RSC Advances

On the hunt for truly biocompatible ionic liquids for lipase-catalyzed reactions

Journal:	*RSC Advances*
Manuscript ID:	RA-COM-11-2014-015021
Article Type:	Communication
Date Submitted by the Author:	21-Nov-2014
Complete List of Authors:	Deive, Francisco; University of Vigo, Chemical Engineering Department Ruivo, Diana; Instituto de Tecnología Química e Biológica – Universidade Nova de Lisboa, Rodrigues, João; Instituto de Tecnologia Química e Biológica - Universidade Nova de Lisboa, Gomes, Cláudio; Instituto de Tecnologia Química e Biológica - Universidade Nova de Lisboa, Sanromán, M.; University of Vigo, Chemical Engineering Department Rebelo, Luís Paulo; Instituto de Tecnología Química e Biológica – Universidade Nova de Lisboa, Esperança, José; Instituto de Tecnología Química e Biológica – Universidade Nova de Lisboa, Rodriguez, Ana; University of Vigo, Chemical Engineering Department

SCHOLARONE™
Manuscripts

Journal Name

RSCPublishing

COMMUNICATION

On the hunt for truly biocompatible ionic liquids for lipase-catalyzed reactions

Cite this: DOI: 10.1039/xoxxoooooox

F.J. Deive‡, [a] D. Ruivo‡, [b] J.V. Rodrigues, [b] C.M. Gomes, [b] M.A. Sanromán, [a] L.P.N. Rebelo, [b] J.M.S.S Esperança*[b] and A. Rodríguez*[a]

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/

One of the challenges in the field of biocatalysis is the search for efficient reaction media avoiding enzyme deactivation. This work shows for the first time that water + hydrophilic ionic liquids mixtures enhance (up to 50%) the enzymatic activity of lipases, opening new opportunities for enzyme extraction and biocatalysis.

Over the last two decades ionic liquids have gained recognition as viable candidates as catalytic media. A unique combination of properties and an easier tunability due to the existence of a plethora of possible cation-anion combinations, leads to an increasing interest of both the scientific community and the industry in thousands of new ionic liquids with distinct properties,[1] environmental risks,[2] and applications.[3]

Bringing together biocatalysis and ionic liquids has led to remarkable results for different types of reaction such as redox, hydrolysis, esterifications in the presence of enzymes like dehydrogenases, lipases, proteases, peroxidases or amylases.[4] Among them, lipases (EC 3.1.1.3 - hydrolases) are one of the most widely used class of enzymes in biotechnology,[5,6] due to their appealing features such as their chemo-, regio- and enantioselectivity, broad substrate specificity and ability to be active in aqueous and organic media. These enzymes are known to perform its catalytic action both in hydrolytic and synthetic reactions like esterifications, transesterifications, alcoholysis, etc.[7] In these latter cases, the pursuit of an optimum non aqueous biocatalytic medium is requested, so ionic liquids have been suggested as a viable alternative.

Recent studies have converged upon the idea that most water miscible ionic liquids act as enzyme-deactivating agents at low water content.[4] Nevertheless, our current environmental awareness is furthering the design of environmentally friendly neoteric solvents. More specifically, the functionalized ammonium-based ionic liquids, e.g. the cholinium (N,N,N-trimethylhydroxyethyl ammonium, $[N_{1\,1\,1\,(2OH)}]^+$) family, can be highlighted.[2,8] These liquid salts fulfil our expectations of biocompatibility and ecotoxicity since this cation is a well-known micronutrient.[8] Although it has been widely assumed that hydrophilic solvents strip off the essential waters from the enzyme active site, [9] rendering the enzyme inactive, the ability of this cation to favour the creation of a water-mimicking H-bonded network has been already addressed.[10] Thus, it is crucial to clearly distinguish between the enzyme activity and structure stability of the native fold. In this way, different scenarios are faced: a) a solvent entailing both the loss of native folding and the biocatalytic potential, b) a solvent involving structural changes, and c) a solvent leading to biocatalytic deactivation. In this study, we have blended both aspects with the purpose of achieving an optimum reaction milieu. Among the possible anions, amino acids can be a suitable choice since they are a natural (they make up the bricks of the protein structure) and reasonably cheap feedstock.[11] Moreover, amino acids have been recognized as stabilizing agents for lipases, showing also a synergistic effect on the activation of a lipase protein coated with an ionic liquid.[12,13]

