic liquids (ILs) are emerging as 'green' alternatives to common solvents because they have no measurable vapor pressure and are able to dissolve compounds of varying polarity. They are simply salts and therefore entirely composed of ions that are liquid below 100 °C. Ionic liquids are also chemically diverse owing to the huge number of possible cation/anion combinations that can be synthesized. Typical ILs are based on organic cations, *e.g.* 1,3-dialkylimidazolium, tetraalkylammonium, *etc.*, paired with a variety of anions that have a strongly delocalized negative charge (*e.g.* [BF_4], [PF_6], *etc.*), which results in colourless, low viscosity and easily handled materials with very interesting properties as solvents. Furthermore the miscibility with water or some organic solvents (*e.g.* hexane) can be tuned by selecting the appropriate cation and anion.[1–2]

One of the most interesting applications of ILs has been on biocatalysis.[2–5] ILs have been used in the preparation of biocatalysts by additive in the immobilization process,[6] coating the enzyme,[7] as solid phase support for immobilization,[8] as solvent substituent how medium engineering,[9] or an additive in organic solvents or aqueous media with interesting improvement of enzyme activity and selectivity in several kind of reactions.[10]

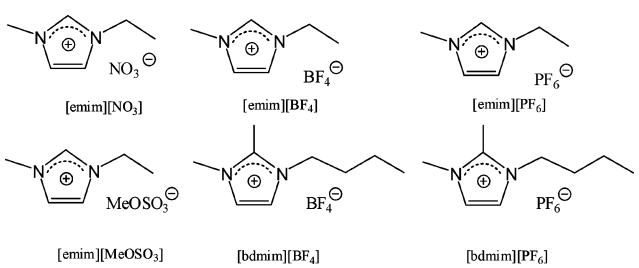
Indeed, some ILs, especially those containing PF_6^-, BF_4^- and $MeOSO_3^-$ anions, were known as positive influences on the enzyme stability,[11] activity and selectivity.[12]

Particularly, in recent years many examples of the application of ILs in biotransformations with lipases have been reported.[13]

This is because of the high versatility of these enzymes which recognize a broad range of substrates with high regio- and enantioselectivity.[14–15] The interesting and complex catalytic mechanism of these enzymes and the great flexibility of their active centre have permitted development of strategies to modulate their catalytic properties, such as using immobilization strategies.[16–17] Also small changes in the experimental conditions (*e.g.* co-solvent addition,[18] pH changes,[18] addition of salts,[19] *etc.*) have been observed to introduce interesting modifications of the activity and selectivity of lipases.

Although the lipase catalytic properties have been interpreted in terms of IL properties including polarity, hydrogen-bond basicity, anion nucleophilicity, and viscosity,[20] hydrophobic ILs seem to be the better choice as reaction solvent in term of lipase activity.[21]

Therefore, in the present manuscript, we have studied the effect of six different ionic liquids (Scheme 1) as additive on the activity and regioselectivity of *Rhizomucor miehei* lipase immobilized by different strategies. Furthermore, this study has



Scheme 1 Ionic liquids tested as additives.

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Table 1 Activity, specificity and regioselectivity of different immobilized preparations of RML in the hydrolysis of **1** in the presence of different additives

Support	ILs	Activity[a]	Time/h	C^b (%)	**2**[c] (%)	Other[d]
Octyl	—	0.58 ± 0.01	24	100	100	
	[emim][BF_4]	2.40 ± 0.02	8	100	95	5
	[emim][PF_6]	0.64 ± 0.01	22	100	97	7
	[emim][NO_3]	2.50 ± 0.03	8	100	100	
	[emim][$MeOSO_3$]	1.00 ± 0.01	12	100	100	
	[bdmim][BF_4]	1.30 ± 0.01	9	100	100	
	[bdmim][PF_6]	3.30 ± 0.02	7	100	100	
CNBr	—	0.038 ± 0.001	144	80	80	
	[emim][BF_4]	0.052 ± 0.002	144	88	88	
	[emim][PF_6]	0.038 ± 0.002	144	78	78	
	[emim][NO_3]	0.052 ± 0.003	144	88	88	
	[emim][$MeOSO_3$]	0.038 ± 0.001	144	81	81	
	[bdmim][BF_4]	0.040 ± 0.002	144	82	82	
	[bdmim][PF_6]	0.064 ± 0.002	144	100	100	
Q-Sepharose	—	0.36 ± 0.005	48	100	100	
	[emim][BF_4]	0.11 ± 0.003	72	100	78	22
	[emim][PF_6]	0.36 ± 0.004	48	100	78	15
	[emim][NO_3]	0.10 ± 0.002	72	100	76	24
	[emim][$MeOSO_3$]	0.77 ± 0.003	26	100	92	8
	[bdmim][BF_4]	0.11 ± 0.004	72	100	80	20
	[bdmim][PF_6]	0.11 ± 0.003	72	100	75	25

