Journal of Chromatography B, 879 (2011) 2679–2687

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Design of ionic liquids for lipase purification

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ARTICLE INFO

Article history:
Received 11 February 2011
Accepted 15 July 2011
Available online 24 July 2011

Keywords:
Candida antarctica lipase B
Ionic liquid
Purification process
Partition process

ABSTRACT

Aqueous two-phase systems (ATPS) are considered as efficient downstream processing techniques in the production and purification of enzymes, since they can be considered harmless to biomolecules due to their high water content and due to the possibility of maintaining a neutral pH value in the medium. A recent type of alternative ATPS is based on hydrophilic ionic liquids (ILs) and salting-out inducing salts. The aim of this work was to study the lipase (*Candida antarctica* lipase B – CaLB) partitioning in several ATPS composed of ionic liquids (ILs) and inorganic salts, and to identify the best IL for the enzyme purification. For that purpose a wide range of IL cations and anions, and some of their combinations were studied. For each system the enzyme partitioning between the two phases was measured and the purification factors and enzyme recoveries were determined. The results indicate that the lipase maximum purification and recovery were obtained for cations with a C_8 side alkyl chain, the $[N(CN)_2]$ anion and ILs belonging to the pyridinium family. However, the highest purification parameters were observed for 1-methyl-3-octylimidazolium chloride $[C_8mim]Cl$, suggesting that the IL extraction capability does not result from a cumulative character of the individual characteristics of ILs. The results indicate that the IL based ATPS have an improved performance in the lipase purification and recovery.

1. Introduction

Enzymes are classified according to the type of reaction they catalyse. 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]. Lipases are widely available in nature where their main function is to digest lipids in order to make these available as an energy source for cells [2]. Fungi and bacteria secrete lipases to their surroundings to facilitate nutrient absorption from the external medium. There are many potential applications of lipases, as in leather and cosmetics processing, animal feed, pulp and paper processing and textile [3]. However, the most significant industrial applications of lipases are mainly found in the pharmaceutical sector, in food and detergents [4]. The number of enzymes commercially available and their range of applications are gradually increasing. There are many reasons for the growing interest in enzyme-mediated reactions compared to chemical processes, including the high degree of specificity, the mild reaction conditions and a lower probability of occurring side reactions. Furthermore, enzyme-mediated processes are energy saving and reduce the extent of thermal degradation. A very useful trait of lipases is their enantio-selectivity, a very useful characteristic in the pharmaceutical area [5]. Some other attributes are also highlighted in literature, such as, their regio-selectivity and broad substrate specificity [6].

The microbial lipases are produced through a fermentation process, in which secondary or intermediate products are also generated. These frequently prevent the use of the fermented broth in industrial procedures or are deleterious to the enzyme. For the success of commercial production of enzymes and proteins, efficient downstream processing techniques are essential. Moreover, when these processes are applied to biological materials, rigorous purification steps, delicate enough to preserve the biological activity, are required. The most common techniques used are the ammonium sulphate precipitation [7], ionic and affinity chromatography [8,9], dialysis, filtration, electrophoresis [10–13] and reverse micelles approaches [14,15]. Nevertheless, some of these separation processes are costly and time consuming, and are not easily scalable [7]. Among the various separation techniques, liquid–liquid (solvent) extraction is one of the best-known, well-established, versatile and easy to use. Moreover, liquid–liquid extraction allows for a good resolution and high yield, a relatively high capacity, easy scale-up and requires low material cost. However, most of the

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doi:10.1016/j.jchromb.2011.07.022

extraction techniques employ organic solvents, which are immiscible with water, volatile, flammable and health hazardous. This makes these systems of extraction inappropriate for the development of environmental-friendly technologies. Another problem with conventional solvents is their limited number, making difficult to find the ideal solvent suited for a particular application, even considering solvent mixtures.

In times of improved molecular engineering and protein design, the development of new and biocompatible extraction methods, for the separation and purification of enzymes and proteins, is gaining increasing importance. An effective and economically viable method for the separation and purification of biomolecules is their partition in an aqueous two-phase system (ATPS). ATPS phase separation occurs on mixing two mutual incompatible solutes in water by which two aqueous phases are formed with different compositions. Solutes distribute themselves between the two phases depending on their relative affinity for each one of the individual phases. This technique is widely used in biomolecules purification where its advantages include favourable selectivity, low cost and adaptability to continuous sample processing and retention of biological activity [16,17]. Nonetheless, most of polymer-based ATPS display high viscosity [18,19]. Finally, these polymer ATPS normally form opaque aqueous solutions, which could interfere with the quantitative and qualitative analysis of extracted compounds.

During the last few years, ILs have been applied for biopurification and bio-extraction processes, and advantages, such as the improvement of enzyme stability, substrate and/or product selectivity, and suppression of side reactions were observed [20–25]. Moreover, IL-based ATPS are substantially less viscous than typical polymer-based ATPS [26,27]. Recently we reported viscosity data for various imidazolium-based ATPS where it was observed that the viscosities of the IL-rich phases ($3.23–8.05\ mPa\ s^{-1}$) are similar to those of the salt-rich phases ($2.00–4.76\ mPa\ s^{-1}$) and much lower than those of polymer based ATPS [26].

Most water-soluble room temperature ILs are salting-in inducing electrolytes (usually known as "chaotropic" salts) that can form a second aqueous phase in the presence of aqueous solutions of salting-out inorganic salts ("kosmotropic" salts) resulting in the formation of ATPS [28–33]. In aqueous systems composed of IL, inorganic salt and water, the driving force for phase separation is the competition between the IL and the salt for water molecules. The higher affinity of the inorganic salt for water induces a migration of water away from the IL ions decreasing their hydration and reducing the ILs solubility in water [34,35]. The inorganic salts commonly employed are ammonium-, potassium- or sodium-based salts of multiply charged anions, such as phosphate [21,36,37], sulphate [37], carbonate [36,37], or citrate [38] (strong salting-out inducing anions). In the past few years, the applications of IL-based ATPS included extraction studies with a significant number of low molecular mass compounds, such as tryptophan [33,39], vanillin [26], alkaloids [27,36,40] and steroids, such as testosterone and epitestosterone [41]. The partition coefficients for these systems range from 10 to 120 with recoveries up to complete extraction in a single step extraction [40]. Moreover, the IL-rich aqueous phase is compatible with liquid chromatography and detection limits of spectroscopic techniques, and thus suitable for analyzing biological fluids, as human urine samples [40]. Besides the studies involving ILs and inorganic salts, recently an imidazolium-derivatized poly(ethylene glycol) (PEG) was used in the ATPS formation aiming the extraction of penicillin (recovery around 96%) [42]. Recently, the use of ILs as adjuvant promoters of polymer-based ATPS has been suggested [43].

