Supplementary Material:

To: "Epoxidation and Baeyer-Villiger oxidation using hydrogen peroxide and lipase dissolved in ionic liquids"

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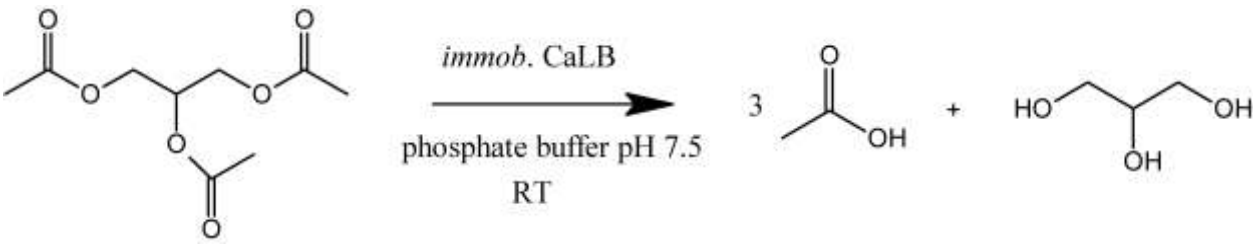
Dissolution of enzymes in hydrogen-bond donating (HBD) ionic liquids

Studies on enzyme dissolution in ionic liquids revealed that ionic liquids that form strong hydrogen bonds such as alkylmethylimidazolium $[AlkylMIm]^+$, with nitrate, acetate or dicyanimide as anion dissolve *Candida antarctica* Lipase B (CalB) free enzyme. In this case dissolution was accompanied by nearly complete or temporary activity loss.[1] On the basis of IR spectroscopy, this activity loss was attributed to interference with the H-bonds that maintain the protein secondary and tertiary structure.[2]

In this study, we embarked on the screening of a novel class of ionic liquids, namely OH-bond containing ILs. These solvents are proposed to provide a balance of mild hydrogen bond-accepting and donating properties. Their H-bond donating properties could possibly contribute to the stability of enzymes in solution.[1]

The range of solvents studied is presented in Fig. 2 of the main paper, and consists of $[HOPMIm]^+[NO_3]^-$ **1**, $[BMIm]^+[NO_3]^-$ **2a,** choline nitrate $[TMEOA]^+[NO_3]^-$ **3,** and triethanolammonium nitrate $[TEOA]^+[NO_3]^-$ **4.** All these ionic liquids are hydrophilic, and for comparison the ionic liquid with weakly coordinating anion **2b** $[BMIm]^+[BF_4]^-$ was included in the study.

We thus wanted to investigate whether immobilized lipase is stable in these solvents, and whether activity can be maintained, either immobilized or dissolved. As activity assay the hydrolysis of triacetin was used (Scheme S1). As immobilized formulations of CaLB, both Novozym 435 as well as cross-linked enzyme aggregates of lipase, CaLB CLEA © were tested.[3]



Scheme S1. Natural reaction: Hydrolysis of triacetylglycerol (triacetin)

In Fig. S1 the activity is given for Novozym 435 in the range of solvents studied. As reference phosphate buffer was used. In this case the reference batch of Novozym 435 used had an activity of 388 U/g.

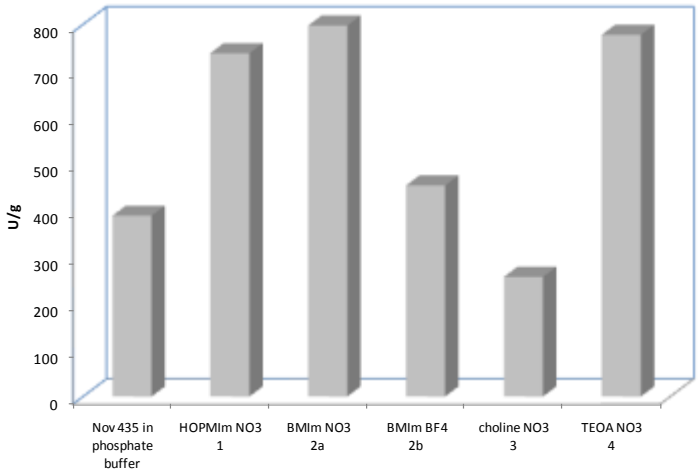


Fig. S1. Activity of Novozym 435 in hydrophilic ionic liquids using triacetin hydrolysis as assay