Hereafter, we have tackled the biocatalytic behaviour of a model lipase from *Thermomyces lanuginosus* (*Tl*L) in the presence of aqueous solutions of amino acids (alanine –Ala-, glycine –Gly- and lysine –Lys-) and cholinium chloride as controls, and amino acid based ionic liquids (ChAA) (cholinium alaninate, cholinium glycinate and cholinium lysinate) (Fig. 1) at different concentrations (0, 0.5, 1, and 2 M, except for alanine where concentrations of 0.25, 0.5 and 1 M were tested, due to its low solubility in water). This study presents an innovative approach by using a biocompatible cation paired with amino acid-based anions with the aim of undertaking an enzyme activity study.

This journal is © The Royal Society of Chemistry 2012

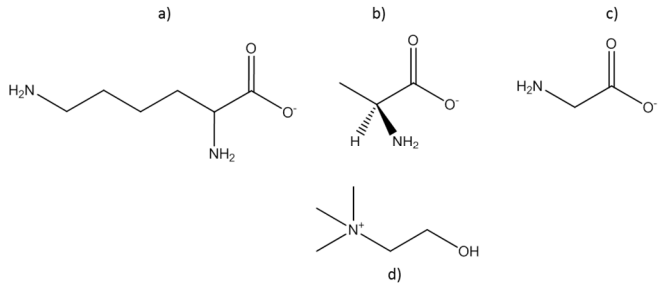

Fig. 1 Structure of the selected anions: a) lysinate, b) glycinate, c) alaninate, and cation: d) cholinium.

The amino acids were selected on the basis of their polar (Lys) and apolar (Ala and Gly) character. TlL was chosen since it is commercially available, and with applications in many biocatalytic applications as recently reviewed.[14] The lipolytic activity data of the selected enzyme in the presence of these compounds was then evaluated by monitoring the hydrolysis of *p*-nitrophenyl esters at the optimum temperature and pH (see Supporting Information-SI). These data are presented in Fig. 2.

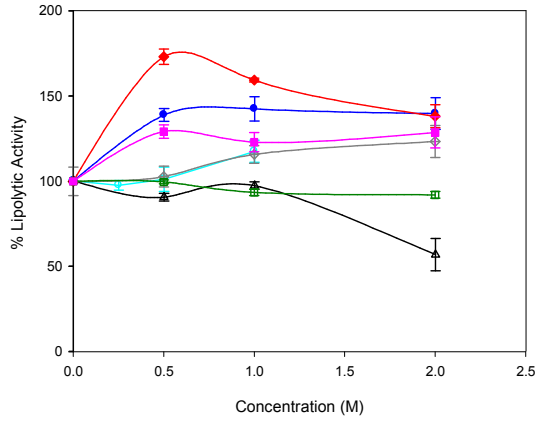

Fig. 2 Lipolytic activity (in percentage regarding the activity in water) of *Tl*L in the presence of different concentrations of the selected amino acids and ionic liquids: ChCl (black); Ala (cyan); Lys (green); Gly (grey); ChAla (blue); ChLys (pink); ChGly (red).

