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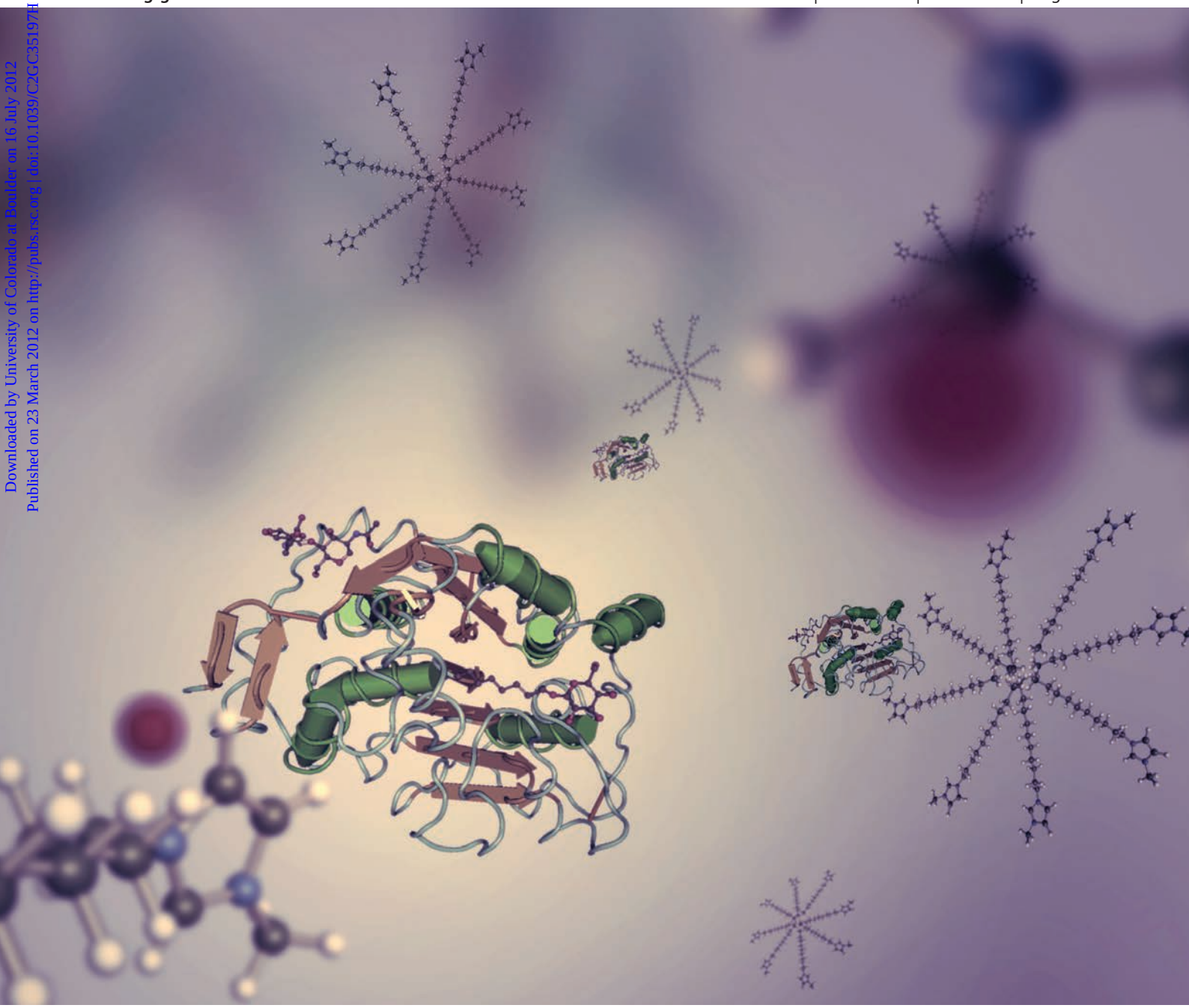

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PAPER

Ionic liquids microemulsions: the key to *Candida antarctica* lipase B superactivity†