[a] The initial rate in μmol mg_{prot}^{-1} h^{-1}. It was calculated at 10–20% yield. IL without enzyme was tested and no conversion was detected. The experiments were performed by triplicate. [b] C = conversion [c] Yield of monodeprotected product **2**. [d] The compound corresponds to a bi-hydrolyzed product detected by MALDI-TOF, $[M+Na]^+$: 499.15, found: 499.13.

been focused on the regioselective hydrolysis of peracetylated lactal **1**, a quite interesting building block in biological active oligosaccharides synthesis.[22]

Results and discussion

The specific activity displayed by three different immobilized preparations of RML in the regioselective hydrolysis of peracetylated lactal **1** in aqueous solution containing 5 eq.‡ of different ILs (Scheme 1) is shown in Table 1. The influence of different cations or anions in the IL was evaluated and the results were quite different depending on the immobilization protocol used.

Firstly, the influence of different anions of ILs on the lipase properties was analyzed. Thus, four ionic liquids – constituted by [emim] as cation – were evaluated.

In all cases, the addition of the IL improved the catalytic activity on the lipase immobilized on octyl-agarose beads (by interfacial adsorption), although ILs with BF_4^- or NO_3^- permitted to enhance the activity in almost 4-fold. The addition of [emim][$MeOSO_3$] improved the enzymatic activity 2-fold whereas [emim][PF_6] just slightly increase the activity value (Table 1).

When RML immobilized on CNBr-agarose (by covalent attachment) was used as biocatalyst, a similar effect was observed but with less activity increment (Table 1). [emim][BF_4] or [emim] [NO_3] increased the activity in 1.4-fold whereas the rest did not affect to the activity.

Different behavior was observed when these ILs were added in the reaction catalyzed by RML immobilized on Q-Sepharose (by anionic exchange).[23] [emim][$MeOSO_3$] was the best IL increasing 2-fold the enzyme activity. ILs with BF_4^- or NO_3^- were the worst ones (the best ones with the other RML preparations), causing a more than 3-fold decrease in the activity. Addition of [emim][PF_6] did not produce any different in the biocatalyst activity (Table 1).

Secondly, the effect of the anion in ILs was evaluated, by substitution [emim] for [bdmim] (Table 1).

Using a more hydrophobic anion, the behavior on the specific activity varied. The best result in term of activity enhancement was achieved by using an IL bearing [PF_6] – the worst cation in emim ILs. The addition of [bdmim][PF_6] enhanced the activity of octyl-RML 6-fold in the hydrolysis of 1. Using [bdmim][BF_4] an improvement of 2-fold was observed (Table 1).

The presence of [bdmim][PF_6] also gave the best activity improvement on the CNBr-RML, 1.68-fold compared without additive or [emim][PF_6] (Table 1). With [bdmim][BF_4] the enzyme slightly improved its activity.

Using Q-Sepharose-RML, the addition of these ILs caused a decrease on the activity (Table 1).

Using NaCl as typical salt as additive, almost no effect on the activity for the different immobilized RML preparation was observed (see ESI†).

The kinetic parameters for the octyl-RML immobilized preparation were calculated in order to understand the nature of the effect (Table 2).

Table 2 Kinetic parameters for octyl-RML with **1** as substrate

ILs	K_m/mM	V_{max} (nmol min^{-1} mg^{-1})	K_{cat}/ms^{-1}
—	5.02 ± 0.09	4.47 ± 0.1	0.0051 ± 0.0003
[emim][PF_6]	3.33 ± 0.05	2.51 ± 0.08	0.0287 ± 0.0009
[bdmim][PF_6]	1.66 ± 0.02	25.5 ± 1.2	0.0029 ± 0.0001

The K_m value of the immobilized enzyme was lower in the presence of [bdmim][PF_6] (1.66 mM) compared with the value of the immobilized preparation without ionic liquid (5.02 mM). The K_m value for the biocatalyst in the presence of [emim][PF_6] was 3.33 mM, lower than the value without additive, showing that the affinity of the enzyme for the substrate increased in the presence of both ILs. However, the influence of the presence of IL was different on the K_{cat}. The turnover number was almost 6-fold higher with the addition of [bdmim][PF_6] and almost 2-fold lower with the addition of [emim][PF_6] (Table 2). This result seems to indicate that the effect on the activity of the immobilized preparation in the presence of [emim][PF_6] is exclusively based on a decrease of the k_{cat} whereas the improvement on the catalytic activity for the octyl-RML preparation in the presence of [bdmim][PF_6] is based on an influence on both parameters (a better substrate affinity and an increase of turnover number).