The analysis of the activity values in the presence of the selected amino acids reveals that none of them entails a deleterious effect for concentrations lower than 2 M. The data presented in Fig. 2 evidence the existence of some ions specific effects at high ionic liquid concentration in the particular case of ChCl, where a decrease in the lipolytic activity to about 50% occurs at a 2 M salt concentration. It is outstanding that amino acid-based ionic liquids always lead to activity levels higher than 100%, and in some cases the biocatalytic potential turned out to overcome in approximately 50% that of aqueous solutions of enzyme at optimum pH. To the best of our knowledge, this is the first time where an hydrophilic ionic liquid is used in solution to substantially enhance the biocatalytic activity of enzymes. The obtained results open up new opportunities both for enzyme extraction and biocatalysis.

A possible explanation of the observed trends could be well-known factors such as ionic liquid solution viscosity and pH. However, the viscosity of the aqueous solutions of the selected ionic liquids is close to that of water and cannot explain the specific effects observed. In relation to the pH, in our particular case there is a need to prevent acid pH values, since it has been demonstrated that *Tl*L is active at neutral and alkaline pHs.[15] Thus, aqueous pKa data of the amino acid can be a valuable starting point to predict the pH of its derived ionic liquid. The pH of aqueous solutions of the compounds used in this work is shown in Table 1. All the amino acid-based ionic liquids satisfy the alkalinity requirement to be considered as effective enzyme medium. On the other hand, ChCl at 2 M reaches acidic values, which explains the reduction in the observed values of lipolytic activity. The effect of the selected ionic liquids on *Tl* lipase was studied without any buffer, to avoid "extra" effects disturbing a pure analysis of the enzyme activity in the presence of the newly synthesized solvents. Besides, when working with this kind of ionic liquids, the pH of the medium cannot be controlled by simply adding a "buffer mixture" of salts, because the intrinsic proton activity of the ionic liquid will bog down the possible buffering action.[16] pH control under high ionic liquids concentration is a topic of undoubted interest when working with proteins in the presence of ionic liquids, but the buffering action could be done by an appropriate selection of these liquid salts. Therefore, adding a conjugate acid (or base) species to the ionic liquid media could be one future solution to consider when working with enzymes with distinct optimum pH values.

Table 1. pH for the aqueous solutions containing different concentrations (in molarity) of the selected amino acids and ionic liquids.

M	Ala	Gly	Lys	ChCl	ChAla	ChGly	ChLys
0.25	6.8						
0.5	6.3	6.7	10.0	5.3	10.9	10.9	9.8
1	6.0	6.5	9.9	5.5	11.0	11.0	9.8
2	-	6.4	9.9	4.7	11.3	11.1	10.0

Notwithstanding our main purpose is to maintain/enhance the biocatalyst activity, the study of the enzyme structural changes in the presence of the selected ionic liquids can shed light on the observed biocatalytic behavior. Hence, we have used Differential Scanning Calorimetry (DSC) and Differential Scanning Fluorimetry (DSF) to elucidate the conformational changes in the enzyme structure.

The DSC technique has been already proposed to unravel the interactions between proteins and ionic liquids.[17,18] Additionally, DSF has been suggested by Rodrigues and coworkers[19] as a rapid and useful means to analyze structural changes for proteins in the presence of cholinium-based ionic liquids. The methods and the results obtained are presented in Table S1 of the SI. Fig. 3 shows one example of the experimental results. A good agreement in the results obtained by these two distinct techniques is observed, although in some cases it is clear that the solvent conditions influence the unfolding pathway. For example, ChAla and ChGly seem to decrease the population of the unfolding intermediate observed at a Tm ~68 °C, whereas thermal unfolding in the presence of ChCl and ChLys seems still to comprise an at least two step reaction. In general terms, despite the deactivating effect observed for ChCl, this ionic liquid is the one entailing the lowest structural disturbance with a decrease in Tm smaller than 5 ºC (Fig. S7 in SI). In contrast, the presence of the amino acid-based ionic liquids induce an alteration of the enzyme structure which is reflected in a maximum decrease of

This journal is © The Royal Society of Chemistry 2012

the Tm of about 14 ºC, almost independently of the ionic liquid concentration. However, it is important to highlight that the observed ChAA-induced structural changes do not compromise at all the enzyme activity, but reinforces it.