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The activity of the commercial enzyme *Candida antarctica* lipase B (CaLB), in aqueous solutions of the ionic liquid 1-decyl-3-methylimidazolium chloride, [C_{10}mim]Cl, at pH 7.0 was investigated. The relative enzyme activity (Act_{IL}/Act_{Bf}) for ionic liquid molar concentrations ranging from 0.000–0.150 mol L^{-1} show an increase up to six fold in Act_{IL}/Act_{Bf}, with the ionic liquid molar concentration. This phenomenon is shown to be related with the formation of micelles originated by the self-aggregation of [C_{10}mim]Cl. The enzyme activation energy is not affected by the ionic liquid presence, suggesting the absence of structural changes in the enzyme induced by the ionic liquid. It is here demonstrated, for the first time, that it is possible to significantly increase the activity of an enzyme simply by using aqueous solutions of ionic liquids.

1. Introduction

Lipases (EC 3.1.1.3) are a sub-class of enzymes within the esterase family whose natural function is to hydrolyse long chain triacylglycerols, such as oils or fats.[1] This sub-class is widely available in nature where its main function is to digest lipids in order to make these available as an energy source for cells.[2] Some of the most significant industrial applications of lipases are mainly found in the pharmaceutical sector, in foods and detergents.[3] Organic solvents are widely used for biocatalysis, due to their excellent functionality, since these solvents can led to an enhancement in enzyme stability.[4,5] The reasons for the great applicability of organic solvents have been discussed in detail by Castro and Knubovets[6] and Halling.[7] These authors reported that organic solvents improve the solubility of hydrophobic reagents or avoid undesired side reactions. However, organic solvents have several disadvantageous characteristics, such as their significant vapor pressure, flammability and toxicity.

In the last decade, room temperature ionic liquids (ILs) have gained particular importance in the biocatalysis field, being nowadays generally recognized as a very promising reaction media.[8–13] Because of their negligible vapor pressures, ILs have been generally employed as substitutes for the traditional organic solvents in catalytic and synthetic reactions.[14,15] These ionic compounds present large organic cations with a variety of organic or inorganic anions being liquid at, or close to room temperatures.[16] In comparison to common organic solvents, ILs have more favorable properties, including a wide liquidus range, low flammability, high ionic and thermal conductivity, good dissolution power towards many substrates, high thermal and chemical stability and a wide electrochemical potential window.[17] In addition, specific characteristics can be tuned by changing the anion, cation and alkyl side chains, making it possible to design a specific IL for a particular reaction or application.[18] Indeed, this feature is a key factor for the performance of successful reactions, since appropriate combinations of the cation and anion might increase the substrates solubility, improve the enzyme selectivity and/or product selectivity,[19,20] enhance the enzyme activity and/or stability, and improve the suppression of side reactions. The results reported in the literature show that enzymes have the same catalytic behavior in water, organic solvents and ILs. However, like the organic solvents, the ILs may decrease, or even completely inactivate the enzymes, since they have the capacity to interact with the water molecules around the enzyme surface[21] and in some cases, with the enzyme itself.[22,23] The number of studies investigating the effect of different ILs on the enzyme activity is growing, as can be seen by the great number of published articles[22,24–33] and reviews on this subject.[21,34–40] These works show that although some ILs, such as those based on the hydrophobic anions, hexafluorophosphate [PF_6], tetrafluoroborate [BF_4] and bis(trifluoromethylsulfonyl)-imide [NTf_2]) are less denaturing than some organic solvents and responsible for higher catalytic activities,[35,41] the hydrophilic ILs, have, in some cases (depending of the anion moiety, cation core and alkyl chain conjugated) and under some conditions (*e.g.* concentration, water activity, and others)[42,43] a deleterious effect on the enzyme activity and stability. In fact, many enzymes show a similar level of activity in ILs as in conventional organic solvents, which are considerably inferior to those in aqueous solutions, this being a major drawback for the industrial