The extraction of biomacromolecules, such as enzymes and proteins, by water-insoluble ILs is limited by their low solubility in ILs and by the possibility of extracting them without activity loss. ATPS containing water-soluble ILs are likely to be more suitable for the isolation of proteins [7,37,44–52]. ATPS formed by ILs and inorganic salts showed higher recoveries of model proteins than methods employing PEG and salt systems [7,37]. Pei et al. [7,51] studied the partitioning behavior of bovine serum albumin (BSA) at different temperatures in the systems 1-butyl-3-methylimidazolium bromide $[C_4mim]Br/K_2HPO_4$ and 1-butyl-3-methylimidazolium dicyanamide $[C_4mim][N(CN)_2]/K_2HPO_4$ obtaining a purification factor of 6.96 and an extraction efficiency of 82.7–100.7%, respectively, although the simultaneous extraction of BSA and saccharides was described [51]. Du et al. [37] and Deive et al. [52] reported some studies with ATPS applied to the extraction of proteins such as BSA [37] and *Thermomyces lauginosus* [52], respectively. Du et al. [37] showed that the enrichment factor of BSA in 1-butyl-3-methylimidazolium chloride $[C_4mim]Cl + K_2HPO_4$ ATPS was found between 5 and 20 with extraction efficiencies higher than 90%. Deive et al. [52] found recuperation values for the lipase *T. lauginosus* at the IL-rich phase between 20% and 80% using the 1-etyl-3-methylimidazolium ethylsulfate $[C_2mimEtSO_4] + K_2CO_3$ system. Dreyer et al. [48,49] reported the partition coefficients for trypsin (23.48), lysozym (17.98), myoglobin (3.17) and an alcohol dehydrogenase from *Lactobacillus brevis* (3.0) for Ammoeng™ $110 + K_2HPO_4/KH_2PO_4$ based ATPS (pH 7.0). The protein penicillin G was used by Liu et al. [44,45], in two different reports, with extraction yields in the range of 90.8–93.7% using 1-butyl-3-methylimidazolium tetrafluoroborate and $NaH_2PO_4{\cdot}2H_2O$ ATPS (pH 4–5). Finally, Cao et al. [47] described the efficiency of extraction for horseradish peroxidase using the system $[C_4mim]Cl + K_2HPO_4$ (more than 80%). Through literature it was observed that the study of different systems was scarce and mainly focused in $[C_4mim]$ as the cation core and Cl as the principal anion. The IL-based ATPS also displayed better process properties, such as little emulsion formation, fast phase separation [7,26,37] and low toxicities. IL-based ATPS are formed using hydrophilic ILs. Since the toxicity of ILs is directly related with their hydrophobicity [53–55], this means that these ILs have, at least, lower toxicities than their more hydrophobic counterparts [56].

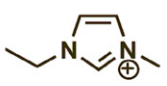
The results obtained so far for the extraction of both low and high molecular mass compounds by IL-based ATPS are very promising and indicate a general potential for wider use. In the present work, the partition coefficients of *Candida antarctica* lipase B (CaLB) in various IL-based ATPS were investigated. The study of the optimization of the IL structural features for the lipase purification was carried out employing imidazolium-, pyrrolidinium-, pyridinium- and piperidinium-based ILs combined with several anions (chloride, dicyanamide, methanesulfonate and triflate). Moreover, the cation alkyl chain length and the addition of functional groups to the cation were also evaluated.

2. Materials and methods

2.1. Material

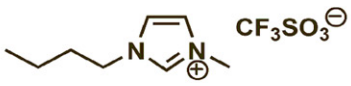
The present study was carried out using aqueous solutions of K_3PO_4, K_2HPO_4 and KH_2PO_4 with purities higher than 98% (w/w). All salts were purchased at Sigma–Aldrich (http://www.sigmaaldrich.com/). All ILs were acquired at IoLiTec (Ionic Liquid Technologies, Germany) with mass fraction purities higher than 98%, confirmed by us using ^1H NMR, ^{13}C NMR and ^{19}F NMR. Their molecular structures and respective names and abbreviations are reported in Fig. 1.

The enzyme used throughout this work was lipase B of *C. antarctica* (CaLB) which was a courtesy from Novozymes (http://www.novozymes.com/en/Pages/default.aspx). The arabic



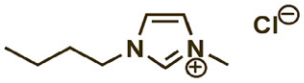
1-ethyl-3-methylimidazolium chloride

[C_2mim]Cl



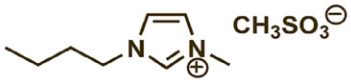
1-butyl-3-methylimidazolium triflate

[C_4mim][CF_3SO_3]



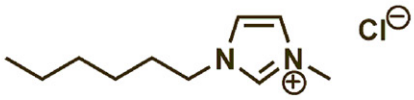
1-butyl-3-methylimidazolium chloride

[C_4mim]Cl



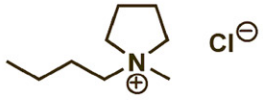
1-butyl-3-methylimidazolium methanesulfonate

[C_4mim][CH_3SO_3]



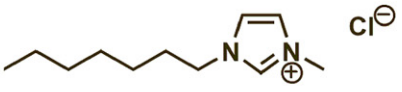
1-hexyl-3-methylimidazolium chloride

[C_6mim]Cl



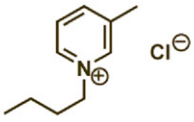
1-butyl-1-methylpyrrolidinium chloride

[C_4mpyrr]Cl



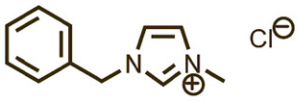
1-heptyl-3-methylimidazolium chloride

[C_7mim]Cl



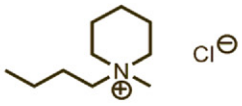
1-butyl-3-methylpyridinium chloride

[C_4mpyr]Cl



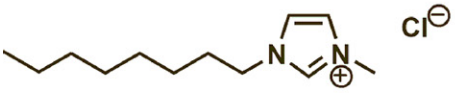
1-benzyl-3-methylimidazolium chloride

[C_7H_7mim]Cl



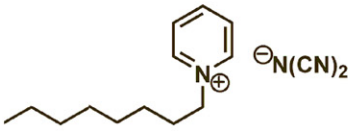
1-butyl-1-methylpiperidinium chloride

[C_4mpip]Cl



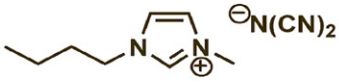
1-methyl-3- octylimidazolium chloride

[C_8mim]Cl



1-octylpyridinium dicyanamide

[C_8pyr][$N(CN)_2$]