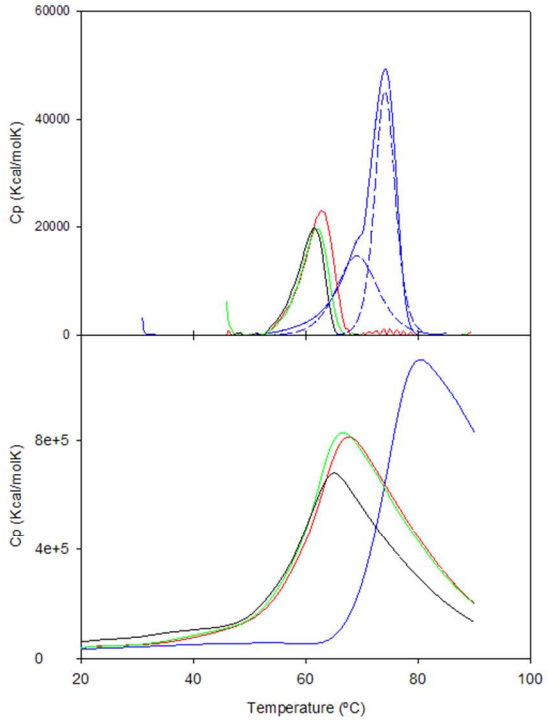

Fig. 3 Thermal denaturation profiles of *Tl*L by DSC (up) and DSF (down) in the presence of different concentrations of the ionic liquid ChAla: blue represents water, red represents ChAla 0.5 M, green represents ChAla 1 M and black represents ChAla 2 M. Blue dashed lines represent the water curves fitted by the non-two-state-model.

The reason behind this behavior can underlie in the different surface charge of the enzyme in the presence of the selected ionic liquids (Fig. 4) induced by the distinct pH of the corresponding ionic liquid media. In this way, ChCl will interact less strongly with the enzyme than the ChAA, since the latter lead to greater interplays with the amino acids sticking out of the main polypeptide chain.

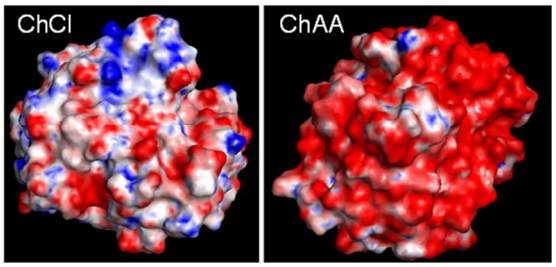

Figure 4. Surface charge distribution for the *Tl*L in the presence of the selected cholinium-based ionic liquids. ChCl (acid pH at 2 M) and ChAA (alkaline pH at 2 M). Negatively charged areas are represented in red, and positively charged areas in blue (Images obtained with PyMOL v0.98 using the APBS tool).

In summary, this work points out that, per se, structural results are not enough to analyze the influence of ionic liquids on the activity of an enzyme. Moreover, it shows for the first time that a new biocompatible amino acid-based hydrophilic ionic liquid can enhance enzyme activity, acting as a special media to be concomitantly used in enzyme extraction and biocatalysis.

Acknowledgements

The authors thank the Spanish Ministry of Economy and Competitivity for financial support through the project CTMQ 2012-31534 and Fundacão para a Ciência e Tecnologia, FCT, through projects PTDC/CTM-NAN/121274/2010 and PEst-OE/EQB/LA0004/2013. FJD acknowledges Xunta de Galicia for funding through the Isidro Parga Pondal program and JMSSE and DR acknowledge FCT for an Investigator FCT contract and a PhD fellowship (SFRH/BD/74081/2010), respectively. The NMR spectrometers are part of the National NMR Facility supported by FCT (RECI/BBB-BQB/0230/2012).