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use of these solvents for biocatalytic reactions. In many cases, the application of enzymes in ILs is limited by their low solubility, activity and also stability. In this context, there is a strong and urgent need to increase the activity and stability of enzymes in ILs, particularly in those potential enzyme-inactivating (hydrophilic) ILs. Some strategies have been considered: (i) the modification of enzymes and (ii) the modification of the solvent environments, ranging from biocatalyst formulations to the tailoring of the reaction medium to a particular reaction.[17,44] The first approach is reported in different works[17,44] suggesting the use of physically or chemically immobilized enzymes, cross-linked enzyme aggregates (CLEAs) by physical or covalent attachment to polymers, three-phase partitioning, enzyme/protein-coated micro-crystals and lyophilization with cyclodextrins. The second category includes the formation of water-in-IL microemulsions, the IL coating, the use of additives in ILs, and the design of enzyme-compatible ILs. The methods in the first category play with the tolerance of the enzyme to those denaturing factors of these ionic solvents (polarity, surfactant effect, hydrogen-bond basicity, anions nucleophilicity, viscosity, ion kosmotropicity and, finally hydrophobicity).[17] The methods in the second category intend to minimize these denaturing properties to improve the compatibility of the enzymes with ILs. However, all these techniques[17,44] require or the manipulation/pre-treatment of the enzymes or the manipulation of the IL medium, increasing the cost of those processes. Moreover, some of these techniques[17,44] require, beyond the use of ILs, the addition of surfactants or organic solvents, increasing the toxicity concerns.

Since lipases are widely available in nature, the large number of reports on the use of ILs in biocatalysis address the use of lipases.[45–48] *Candida antarctica* lipase B (CaLB) is a good example of a largely used lipase, since it is considered as a very stable enzyme, even compared with other lipases. It is routinely used in diverse areas of biocatalysis and does not require a hydration shell to be active. This characteristic, as well as its quite relaxed substrate specificity and its operational stability, make this enzyme a valuable scientific and industrial synthetic tool.

This work aims at developing an IL system that is compatible with lipase, enhancing its activity without any enzyme treatment or manipulation of the IL. It is based on the concept of microemulsions, but without the addition of a surfactant to promote the aggregates formation. Instead, it uses the capacity of long-alkyl chain ILs, namely 1-decyl-3-methylimidazolium chloride, [C_{10}mim]Cl, to promote the formation of micelles by self-aggregation. It will be here shown, for the first time, that a significant increment of the relative CaLB activity (up to six fold) can be achieved, simply using an aqueous IL solution with no change in the reaction mechanism, as judged by the activation energy of CaLB catalytic reaction.

2. Results and discussion

This work reports, for the first time, the use of a simple method based on the IL [C_{10}mim]Cl, to increase the activity of the commercial enzyme CaLB. In this study, the effect of different molar concentrations of this IL, on the relative lipase activity (Act_{IL}/Act_{Bf}) was investigated and is reported in Fig. 1. The results suggest that, for IL molar concentrations up to 0.033 M, a decrease in the lipase activity occurs when compared with the activity of CaLB in the absence of the IL. The decrease of lipase activity in presence of different substituted alkyl chains on the imidazolium ring was previously described by Klähn *et al.*[23] and also studied by us.[32] With the increase of the cation alkyl chain the enzyme activity is progressively more inhibited due to a stronger interaction of the cation with the non-polar residues of