1-butyl-3-methylimidazolium dicyanamide [C_4mim][$N(CN)_2$]

Fig. 1. Chemical structure of the ILs studied.

gum (acacia tree) and the reagent dye coomassie brilliant blue G-250 were acquired from Fluka (http://www.fluka.org/fluka.php). Potassium hydroxide and the protein bovine serum albumin (BSA, purity = 97%) were obtained from Merck (http://www.merck.com/index.html). Finally, the molecular mass standard (LMW-SDS Marker Kit 14–97 kDa) was acquired from GE Healthcare (http://www.gehealthcare.com/worldwide.html).

2.2. Phase diagrams and tie-lines

The binodal data were determined for all the ILs studied using the cloud point titration method [33,39] at 25 (±1) °C. Aqueous solutions of the various inorganic salts and each ionic liquid were prepared. The pH of each aqueous salt solution (used in the determination of the binodal curves) was measured using a pH meter (HI

9321 microprocessor Hanna Instruments). Repetitive drop-wise addition of the aqueous inorganic salt solution to the aqueous solution of IL was carried out until the detection of a cloudy solution, followed by the drop-wise addition of ultrapure water until the detection of a monophasic region (limpid solution). The whole procedure was performed under constant stirring. The ternary system compositions were determined by the weight quantification of all components added within an uncertainty of $\pm 10^{-4}$ g. All these systems were already reported in literature [57,58].

Tie-lines (TLs) were determined by a gravimetric method previously described [33,39] and adapted from Merchuck et al. [59]. A mixture at the biphasic region was prepared, vigorously stirred, and allowed to reach equilibrium by phase separation of both phases for 24 h, at 25 °C, using small ampoules (10 cm^3) especially designed for this purpose. After the separation step, both top and bottom phases were weighed. Each individual TL was determined by application of the lever arm rule [59].

2.3. Partitioning of the lipolytic enzyme

All the partitioning systems were prepared from ATPS composed of 25 wt% of IL + 30 wt% of the phosphate buffer aqueous solution + 0.4 wt% of CaLB (the total mass of the extraction systems prepared is 5.0 g). All systems were prepared in graduated glass centrifuge vials of *circa* 10 cm^3. After the ATPS preparation, the mixture was gently stirred and centrifuged at 2500 rpm. The graduated tubes of centrifuge were placed at 25.00 (±0.01) °C, for at least 12 h to reach equilibrium, using a Julabo F25 water bath. After reaching equilibrium, both phases were carefully separated. The two aqueous phases were then cautiously collected for the determination of their volume and weight.

In this study, the partition coefficient was defined as the ratio of protein concentration or enzyme activity in the bottom and top phases, as described by Eqs. (1) and (2),

$$K_P = \frac{C_T}{C_B} \quad (1)$$

$$K_E = \frac{EA_T}{EA_B} \quad (2)$$

where C_T and C_B are, respectively, the total protein concentration (mg mL^{-1}) in the top and bottom phases, and EA_T and EA_B are the enzyme activity (U mL^{-1}) of the top and bottom phases, respectively. These partition experiments were carried out in triplicate, being the results reported in this work the average of the three assays. It should be remarked that for all studied ATPS, the top phase is the IL-rich phase while the bottom phase is the phosphate-buffer-rich phase. To further evaluate this process as a purification technique, the enzyme specific activity (SA, expressed in U mg^{-1} of protein), the enzyme (R_B^E) and protein (R_B^P) recovery in bottom phase, and the purification factor (PF) were calculated for all systems as,

$$SA = \frac{EA}{C} \quad (3)$$

$$R_B^E = \frac{100}{1 + R_V K_E} \quad (4)$$

$$PF = \frac{SA}{SA_i} \quad (5)$$

$$R_B^P = \frac{100}{1 + R_V K_P} \quad (6)$$

where R_V represents the volume ratio between V_T and V_B that are, respectively, the volumes of top and bottom phases. The enzyme specific activity (SA) (Eq. (3)) can be evaluated for both phases through the ratio of the enzyme activity (EA) and the protein concentration (C) in each one of the phases. The purification factor (PF) was calculated by the ratio between the SA after and before (SA_i) the extraction procedure.

2.4. Enzymatic activity

Lipolytic activities were assayed using the oil emulsion method according to a modification proposed by Soares et al. [60]. The substrate was prepared by mixing 50 mL of olive oil with 50 mL of an aqueous solution of arabic gum (7% (w/v)). The reaction mixture containing 5 mL of substrate, 2 mL of sodium phosphate buffer (100 mM and pH 7.0) and enzyme extract (1 mL) was incubated in a thermostatic batch reactor operating at 37.0 °C, at 100 rpm, and during 5 min. After 5 min of reaction, an aliquot of ≈0.33 g was taken and added to 2 mL of a solution composed of acetone–ethanol–water (1:1:1). The exact weight of each aliquot was determined at the end of the addition procedure. The fatty acids produced were titrated with a potassium hydroxide solution (40 mM) using a phenolphthalein solution. A blank titration was done on a sample where the enzyme was replaced by distilled water. One unit (U) of enzyme activity was defined as the amount of enzyme that produces 1 μmol of free fatty acid per minute (μmol min^{-1}) under the assays established conditions (37 °C, pH 7.0, 100 rpm). The mentioned experimental techniques followed the protocols previously described by Carvalho et al. [61]. The possibility of the hydrolysis of olive oil by the ILs was tested in control essays at the measurement conditions in absence of enzyme and verified to be negligible.

2.5. Protein determination

Total protein concentration was determined by the Bradford's method [62], using a SHIMADZU UV-1700, Pharma-Spec Spectrometer, of 595 nm, and a calibration curve previously established for the standard protein BSA. To cancel the influence of the ILs presence on the protein concentration analysis, a control system for each IL-based ATPS without enzyme was prepared under the same conditions. Equilibrium conditions (24 h, 25 °C) and the phase separation procedure were those previously described.