Notes and references

[a] Department of Chemical Engineering, University of Vigo, 36310 Vigo (Spain), E-mail: aroguez@uvigo.es
[b] Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. República, 2780-157 Oeiras, Portugal

Electronic Supplementary Information (ESI) available: Experimental details and NMR of ionic liquids. See DOI: 10.1039/c000000x/

1. a)M. J. Earle, J. M. S. S. Esperança, M. A. Gilea, J. N. Canongia Lopes, L. P. N. Rebelo, J. W. Magee, K. R. Seddon, J. A. Widegren, *Nature* 2006, **439**, 831; b) L. P. N. Rebelo, J. N. Canongia Lopes, J. M. S. S. Esperança, E. J. Filipe, *J. Phys. Chem. B* 2005, **109**, 6040; c) M. Smiglak, W. M. Reichert, J. D. Holbrey, J. S. Wilkes, L. Y. Sun, J. S. Thrasher, K. Kirichenko, S. Singh, A. R. Katritzky, R. D. Rogers, *Chem. Commun.* 2006, 2554 d) K. J. Baranyai, G. B. Deacon, D. R. MacFarlane, J. M. Pringle, J. L. Scott, *Aust. J. Chem.* 2004, **57**, 145.
2. M. Petkovic, K. R. Seddon, L. P. N. Rebelo, C. S. Pereira, *Chem. Soc. Rev.* 2011, **40**, 1383.
3. N. V. Plechkova, K. R. Seddon, *Chem. Soc. Rev.* 2008, *37*, 123-150.
4. P. Lozano, *Green Chem*. 2010, **12**, 555 (and references therein).
5. R. D. Schmid, R. Verger. *Angew. Chem. Int. Ed.* 1998, **37**, 1608.
6. F. van Rantwijk, R. A. Sheldon. *Chem. Rev.* 2007, **107**, 2757.
7. M. Kapoor, M. N. Gupta, *Process Biochem.* 2012, **47**, 555.
8. R. M. Vrikkis, K. J. Frazer, K. Fujita, D. R. MacFarlane, G. D. Elliott, *J. Biomech. Eng*. 2009, **131**, 074514
9. A. M. Klibanov, *Nature* 2001, **409**, 241.
10. H. Weingärtner, C. Cabrelle, C. Herrmann, *Phys. Chem. Chem. Phys.* 2012, **14**, 415.
11. J. C. Plaquevent, J. Levillain, F. Guillen, C. Malhiac, A. C. Gaumont, *Chem. Rev.* 2008, **108**, 5035.
12. Y. Abe, T. Hirakawa, S. Nakajima, N. Okano, S. Hayase, M. Kawatsura, Y. Hirose, T. Itoh, Adv. Synth. Catal. 2008, 350, 1954
13. K. Yoshiyama, Y. Abe, S. Hayse, T. Nokami, T. Itoh, Chem. Lett. 2013, 42,663
14. R. Fernandez-Lafuente, *J. Mol. Cat. B: Enzym.* 2010, **62**, 197.
15. F. J. Deive, A. Rodríguez, A. B. Pereiro, J. M. M. Araújo, M. A. Longo, M. A. Z. Coelho, J. N. Canongia Lopes, J. M. S. S. Esperança, L. P. N. Rebelo, I. M. Marrucho, *Green Chem.* 2011, **13**, 390.
16. McFarlane et al, Chem. Commun., 2010, **46**, 7703.
17. D. Constantinescu, C. Herrmann, H. Weingärtner, *Phys. Chem. Chem. Phys*., 2010, **12**, 1756.
18. D. Constantinescu, C. Herrmann, H. Weingärtner, *Angew. Chem. Int. Ed*., 2007, **46**, 8887.
19. J. V. Rodrigues, V. Prosinecki, I. Marrucho, L. P. N. Rebelo, C. M. Gomes, *Phys. Chem. Chem. Phys.* 2011, **13**, 13614.