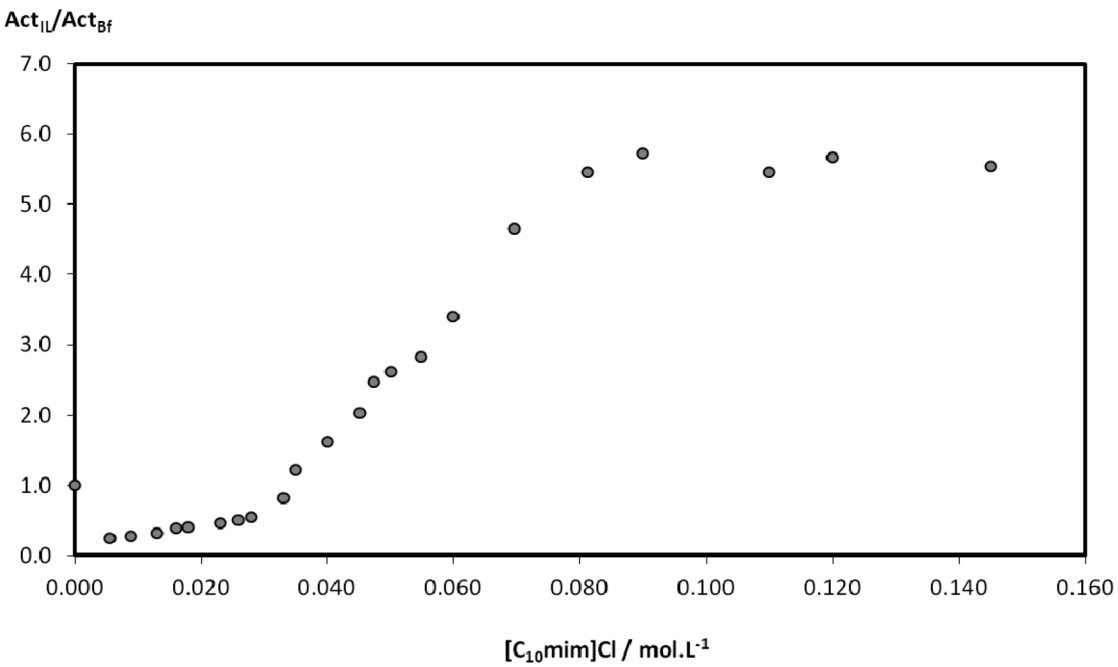

Fig. 1 The relative enzyme activity of CaLB as a function of the concentration of [C_{10}mim]Cl.

the enzyme active site.[23] With the partial or even total obstruction of the active site, which is dependent on the molar concentration of IL, the diffusion into and binding of the substrate to the active site is hindered, with a consequent decrease in the enzymatic activity.[32]

Conversely, for molar concentrations above 0.033 M, the enzyme activity is higher than that observed in the buffer without IL. Moreover, the lipase activity continues increasing with the molar concentration of IL, achieving a maximum six-fold increase for an IL molar concentration of 0.090 M. To the best of our knowledge, an increase of this significance on the activity of any enzyme in presence of an IL solution is here reported for the first time.

The formation of micelles in long chain imidazolium ILs, including the IL used here, was described in recent works by several authors.[49–53] To evaluate if the aggregation of [C_{10}mim]-Cl was related to the observed activity increase, the critical micelle concentration (CMC) of [C_{10}mim]Cl was determined by electric conductivity (mS cm^{-1}) measurements, being these results presented in Fig. 2. Data for [C_{10}mim]Cl in potassium phosphate buffer aqueous solution (pH 7.0) (represented by triangles) allow an estimation for the CMC of this system of 0.048 M. This is in good agreement with the data reported by other authors, measured by different methods that range from 0.04–0.06 M.[54–56] However, as a careful analysis of Fig. 1 shows, the increase in the relative enzyme activity starts for molar concentrations of IL below 0.020 M, which are well below the CMC value determined. To evaluate the effect of the substrate (*p*-NFL) on the aggregation of the IL, the electric conductivity measurements were applied to the IL solutions in potassium phosphate buffer but now in presence of the same amount of substrate (*p*-NFL) used in the enzyme activity measurements (data represented by diamonds in Fig. 2). The results show that the substrate significantly contributes to lower the CMC of the system, as discussed by Łuczak *et al*.[57] for other compounds, such as salts and alcohols. The CMC value measured in presence of the substrate is now around 0.015 M. A comparison of the conductivity (Fig. 2) and the relative enzyme activity (Fig. 1) data shows that the increase of Act_{IL}/Act_{Bf} is observed for molar concentrations of [C_{10}mim]Cl above the CMC for the system with *p*-NFL.