In the case of ionic liquids **1**, **2a** and **4** the enzyme displayed a twofold activity enhancement compared to Novozym 435 in phosphate buffer. Thus, in ionic liquids with the strongly coordinating nitrate anion **2a** $[BMIm]^+[NO_3]^-$ and **1** $[HOPMIm]^+[NO_3]^-$ the activity is enhanced markedly. In contrast, **2b** $[BMIm]^+[BF_4]^-$ which contains only a weakly coordinating anion gave comparable activity to phosphate buffer, thus underlining the importance of nitrate. In the case of tertiary ammonium based ionic liquids, with the Novozym 435 activity is significantly better in **4** $[TEOA]^+$, compared to **3** (choline cation)**.** Although both salts contain free alcohol groups (three ethanolic groups for **4**, compared to one ethanolic group in **3**) and nitrate as counter anion, **4** was a much better solvent, and thus triethanolammonium a much better cation.

Dissolution of Novozym 435 in ionic liquids.

Previous tests revealed that ILs could dissolve free CaLB, and could also desorb (leach) CaLB from the polymeric support in Novozym 435. In order to study the stability of immobilized lipase, the same activity assay was applied to the supernatant of reactions conducted in ionic liquids. In this case the solution of CaLB with ionic liquids was filtered after incubation for 1 hour in order to remove the resin. Next the standard activity test was applied (Fig. S2).

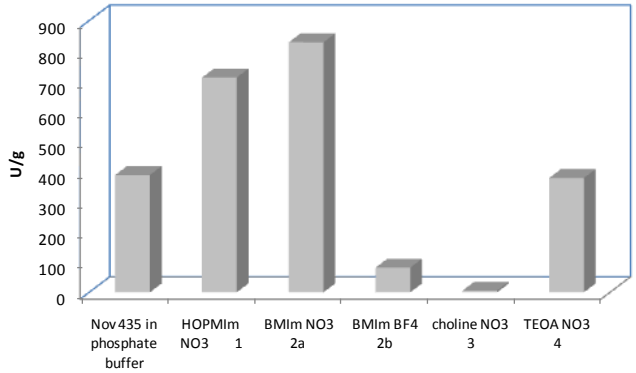


Fig. S2 Activity of filtered Novozym 435 solution in ionic liquid (triacetin hydrolysis assay)

Roughly the same activity (96-100%) was observed with **1** $[HOPMIm]^+[NO_3]^-$ and **2a** $[BMIm]^+[NO_3]^-$ and compared to the starting heterogeneous preparation. In other words the enzyme dissolved and remained active in the homogeneous IL-solution. This indicates that the activity previously observed in **1** and **2a** was completely due to dissolved enzyme. It is therefore safe to conclude that most of the enzyme was desorbed from the support. For **4** $[TEOA]^+[NO_3]^-$ 49 % of the activity was still present, indicating significant dissolution of enzyme as well. $[BMIm]^+[BF_4]^-$ **2b** with its weakly coordinating anion, was not very effective in dissolving lipase CaLB. Only 18 % of total activity was found in the hydrolytic test. In the case of **3** $[TMEOA]^+[NO_3]^-$, hardly any dissolution occurred (2-5 % of activity was observed).

CaLB CLEA©

The technique of Cross Linked Enzyme Aggregates is another way of immobilizing enzyme and allows for a broad application of enzymes under a variety of conditions.[4] The same series of experiments was applied to CaLB CLEA©. In the activity assay for our CLEA-preparation in phosphate buffer, an activity of 1001 U/g was measured. Filtered solutions of CaLB CLEA© in our range of ionic liquids displayed however very low activity (ca. 8% of activity in aqueous solution). This demonstrates that CaLB CLEA© is stable in a range of ionic liquids. Subsequently, CaLB CLEA© was dispersed and its activity measured as previously described (Fig. S3).