2.6. Polyacrylamide gel electrophoresis

Equivalent amounts of CaLB from each sample were subjected to electrophoresis on a 12.5% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and overlaid with a 4% stacking gel as described by Laemmli [63] on a Bio-Rad mini-PROTEAN electrophoresis system. Gels were then stained with colloidal comassie blue. Digital gel images were acquired in a GelDoc system (Bio-Rad http://www.bio-rad.com/). Those assays were performed at 160 V for 1 h. The molecular mass standard (LMW-SDS Marker Kit 14–97 kDa) was composed of phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

3. Results and discussion

3.1. Phase diagrams

Phase diagrams are required for the design of aqueous two-phase extraction processes and for the development of models able to predict the partitioning of solutes between the two phases. Aiming at a better definition of the biphasic region compositions, adequate for enzyme partitioning and purification, the experimental phase diagrams at 25 °C and atmospheric pressure, for the 15 systems composed by inorganic salts + ILs + H_2O were determined before [57,58] and are presented in Figs. 2–5. These diagrams allow for a comprehensive understanding of the impact of the salts and

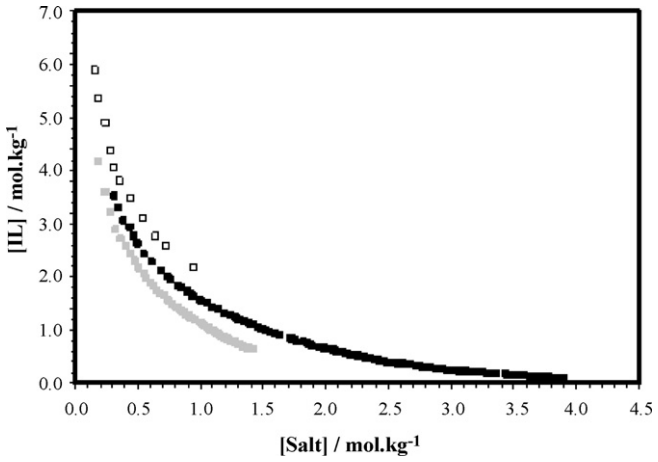


Fig. 2. Phase diagrams for the [C_4mim]Cl and different potassium-phosphate-based salts ATPS at 298 K. □, K_2HPO_4/KH_2PO_4 (pH = 7.0); ■, K_2HPO_4 and ▩, K_3PO_4.

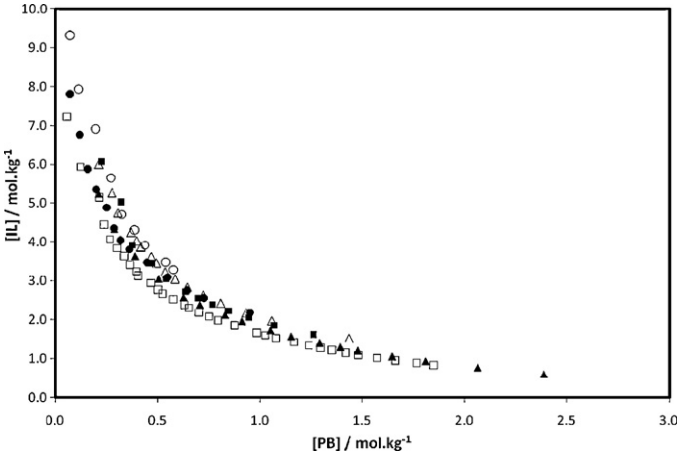


Fig. 3. Phase diagrams for [$C_n$mim]Cl and K_2HPO_4/KH_2PO_4 ATPS (pH = 7.0) at 298 K. ○, [C_2mim]Cl; ●, [C_4mim]Cl; □, [C_6mim]Cl; △, [C_7mim]Cl; ▲, [C_8mim]Cl and ■, [C_7H_7mim]Cl.

ILs nature on the formation of ATPS. The mass fraction data for binodal curves and respective tie-lines are provided in literature [33,39,57–58].

The binodal curves of the ATPS formed by the various phosphate-based salts, K_3PO_4, K_2HPO_4 and phosphate buffer solution (PB or KH_2PO_4/K_2HPO_4) with the IL [C_4mim]Cl are presented in Fig. 2. Despite their higher solubility in water and thus higher ability to promote the phase separation, the aqueous solutions formed by K_3PO_4 and K_2HPO_4 presented higher pH values, 13.7 and 9.1, respectively, which are undesirable when the objective is their application to enzymatic processes, due to the deleterious effect of the pH in some enzyme structures which could cause the decrease of their activity or even their inactivation [19]. Because KH_2PO_4 exhibits a weaker interaction with water molecules than the other phosphate-based salts, it is not capable by itself to promote the formation of IL-based ATPS [41]. Instead, a buffer composed of K_2HPO_4/KH_2PO_4, to achieve pH 7.0, was used and showed to be able to induce ATPS in several IL aqueous solutions. The influence of the three salts in the binodal curves with the same IL follows the Hofmeister series: $K_3PO_4 > K_2HPO_4 > K_2HPO_4/KH_2PO_4$. Strong salting-out inducing anions, PO_4^{3-} and HPO_4^{2-}, exhibit a better capability for creating ion-hydration complexes by excluding water from the IL-rich phase, favoring the formation of ATPS. The K_2HPO_4/KH_2PO_4 buffer has a lower ability for the formation of ATPS but was used preferentially in this study due to the neutral pH value achieved.

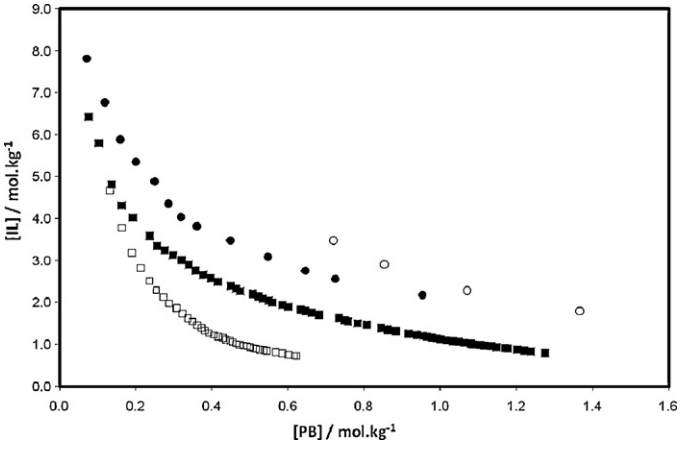


Fig. 4. Phase diagrams for [C_4mim]-based ILs and K_2HPO_4/KH_2PO_4 ATPS (pH = 7.0) at 298 K. ○, [C_4mim][CH_3SO_3]; □, [C_4mim][CF_3SO_3]; ■, [C_4mim][$N(CN)_2$]; ●, [C_4mim]Cl.