In order to confirm the formation of aggregates, dynamic light scattering measurements were performed for systems composed by the potassium phosphate buffer solution (pH 7.0) the substrate *p*-NFL at the concentration used in the enzyme activity measurements and different ILs at various molar concentrations. The ILs tested were [C_4mim]Cl, [C_8mim]Cl, and [C_{10}mim]Cl. The results (see Fig. S1 from ESI†) show the presence of aggregates in the 1–10 nm size range only in presence of the higher concentrations of the IL with the longest alkyl chains [C_{10}mim]Cl.

The significant increase in activity observed (six fold), relative to the aqueous system is thus explained by the formation of micelles promoted by the self-aggregation of [C_{10}mim]Cl. The self-aggregation of the IL leads to the formation of several micelles, which are responsible for the increase in the IL–water interface, and consequently, the increase in the enzyme activity. This behaviour was previously observed for microemulsions formed by surfactants and is described in the review of Stamatis *et al*.[58] In this work, the author explains that the presence of microemulsion systems provides a large increase in the interfacial area increasing the contact between the substrate molecules and the enzyme active site. Moreover, it is well known that some enzymes, such as the lipases, are normally located and are more active at the interfacial region of the aggregates.[58] This significant increase in an enzyme activity is known in the literature as superactivity and it was observed in microemulsion systems based on several surfactants.[58,59]

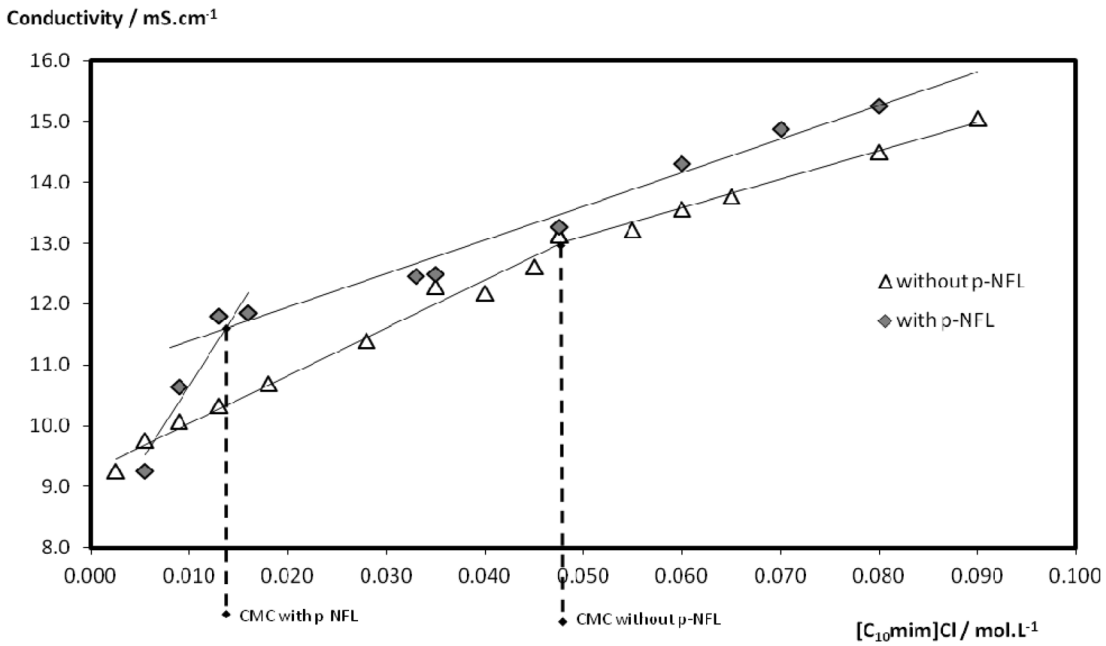

Fig. 2 The effect of the substrate (*p*-NFL) on the electric conductivity and CMC of [C_{10}mim]Cl aqueous solutions.