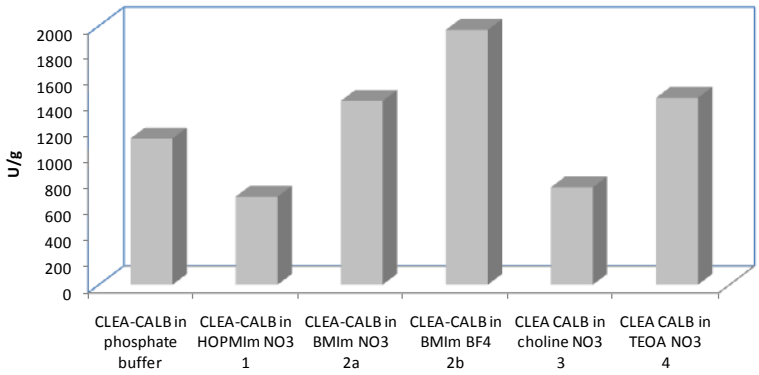


Fig. S3 CaLB CLEA© activity in dispersed ILs

Analogous to Novozym 435, an increase in activity could be detected using ionic liquids, rather than phosphate buffer as the reaction medium. In this case, a different order was observed, reflecting the activity of heterogeneous CaLB in these solvents. In this case weakly coordinating **2b** gave almost a doubling in activity, followed by the strongly coordinating **2b** and **4**. The previously found high performance of **1** $[HOPMIm]^+[NO_3]^-$ as solvent could not be repeated here. Apparently, this solvent is more effective in stabilizing CaLB upon dissolution.

Stability and ageing of the enzymes in ILs.

The stability and ageing of CaLB dispersed in ILs was studied. Both Novozym 435 and CaLB CLEA© were kept in ILs (**1** $[HOPMIm]^+[NO_3]^-$, **3** choline nitrate, **4** $[TEOA]^+[NO_3]^-$), continuously for three months at ± 5 ^oC. Afterwards, the solutions were subjected to the standard activity test. It turned out that for all ionic liquids **1**, **3**, and **4** no change in the hydrolytic activity was observed.

Notably, the catalytic activity of the enzyme for other (anhydrous) reactions, either dispersed or dissolved in ionic liquid, will probably differ from the activity measured in the standard hydrolysis test: In a previous study, the transesterification of ethyl butanoate with *n*-butanol was applied as a test reaction to evaluate the activity of CaLB in a range of ionic liquids.[1] For the transesterification reaction, both hydrophilic **2b** $[BMIm]^+[BF_4]^-$, as well as hydrophobic **2c** $[BMIm]^+[PF_6]^-$ were excellent solvents. Apparently, for transesterification, a low coordinating character of the anion is beneficial.[1,5]

Comparison of CaLB CLEA© and Novozym 435 in the epoxidation reaction

For epoxidation reactions, run at room temperature, the use of Novozym 435 was compared to that of CaLB CLEA© (Table S1). Epoxidation of cyclooctene was studied in this case. Nearly identical results were obtained for all solvents when using CaLB CLEA© instead of Novozym 435 as the source of lipase (see Table S1). Note that in the case of CaLB CLEA© 2.5 times as much units of lipase were present (1000 *vs*. 400 units/g in sample). Apparently neither more lipase (as for CalB CLEA©), nor dissolution of the lipase (as for Novozym 435 in **1**) is able to increase the reaction rate. This leads to the conclusion that the rate-determining step of the cascade reaction, is not influenced by lipase, and is therefore most likely step 2 *i.e.* the epoxidation of olefin with peracid (see Scheme 2, main text).

Table S1. CaLB CLEA © *vs*. Novozym 435 in chemo-enzymatic epoxidation of cyclooctene in ionic liquids after 24 hours

	Yield of cyclooctene oxide (%)		
ILs	$[HOPMIm]+[NO_3]-$ **1**	$[BMIm]^+[BF_4]^-$ **2b**	$[BMIm]^+[PF_6]^-$ **2c**
Novozym 435	69	39	38
CaLB CLEA©	72	41	47

Conditions: cyclooctene 1.48 mmol, 50 % aq. hydrogen peroxide 2.6 mmol (5x added with intervals of 1 h), octanoic acid 0.2 mmol, enzyme CaLB (Novozym 345 or CaLB CLEA©, both 10 mg), ionic liquid 1 mL, RT, 24 h.

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3 O. Kirk, M. W. Christensen, *Org. Proc. Res. & Developm.*, 2002, **6**, 446.
4 P. López-Serrano, L. Cao, F. van Rantwijk, R. A. Sheldon, *Biotechnol. Lett.*, 2002, **24**, 1379.
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