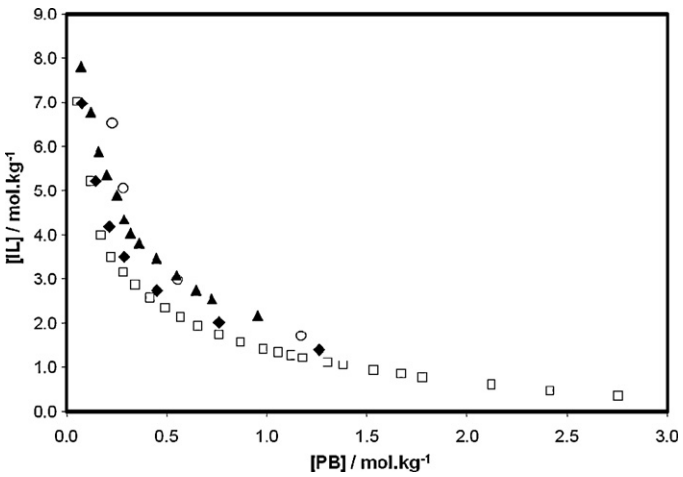


Fig. 5. Phase diagrams for chloride-based ILs and K_2HPO_4/KH_2PO_4 ATPS (pH = 7.0) at 298 K. ○, [C_4mpyrr]Cl; ♦, [C_4mpip]Cl; □, [C_4mpyr]Cl; ▲, [C_4mim]Cl.

Fig. 3 shows the influence in the ATPS formation of several imidazolium-based ILs, with variable alkyl side chain lengths (C_2–C_8), in the presence of K_2HPO_4/KH_2PO_4 buffer solution [57]. The [C_7H_7mim]Cl was also studied aiming at understanding the effect of the aromatic nature of the IL in the formation of ATPS [57]. The results indicated that the increase in the alkyl chain length leads to a better phase separation due to the increase in the hydrophobic character of the IL, and the consequent lower affinity of the IL for water molecules [33,39,64]. This behavior is a characteristic of ILs with alkyl side chains up to 6 carbon atoms ([C_6mim]Cl). However, when $n > 6$ for [$C_n$mim]Cl, the capacity of ATPS formation decreases due to the possibility of the ILs self aggregation. Najdanovic-Visak et al. [65] showed that self aggregation of the series [$C_n$mim]Cl in aqueous solution occurs for systems containing cations with $n > 6$ (number considered as transitional by Blesic et al. [66]). The possibility of ILs with longer alkyl chains to undergo micelle formation and self aggregation seems to have an impact on the efficiency of these compounds to form ATPS. Indeed, this turnover on the phase diagrams with the IL cation alkyl chain length at a critical length of the side chain was also observed previously with IL-K_3PO_4-based ATPS [67]. The ILs ability to form ATPS at ≈0.9 mol kg^{-1} of K_2HPO_4/KH_2PO_4 followed the order: [C6mim]Cl > [C4mim]Cl ≈ [C8mim]Cl > [C7H7mim]Cl ≈ [C7mim]Cl > [C2mim]Cl. Concerning the influence of one additional aromatic

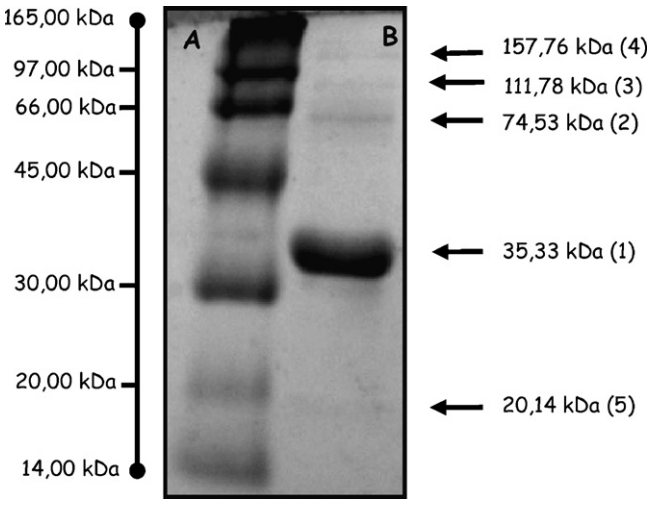


Fig. 6. Sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) patterns of CaLB. Lane A: molecular mass standard (14–97 kDa), Lane B: CaLB; 12% acrylamide gel stained with coomassie blue R-250.

ring to the imidazolium cation, $[C_7H_7mim]Cl$, the binodal curve data suggests that the presence of the benzyl group induced a slight increase in the ability to form IL-based ATPS when compared with $[C_7mim]Cl$. Nevertheless, comparing $[C_7H_7mim]Cl$ with $[C_6mim]Cl$ the ability to form ATPS increased with the former, as previously observed for similar systems with K_3PO_4 [33].

The effect of the IL anions in ATPS formation was studied with $[C_4mim]$-based ILs, namely $[C_4mim]Cl$, $[C_4mim][CH_3SO_3]$, $[C_4mim][CF_3SO_3]$ and $[C_4mim][N(CN)_2]$. The results regarding the IL anion influence are displayed in Fig. 4 and in recent literature [58]. The ILs sequence regarding their ability for ATPS formation with the K_2HPO_4/KH_2PO_4 buffer can be described as follows: $[C_4mim][CF_3SO_3] > [C_4mim][N(CN)_2] > [C_4mim]Cl > [C_4mim][CH_3SO_3]$. This order closely followed the IL anions rank reported before with ATPS involving the inorganic salt K_3PO_4 with only a small deviation observed between the chloride and methanesulfonate anions [39]. A careful analysis of the ternary aqueous phase diagrams of $[C_4mim]Cl$ and $[C_4mim][CH_3SO_3]$ with K_3PO_4 shows that these binodal curves are very close to each other in the molality range evaluated [39]. This slight change in the anion order was thus probably related with the difference in the ionic strength of the inorganic salt solutions. For the remaining anions, the ability to promote the phase split increased with the hydrogen-bond basicity (β) [68] as previously observed [39]. The hydrogen-bond basicity parameter is related with the probability and strength of ion-water type interactions [39]. The capability of a specific anion to be preferentially hydrated depends on the anions' hydrogen bonding accepting strength, and thus the ATPS formation is closely related with such parameter. An increase in the hydrogen bond basicity (β) reflects an increase in the hydrogen-bond accepting strength of the IL anion.