The enzymatic activity increase observed could also be the result of modifications of the reaction mechanism, caused by structural alterations of the enzyme due to the interaction between the ILs and the enzyme surface.[22,23] To evaluate if this effect was also taking place in the system under investigation, the enzyme activity was studied at different temperatures and for the system without IL and in presence of 0.0230 and 0.0550 M of [C_{10}mim]Cl. The results are reported in Fig. 3 and Table S1 from ESI.† An increase of the enzyme activity was observed at all temperatures. Using these data, the activation energy of the lipase was calculated in the three systems. The results shown (Fig. 3) indicate that the activation energy of the enzyme is independent of the presence of IL, which proves that in spite of the large increase in the activity observed in the presence of the IL, the enzymatic reaction mechanism is not modified by the presence of the IL. The effect on the enzyme activity in the presence of [C_{10}mim]Cl, seems to be caused only by the formation of the micelles, due to the self-aggregation of the IL and not by structural changes in the enzyme.

As previously discussed, various studies were carried using ILs to stabilize or activate enzymes.[17,44] Despite the increase in the activity or selectivity promoted by these methods previously reported in literature[17,44] they use more sophisticated, expensive and/or toxic methodologies to achieve the increase in the stability and activity of the generality of enzymes. Concerning the use of microemulsions, Goto and collaborators[60] reported the use of water-in-IL microemulsions as a new medium for dissolving various enzymes and proteins, namely horseradish peroxidase, being the enhancement of the enzyme activity observed when compared with the same system using an organic solvent instead of the IL. A three-fold enzyme activity enhancement of *Burkholderia cepacia* lipase using water-in-IL microemulsions based on the hydrophobic 1-octyl-3-methyl imidazolium bis (trifluoromethylsulfonyl)imide ([C_8mim][NTf_2]) was reported in comparison with the water-in-isooctane microemulsions.[61] These results show that water-in-IL microemulsions are capable of activating the enzymes when compared with organic solvents but to a much lesser extent than the results reported here for which an enhanced activation is achieved in comparison with aqueous solutions. Thus, only through the use of surfactants was the superactivity of lipase previously observed,[62,63] being here reported for the first time with ILs.

3. Experimental section

3.1. Material

The IL used in this study was 1-decyl-3-methylimidazolium chloride – [C_{10}mim]Cl. This IL was acquired at IoLiTec (Ionic Liquid Technologies, Germany) with mass fraction purity higher than 99%, confirmed by us using ^{1}H-NMR and ^{13}C-NMR. The enzyme used, *Candida antarctica* lipase B (EC 3.1.1.3), was kindly offered by Novozymes. For the determination of the enzyme activity by the spectrophotometric method, *p*-nitrophenyl laurate (*p*-NFL) from Fluka (purity ≥98.0% by GC), dimethyl sulfoxide (DMSO) from Lab-Scan and *p*-nitrophenol (*p*NF) from Sigma-Aldrich, were used.

3.2. Methods

3.2.1. Enzymatic activity. The lipase activity was assayed spectrophotometrically using a SHIMADZU UV-1700, PharmaSpec Spectrometer. The substrate solutions composed of *p*-NFL were prepared by mixing 0.018 g of *p*-NFL in 1 mL of DMSO. This solution was then diluted 100 times in a potassium phosphate buffer solution (50 mM and pH 7.0). The enzymatic solution was prepared by adding 25 μL of CaLB to 300 μL of the potassium phosphate buffer solution (50 mM and pH 7.0). A specific amount of IL, dependent on the final IL molar concentration required, was then added to this enzymatic solution. To measure the lipase activity, 200 μL of the enzymatic solution (containing different amounts of the IL or the potassium