Analyzing the reaction course for octyl-RML (Fig. 1), all ILs added in this enzymatic process decreased the time for a full conversion of the product. The reaction without additive was finished at 24 h whereas using [bdmim][PF_6] the substrate was completely consumed in 7 h. The specificity and regioselectivity of the biocatalyst was maintained in the presence of most ILs producing exclusively the deprotection of the acetyl group on *C*-3 (Scheme 2, Table 1). Only a 5–7% bi-hydrolyzed product was observed using [emim][PF_6]or [emim][BF_4] (Table 1).

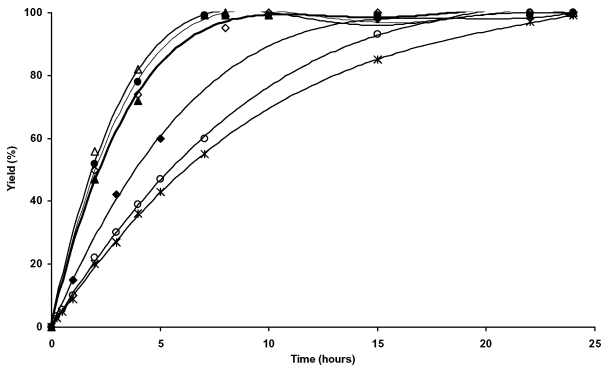
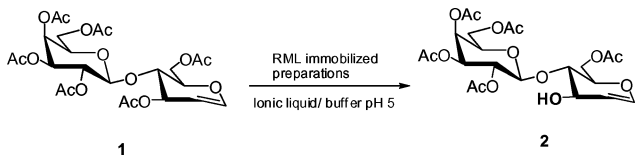


Fig. 1 The influence of addition of different ionic liquids on the hydrolysis of **1** catalyzed by octyl-RML. Without additive (asterisks), [emim][$MeOSO_3$] (rhombus), [emim][NO_3] (circles), [emim][PF_6] (empty circles), [emim][BF_4] (empty rhombus), [bdmim][BF_4] (triangles), [bdmim] [PF_6] (empty triangles).



Scheme 2 Regioselective hydrolysis of per-*O*-acetylated lactal **1** catalyzed by different RML immobilized preparations in aqueous media containing ionic liquids.

The profile for the CNBr-RML preparation was different (Fig. 2), where only a 100% conversion was achieved after 144 h by the addition of [bdmim][PF_6]. Others ILs almost did not affect on the rate.

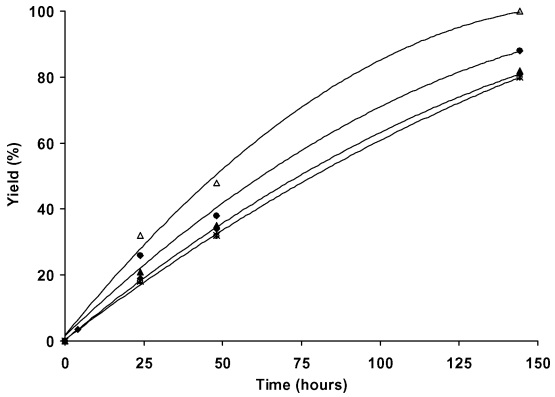


Fig. 2 The influence of addition of different ionic liquids on the hydrolysis of **1** catalyzed by CNBr-RML. Without additive (asterisks), [emim][$MeOSO_3$] (rhombus), [emim][NO_3] (circles), [emim][PF_6] (empty circles), [emim][BF_4] (empty rhombus), [bdmim][BF_4] (triangles), [bdmim] [PF_6] (empty triangles).

The specificity and regioselectivity of this biocatalyst was also maintained after the addition of ILs.

Using Q-Sepharose-RML preparation the effect of the IL on the reaction course was quite different, especially in the final yield of product. In this case the addition of ILs caused a change in the regioselectivity of the enzyme, producing another substance (a bi-hydrolyzed product analyzed by HPLC and MALDI-TOF, see ESI) in different yields together the hydrolysis in *C*-3 (Table 1). The ratio between **2** and the other peak was different depending on the ionic liquids. In the presence of [emim][$MeOSO_3$], 100% conversion is achieved in 26 h with 92% of 2 whereas with the other ILs the full conversion is achieved in 72 h and around 70–78% yield of 2 is obtained (Fig. 3).

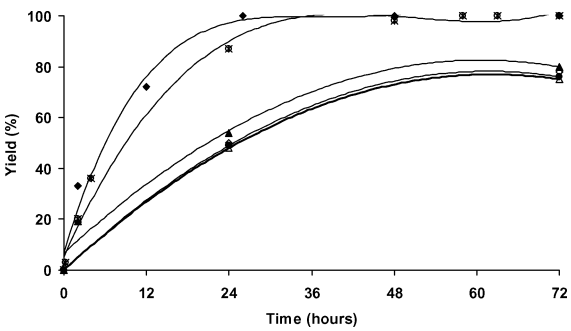


Fig. 3 The influence of addition of different ionic liquids on the hydrolysis of **1** catalyzed by Q-Sepharose-RML. Without additive (asterisks), [emim][$MeOSO_3$] (rhombus), [emim][NO_3] (circles), [emim][PF_6] (empty circles), [emim][BF_4] (empty rhombus), [bdmim][BF_4] (triangles), [bdmim] [PF_6] (empty triangles).