Fig. 5 shows the influence of the IL cation type on ATPS formation already shown in literature [57]. According to these results, the sequence for the ability of phase split formation regarding the various IL cations was as follows: $[C_4mpyrr]Cl \approx [C_4mim]Cl < [C_4mpip]Cl < [C_4mpyr]Cl$.

3.2. Lipase partition on IL based ATPS

The ATPS discussed above were used to study the partitioning of *C. antarctica* lipase B – CaLB (Lipozyme CALB). The 2D electrophoresis analysis, reported in Fig. 6, shows the presence of residual contaminant proteins on the commercial enzyme used. The two lanes of this SDS-PAGE correspond to the molecular mass standards (Lane A) and CaLB (Lane B). The presence of multiple light bands confirms the presence of the CaLB enzyme (numbered with 1 in Fig. 6) and further contaminant proteins, represented by the remaining bands (numbered with 2–5 in Fig. 6). The enzyme used in this work has a molecular weight of 35.3 kDa, that is consistent with what has been reported in literature by Ooi et al. [70] (39.5 kDa for lipase by *Burkholderia pseudomallei*) and Snellman et al. [71] (33 kDa for lipase by *Acinetobacter* sp.). Since the commercial enzyme was semi-purified, the expectable purification is thus limited. Nevertheless, the improvement in the purification factor using the ATPS here studied is reported in Tables 1–3 along with the partition coefficient of the enzyme, as well as the total protein, between the two phases.

The partition coefficients and purification efficiencies were determined at the biphasic region of each ATPS at the following composition: 25% (w/w) of IL + 30% (w/w) of KH_2PO_4/K_2HPO_4 + 45% (w/w) of water. The results shown in the last column of Tables 1–3 suggest that the main ionic liquid feature that controls the enzyme purification is the ionic liquid cation alkyl chain length, followed by the anion and finally, by the cation core. Table 1 shows that the increase in the alkyl chain length induces a stronger increase in K_P than on K_E that remains consistently very low. The increase in the alkyl chain length makes the ionic liquid more hydrophobic, decreasing the Coulombic interactions and increasing the dispersive forces that occur between the proteins and the ionic liquids at the IL-rich phase. These factors seem to favor the partitioning of the contaminant proteins into the IL-rich phase. Due to its low isoelectric point (pI = 6.0), the lipase is negatively charged at these pH conditions (pH 7.0) [72,73] which results in the increase of its hydrophilic character, creating a higher affinity of the enzyme for the salt-rich phase. The differences observed in the partitioning behavior between the contaminant proteins and the enzyme, must result from differences in their hydrophobicity/hydrophilicity that will allow the partition of the enzyme and contaminant proteins into different phases. The highest value observed for the purification factor was 2.6 ± 0.1 (for $[C_8mim]Cl$). The enzyme recovery efficiencies obtained for all systems were consistently above 96%. Note that in this work the increase in purity and the PF is entirely defined by the increase in the enzyme activity. The presence of IL in the medium is reported to have a deleterious effect, though not very significant at this concentration level, on the enzyme activity [74]. The increase in the PF must thus results mainly from the removal of the contaminant proteins, which are acting as enzymatic inhibitors.

The effects of both the IL anion and cation nature in the enzyme partitioning are described in Tables 2 and 3. Both the anion and cation nature were found to promote a small effect in the enzyme purification. However, the most effective anion studied was $[N(CN)_2]$ with $K_p = 0.36 \pm 0.07$ and $PF = 1.35 \pm 0.08$, while the most efficient cation evaluated was $[C_4mpyr]$ with $K_p = 0.24 \pm 0.02$ and $PF = 1.28 \pm 0.08$. The higher K_p and PF results are related with the enhanced hydrophobic character of IL-rich phases composed by such ions. The hydrogen bonding basicities (β) of ILs [68] are helpful in the interpretation of the anion influence results reported in Table 2. The increase in β values promotes the crescent organization of water molecules around the ionic liquid which in turn leads to an increase in the water content at the IL-rich phase (also visible in Fig. 4) and in the volume ratio (Table 2). In opposition to the volume ratio, the partition coefficients do not closely follow the ILs hydrogen-bond basicity. Those differences could be explained by the difference in the hydrophobic nature of the two phases, and on the IL anion ability to interact with the remaining components of the system. In what concerns the purification factor, the results suggest that the IL anion influence was not highly significant as stated before. Nevertheless, $[N(CN)_2]$ was found to be the most effective anion, among the studied IL anions. In this case, the partition sys-

Table 1
Effect of the IL cation side alkyl chain length in the partition and purification of CaLB, using 25% (w/w) of IL and 30% (w/w) of potassium phosphate buffer solution, at pH 7.0 and 25 °C.

Ionic liquid	$R_V \pm$ std	$K_P \pm$ std	$R_B^P \pm$ std	$K_E \pm$ std	$R_B^E \pm$ std	PF ± std(fold)
[C_2mim]Cl	1.6 ± 0.3	0.07 ± 0.02	89 ± 3	0.006 ± 0.002	99.096 ± 0.06	1.05 ± 0.04
[C_4mim]Cl	1.4 ± 0.2	0.133 ± 0.009	85 ± 1	0.02 ± 0.01	98 ± 1	1.10 ± 0.03
[C_6mim]Cl	1.1 ± 0.2	0.184 ± 0.003	90.954 ± 0.06	0.015 ± 0.002	98.1 ± 0.2	1.068 ± 0.004
[C_7mim]Cl	1.1 ± 0.1	0.5 ± 0.1	65 ± 6	0.017 ± 0.003	98.2 ± 0.4	1.48 ± 0.05
[C_8mim]Cl	0.9 ± 0.1	1.6 ± 0.2	40 ± 3	0.046 ± 0.001	95.9 ± 0.2	2.6 ± 0.1
[C_7H_7mim]Cl	0.97 ± 0.03	0.16 ± 0.01	85.1 ± 0.7	0.031 ± 0.003	97.1 ± 0.2	1.14 ± 0.03

Table 2
Effect of the IL anion nature in the partition and purification of CaLB, using 25% (w/w) of IL and 30% (w/w) of potassium phosphate buffer solution, at pH 7.0 and 25 °C.