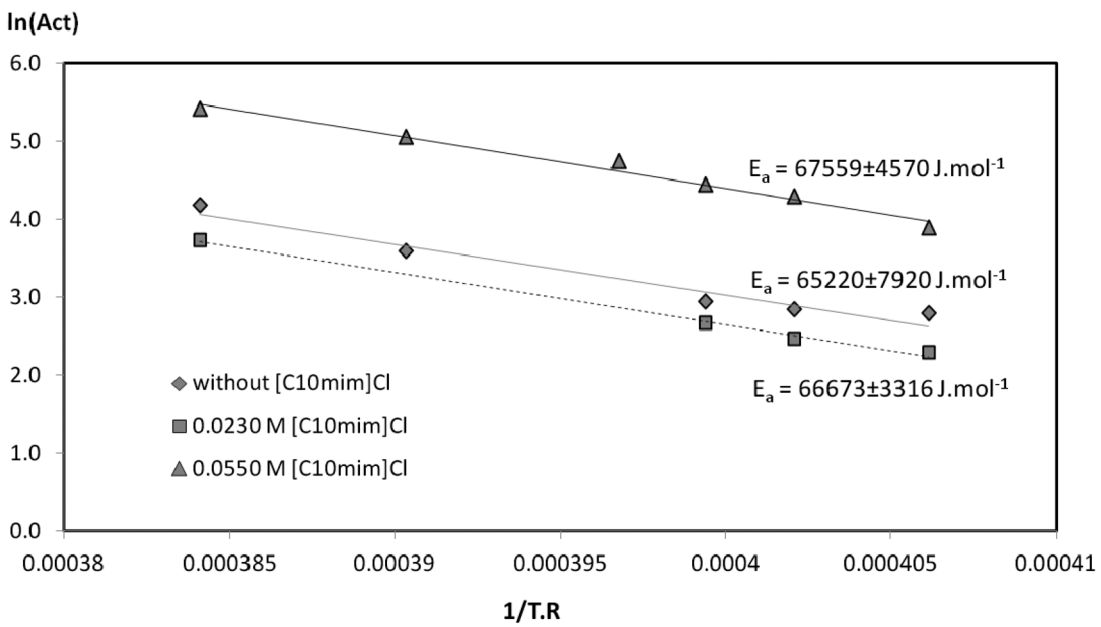

Fig. 3 The temperature dependency of the activity and activation energy of CaLB for various concentrations of [C_{10}mim]Cl.

phosphate buffer solution) was added to 1.8 mL of substrate solution (0.56 mM), previously acclimatized at 37 °C for 15 minutes, and the reaction was left to proceed at 37 °C. To quantify the enzyme activity, the absorbance variation at 410 nm was registered during 150 seconds (ΔAbs min^{-1}), over the linear range of absorbance variation with time. The lipase activity was calculated in U L^{-1}, one unit being defined as the amount of enzyme that hydrolyzed 1 μmol of *p*-NFL per min (the amount of *p*-NFL hydrolyzed was quantified based on a calibration curve previously determined). In this work, the results are reported as the ratio between the activity of the enzyme with IL (Act_{IL}) and the enzyme activity in phosphate buffer without IL (Act_{Bf}), denoted in this work by relative lipase activity (Act_{IL}/Act_{Bf}).

3.2.2. Effect of temperature on the enzyme activity. The effect of temperature was also assessed in this work. The enzyme activity was determined for several reaction temperatures ranging from 23–40 °C, according to the same method already described in section 2.2.1. Finally, the activation energy for the hydrolytic reaction of the enzyme was calculated using the Arrhenius equation:

$$\ln(\mathrm{Act}) = \ln(A) - (E_a/RT) \quad (1)$$

where A is the pre-exponential Arrhenius factor; T is the temperature (K), E_a is the activation energy (J mol^{-1}) and R is the universal gas constant (8.314 J mol^{-1} K^{-1}).

3.2.3. Electric conductivity measurements. The conductivity of the samples was measured using a SevenMulti™ (Mettler Toledo Instruments) at 298 K, within an uncertainty of ± 0.01 mS cm^{-1}.

3.2.4. Dynamic light scattering (DLS). To determine the size of aggregates formed in the ILs–aqueous buffer mixtures, dynamic light scattering (DLS) was employed, using a Nano-ZS, ZetaSizer from Malvern Instruments.

4. Conclusion

This work shows that it is possible to induce superactivity in *Candida antarctica* lipase B by using the long alkyl chain ionic liquid ([C_{10}mim]Cl). The results demonstrate that the activity increment does not result from changes in the reaction mechanism or enzyme structural alterations induced by the ionic liquid, but instead may be explained by the formation of microemulsions due to the ionic liquid alkyl chain self-aggregation. The behavior here reported could be the basis for novel methodologies of enzyme activation using aqueous solutions of ionic liquids.

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