The addition of the same equivalents of NaCl affected the reaction catalyzed by CNBr-RML and Q-Sepharose-RML, where 30% of undesired product was produced (see ESI).

Conclusion

In this paper, we have described the influence of small amounts of different ionic liquids on the catalytic activity and regioselectivity of three different immobilized preparations of

RML – besides on different manner – catalyzing the hydrolysis of hexa-*O*-acetyl lactal **1** in aqueous media.

We have observed how the enzymatic activity of the different RML preparations varied depending on cation or anion constituting the IL. The activity of octyl-RML was rise with all ILs, the CNBr-RML activity increased with some ILs whereas it was not affected with others. Two ILs improved the activity of Q-Sepharose-RML while the rest caused a decrease. The regioselectivity in most cases remained unaltered, although ILs changed this property for Q-Sepharose-RML, producing an undesired bi-hydrolyzed product from 5–25%.

Therefore, the addition of [bdmim][PF_6] enhanced 6-fold the activity of octyl-RML preparation keeping a high regioselectivity, producing 3-hydroxy product 2 in high overall yield (>95%), key intermediate in the synthesis of different glycoderivatives.

The origin of the effects observed could be explained by considering the nature of the immobilized enzyme. The enzyme immobilized on octyl-agarose exists on a fixed open conformation with a high hydrophobic pocket surrounding the active site. The best results for this lipase immobilized preparation were found by using the most hydrophobic ionic liquids, probably because they can penetrate easily changing the active site environment. The study of the kinetic parameters demonstrated that the affinity of the substrate was better (lower K_m) and the turnover number (K_{cat}) was increased. In the case of the lipase immobilized on an ionic exchanger Q-Sepharose, the most hydrophilic ILs gave the best results; in this case considering the most hydrophilic area around the active site. Also the alteration of ionic charges on the protein and the influence on the conformational changes, in the same way as previously described for pH change[18] could be also another artifact.

Experimental

Materials

The lipase from *Rhizomucor miehei* (RML) was from Novozymes (Denmark). Q-Sepharose, octyl-agarose (4BCL) and cyanogen bromide (CNBr-activated Sepharose 4BCL) were purchased from GE-Healthcare (Uppsala, Sweden). The purification and immobilized preparations [containing 4 mg lipase per g support] (octyl-RML, CNBr-RML, Q-Sepharose-RML) were performed as previously described.[23] Hexa-*O*-acetyl-1,5-anhydro-2-deoxy-4-*O*-β-D-galactopyranosyl-D-arabinohex-1-enitol (**1**) were from TCR Toronto (Canada). 1-Ethyl-3-methylimidazolium methyl sulfate [emim][$MeOSO_3$], 1-Ethyl-3-methylimidazolium nitrate [emim][NO_3], 1-Ethyl-3-methylimidazolium hexafluorophosphate [emim][PF_6], 1-Ethyl-3-methylimidazolium tetrafluoroborate [emim][BF_4], 1-Butyl-2,3-dimethylimidazolium tetrafluoroborate [bdmim][BF_4] and 1-Butyl-2,3-dimethylimidazolium hexafluorophosphate [bdmim] [PF_6] were from Fluka.

Enzymatic hydrolysis of peracetylated lactal

(0.01 mmol, 4.8 mg) was added to 2 mL solution of 25 mM acetate buffer with 3% acetonitrile and 5 eq. of ionic liquid at pH 5, 25 °C, and the reaction was initialized by adding 0.4 g of biocatalyst. The hydrolytic reaction was carried out under mechanical stirring, and the pH value was controlled by automatic titration. Hydrolysis reactions were followed by HPLC.

Analytical method

HPLC analyses were performed using HPLC-spectra P100 (Thermo Separation products). The column was a Kromasil-C_{18} (250 × 4.6 mm and 5 μm) from Analisis Vínicos (Tomelloso, Spain). Analyses were run at 25 °C using an L-7300 column oven and UV detector L-7400 at 215 nm. The eluent was an isocratic mixture of 40% acetonitrile in 10 mM ammonium phosphate buffer at pH 3.8; flow rate 1.0 mL min^{-1}. The retention time of the product **1** and **2** were 18.50 min and 11.10 min respectively in these conditions. Compound **2** has been recently characterized.[24]

Acknowledgements

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Notes and references

‡ 10, 15 and 20 eq. of ionic liquids were also assayed and no significant differences were observed.

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