Ionic liquid	$R_V \pm$ std	$K_p \pm$ std	$R_B^P \pm$ std	$K_E \pm$ std	$R_B^E \pm$ std	PF ± std(fold)
[C_4mim][CF_3SO_3]	0.50 ± 0.04	0.10 ± 0.03	95 ± 1	0.017 ± 0.003	98.6 ± 0.4	1.05 ± 0.05
[C_4mim][$N(CN)_2$]	0.76 ± 0.02	0.36 ± 0.07	75.1 ± 0.8	0.011 ± 0.002	99.5 ± 0.5	1.35 ± 0.08
[C_4mim][CH_3SO_3]	1.20 ± 0.05	0.18 ± 0.04	92.6 ± 0.3	0.028 ± 0.003	98.4 ± 0.1	1.11 ± 0.07
[C_4mim]Cl	1.4 ± 0.2	0.133 ± 0.009	85 ± 1	0.02 ± 0.01	98 ± 1	1.10 ± 0.03

Table 3
Effect of the IL cation core in the partition and purification of CaLB, using 25% (w/w) of IL and 30% (w/w) of potassium phosphate buffer solution, at pH 7.0 and 25 °C.

Ionic liquid	$R_V \pm$ std	$K_p \pm$ std	$R_B^P \pm$ std	$K_E \pm$ std	$R_B^E \pm$ std	PF ± std(fold)
[C_4mpyrr]Cl	1.63 ± 0.09	0.15 ± 0.02	80.8 ± 0.8	0.0152 ± 0.0002	97.5 ± 0.1	1.15 ± 0.05
[C_4mim]Cl	1.4 ± 0.2	0.133 ± 0.009	85 ± 1	0.02 ± 0.01	98 ± 1	1.10 ± 0.03
[C_4mpyr]Cl	1.64 ± 0.07	0.24 ± 0.02	71.3 ± 0.5	0.009 ± 0.002	98.0 ± 0.9	1.28 ± 0.08
[C_4mpip]Cl	1.67 ± 0.04	0.20 ± 0.03	80 ± 4	0.0076 ± 0.0003	98.2 ± 0.9	1.16 ± 0.05
[C_8pyr][$N(CN)_2$]	0.6 ± 0.0	0.00 ± 0.00	100.00 ± 0.00	0.003 ± 0.002	99.8 ± 0.2	0.998 ± 0.002

tem was represented by $K_E \ll 1$, a high K_p value and an enzyme bottom recovery of (99.5 ± 0.5)%.

Table 3 shows the results for the influence of distinct cation types. One more time, the results of the purification factors shows the minor effect of the cations ($0.998 \pm 0.002 < PF < 1.28 \pm 0.08$), indicating a mild removal of contaminants proteins from the enzyme solution. Despite the low purification values, the most effective cation was [C_4mpyr]. In what concerns the partitioning parameters, all the studied cations led to $K_E \ll 1$ indicating the higher affinity of the enzyme for the bottom phase (inorganic-salt-rich phase). This was also reflected on the high enzyme recovery at this phase ($97.5 \pm 0.1 < R_B^E \approx 98.2 \pm 0.9$)%.

From the aforementioned ILs, the structural characteristics, which maximize the purification factor with high recuperations were selected and further investigated: the pyridinium cation core, the dicyanamide anion and the alkyl side chain with 8 carbons, which were tailored as [C_8pyr][$N(CN)_2$] (Figs. 7 and 8 and Table 3). The first step was to determine the binodal curve for the system [C_8pyr][$N(CN)_2$] + H_2O + K_2HPO_4/KH_2PO_4 (see supporting information – Tables S1). In this context, this binodal curve was compared in Fig. 7 with some other curves previously determined [57,58]. The ILs sequence regarding their ability for ATPS formation (Fig. 7) with the K_2HPO_4/KH_2PO_4 buffer can be described as follows: [C_4mim]Cl < [C_8mim]Cl < [C_4mpyr]Cl < [C_4mim][$N(CN)_2$] < [C_8pyr][$N(CN)_2$]. To complete the phase diagram for the system containing [C_8pyr][$N(CN)_2$], three different tie lines were additionally determined (Fig. 8 and Tables S2 and S3 from supporting information). The results indicate that, as previously observed for other systems [33,39,43], for shorter tie-line length (TLL) the TLs were approximately parallel, whereas for longer TLLs the tie-line slopes started to deviate. These deviations in the TLs slopes were in agreement with literature [33,39,43] and were related with the fact that the salt-rich phase was increasingly free of IL at longer TLLs.

In what concerns the ability for the lipase partitioning, surprisingly, this IL ([C_8pyr][$N(CN)_2$]) presenting all the optimized features previously studied, performed very poorly in the extrac-

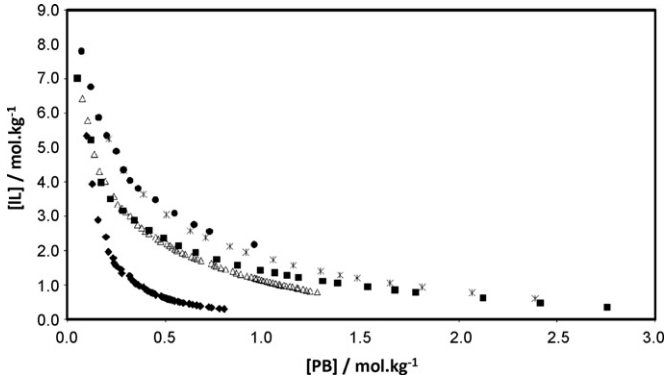


Fig. 7. Phase diagrams for different ILs and K_2HPO_4/KH_2PO_4 ATPS (pH = 7.0) at 298 K. ∗, [C_8mim]Cl; ●, [C_4mim]Cl; ■, [C_4mpyr]Cl; △, [C_4mim][$N(CN)_2$]; ♦, [C_8pyr][$N(CN)_2$].

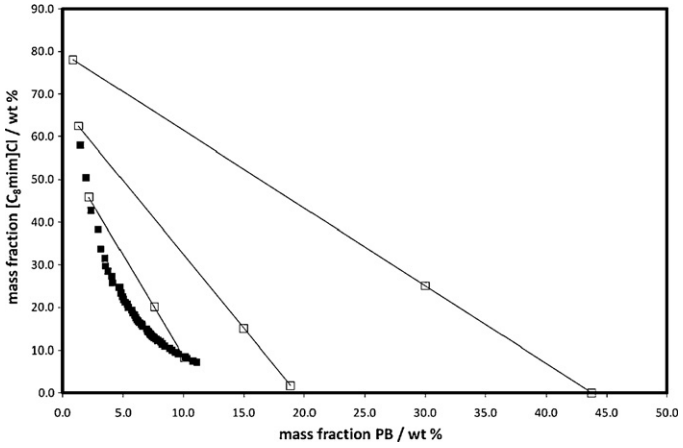


Fig. 8. Phase diagram for the [C_8pyr][$N(CN)_2$] and K_2HPO_4/KH_2PO_4 ATPS (pH = 7.0) at 298 K: ■, binodal curve data; □, TL data.

tion/purification of lipase. Though the lipase partition into this IL is, as expected, very small, the unexpected behavior results from the extremely low partition observed for the contaminant proteins. The formation of self aggregates for ILs with sufficiently long chains changes the ATPS formation aptitude, as discussed above and shown in Fig. 3. It also seems to have a strong impact on the partition of the biomacromolecules and on their purification efficiencies. The optimization of the IL alkyl chain length based on the imidazolium cation (and probably other IL structural features) does not seem to be transferable to different cations. For reasons that are not entirely clear at present, but that may be related with cohesion forces, contrarily to what is observed for [$C_n$mim]Cl, the formation of aggregates in [C_8pyr][$N(CN)_2$] seems to reduce the interaction with the contaminant proteins leading thus to a negligible extraction into the IL-rich phase and to a poor purification factor. This behavior was also previously observed by us for the extraction of small molecules (nicotine and caffeine [40], and vanillin [26]). A change in the partition coefficients was observed when the number of carbons of the IL alkyl chain increased from 6 to 8 probably due to micelle formation. In fact, recent results suggested that ILs composed by the [$N(CN)_2$] anion exhibit a higher capability for micelle formation when compared with the chloride anion for a common cation [75]. This difference is closely related with the size of the counterion [75,76]. Moreover, it was suggested that large anions, as dicyanamide, favor the ILs aggregation since they decrease the charge repulsion at the micelle [75,76]. On the other hand, strongly hydrated ions, such as chloride, are partially screened by the surrounding polar water molecules, and therefore, they are less effective at reducing charge repulsion [75,76]. Regarding the cation core, it was also shown that critical micelle concentrations of imidazolium-based ILs are higher than the ones observed with pyridinium-based ILs (with similar anions and alkyl side chain lengths) [66,77,78]. As a result, [C_8pyr][$N(CN)_2$] will have a higher aptitude for micelle formation when compared to [C_8mim]Cl. The higher aptitude of [C_8pyr][$N(CN)_2$] for self aggregation compared to [C_8mim]Cl may be responsible for the unexpected poor results obtained with the pyridinium-based IL. Using the partition coefficients and purification factors obtained in this work, a comparison was attempted with other literature data to evaluate the suitability of ATPS based in ILs for protein purification. In the majority of the works described in Section 1, the PF values were all higher [7,37,48,49] than those found in this work. However, a direct comparison with the results obtained in this work cannot be done due to the different nature of the proteins of the ATPS studied. More extensive and directly comparable data regarding the purification of lipase using polymer-based ATPS are available by Nandini and Rastogi [79] and Souza et al. [80]. Nandini and Rastogi [79] considered the same conditions of temperature and pH of the present work, but used instead the PEG 6000 + Na_2HPO_4 + H_2O ternary system. This system presented a PF = 1.56 but a recovery of only 88.45%. Souza et al. [80] presented results for the partitioning and purification of the porcine pancreatic lipase considering the same conditions of temperature, type of salt solution and pH as in this work carried here. Souza et al. [80] reported the application of ATPS composed of 20% (w/v) of PEG 8000 + 18% (w/v) of phosphate buffer solution (PB) + H_2O, with a purification factor of the enzyme of 1.13 ± 0.09 (fold) and a recovery yield of 83.40%. According to the results described above, more than 97% of the enzyme is recovered in the salt rich phase what makes possible to apply conventional techniques such as dialysis [80,81] for the enzyme separation from the salt solution. The results here obtained for the purification and recovery of lipase suggest that by using ATPS based in ILs, and with an appropriate choice of the IL structural features, it is possible to achieve improved purification coefficients and enhanced recoveries.

4. Conclusion

ILs have been shown to be able to induce aqueous phase separation in the presence of potassium phosphate buffer forming ATPS.

Various IL structural features were studied in the partitioning and purification of *C. antarctica* lipase B. The capacity of IL-based ATPS as prospective extraction and purification media in biotechnological processes was demonstrated since it was possible to increase the purity degree of the commercial enzyme, despite its high commercial purity level. The results obtained indicate that the higher purification factor values were found with an octyl side chain in the imidazolium cation, with the [$N(CN)_2$] anion and with the [C_4mpyr] cation as isolated effects. However, the additive properties were not verified for the extraction parameter, since the tailored [C_8pyr][$N(CN)_2$] was further tested, and its purification capacity was found to be almost inexistent ($PF = 0.998 \pm 0.002$). Nevertheless, a very large purification of the commercial enzyme was achieved with [C_8mim]Cl with a purification factor of (2.6 ± 0.1) and an enzyme recovery at the bottom phase of (95.9 ± 0.2)%. The results obtained show that IL-based ATPS possess excellent ability for lipase purification and recovery.

Acknowledgements

The authors are grateful for financial support from Fundação para a Ciência e a Tecnologia for the Ph.D. grant SFRH/BD/37830/2007 of S.P.M. Ventura and from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the post-doctoral scholarship of A.S. Lima (process number 2185/09-1).

The authors also thank the support from Novozymes for the supply of the enzyme and Rita Ferreira for the electrophoresis analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.07.022.

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