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ORIGINAL PAPER

Concentration effect of hydrophilic ionic liquids on the enzymatic activity of *Candida antarctica* lipase B

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Abstract A systematic study of the effects of hydrophilic ionic liquids concentration and nature (alkyl chain length and type of anion) on the activity of *Candida antarctica* lipase B is here reported. The increase in the concentration of the studied ionic liquids is shown to cause a decrease of the enzyme activity, but the effect is dependent on the ionic liquid used. This behavior is partially due to the ionic liquid impact on the thermodynamic water activity, but direct interactions between the hydrophilic ionic liquid and the enzyme are also disclosed. Cations with longer alkyl chains decrease the enzyme activity by obstruction of its non-polar active site, while direct interactions established between the enzyme and the anions, dominated by dispersion forces and hydrogen-bonding, contribute also for the loss of activity observed.

Keywords Hydrophilic ionic liquids · *Candida antarctica* lipase B · Enzymatic activity · Ionic liquid concentration · Ionic liquid features · Water activity

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Introduction

It is well established that many enzymes are active in solvents other than water, as hydrophobic organic solvents (Zaks and Klibanov 1983) and ionic liquids (Kragl et al. 2002). Lipases have been shown to remain active even in anhydrous organic solvents. One of the most important reasons for applying lipases under such conditions is to avoid hydrolysis when non-hydrolytic transformations are performed, which constitute an increasing number of industrial applications (Schmidt et al. 2001). The minimum water requirement is enzyme-dependent and ranges from a few tightly bound water molecules per molecule of enzyme (Dolman et al. 1996) to a nearly intact hydration shell. The enzyme *Candida antarctica* lipase B (CaLB) is very stable, even when compared with other lipases. CaLB is routinely used in anhydrous organic media and does not require a hydration shell to be active. This characteristic, as well as its quite relaxed reactant specificity and its operational stability, make of CaLB a valuable tool for various synthesis (Anderson et al. 1998).

Ionic liquids (ILs) have recently emerged as alternatives to classical organic solvents for a wide variety of biocatalytic (Kragl et al. 2002; Baumann et al. 2005; Barahona et al. 2006; Lutz-Wahl et al. 2006; Chiappe et al. 2007; Sgalla et al. 2007) and purification (Ventura et al. 2011, 2012) processes. The appropriate combination of the cationic and anionic parts of the solvent might increase substrate solubility, improve the enzyme selectivity or enhance the enzyme activity and/or stability. As in any other organic solvent, the enzyme requires a micro-aqueous phase surrounding its structure. One of the major problems using ILs is that they might decrease, or even destroy, the water layer around its surface, or hinder the access to its active site, decreasing its activity and/or stability. In fact,

some studies about the influence of different ILs on the activity of lipases (Soares et al. 1999; Kaar et al. 2003; Houde et al. 2004; Irimescu and Kato 2004; Kamal and Chouhan 2004; Lau et al. 2004; De Diego et al. 2005; de Maria et al. 2006; Yuan et al. 2006; Dang et al. 2007; de los Rios et al. 2007a, b; Dhake et al. 2009; Hernandez-Fernandez et al. 2009; Zhao et al. 2009; Nandini and Rastogi 2011), have been reported. Recently, some interesting works have been published reviewing these issues (Yang and Pan 2005; Zhao 2005; Zhao et al. 2006a, b; van Rantwijk and Sheldon 2007; Yang et al. 2009; Hernandez-Fernandez et al. 2010). One of the principal conclusions retained is that an IL plays the same role as an organic solvent (Yang and Russell 1996; Berberich et al. 2003; De Diego et al. 2004; Yang et al. 2008) affecting the enzyme performance, by: (1) stripping off the essential water associated with the enzyme; (2) penetrating into the micro-aqueous phase to interact with the enzyme by changing the protein dynamics, the protein conformation, and/or the enzyme's active site; and (3) interacting with the substrates and products by either direct reactions with them or by altering their partitioning between the aqueous and non-aqueous phases (Yang 2009).

Several works have shown the high sensitivity of the enzymes to the ILs structural features (van Rantwijk and Sheldon 2007; Moniruzzaman et al. 2010). The enzyme activity has been reported to gradually increase with the cation alkyl chain length (de los Rios et al. 2007a, b; De Diego et al. 2009). However, this trend is not generally accepted since other authors report the opposite effect (Attri et al. 2011) remaining this an issue that needs to be clarified. Nevertheless, most authors seems to agree that, although hydrophilic ILs and their aqueous solutions dissolve enzymes, most of them establish strong interactions (via hydrogen-bonding) resulting in enzyme deactivation. For lipases, it has been found that nitrate or lactate hydrophilic ILs, for example van Rantwijk et al. 2006 and de los Rios et al. 2007a, b, led to the deactivation of lipases, while a high activity was observed in hydrophobic ILs containing hydrophobic anions (Kaar et al. 2003; de los Rios et al. 2007a, b).

To understand the dynamics of the phenomena occurring, the influence of the system' physical properties should be addressed, being the water activity of the system, pH, excipients and impurities some of the most important (Yang and Pan 2005). Strong interactions of anions with lipases seem to impair the enzyme activity. Recently, the hydration of enzymes when exposed to the ILs with different structures has been studied, and shown that its hydration level is a crucial condition to maintain its best performance, both in terms of stability and activity (Eckstein et al. 2002; Berberich et al. 2003; Kaar et al. 2003; Barahona et al. 2006). However, due to the limited number of ILs investigated, more comprehensive studies aiming at achieving a deeper understanding of these phenomena are required.

In this context, the main aim of this study is to significantly enlarge the range of hydrophilic ILs tested on their effects on the CaLB activity. All the ILs here studied belong to the imidazolium family. Thus, the imidazolium head was conjugated with several alkyl chains (C2–C8) and hydrophilic anions (chloride Cl, bromide Br, trifluoracetate [CF_3COO], methanesulphonate [CH_3SO_3], hydrogenosulphate [HSO_4], dicyanamide [$N(CN)_2$], trifluoromethanesulfonate [CF_3SO_3], and acetate [CH_3COO]). A range of concentrations was also investigated for all the ILs tested. Finally, the level of hydration and binding state of hydration water, represented by the thermodynamic water activity (a_w), was estimated and studied for some of the hydrophilic anion-based ILs, aiming at understand the influence of the free water molecules around the enzyme activity. The loss of enzyme activity was also correlated for the first time with the solvatochromic parameters of the ILs aiming at understanding the impact of the IL-enzyme interactions on the activity loss.

Material and methods

Material

The ionic liquids used in this study were: 1-ethyl-3-methylimidazolium chloride-[C_2mim]Cl, 1-methyl-3-octylimidazolium chloride-[C_8mim]Cl 1-butyl-3-methylimidazolium methanesulfonate-[C_4mim][CH_3SO_3], 1-butyl-3-methylimidazolium trifluoromethanesulfonate-[C_4mim][CF_3SO_3], 1-butyl-3-methylimidazolium acetate-[C_4mim][CH_3COO], 1-butyl-3-methylimidazolium chloride-[C_4mim]Cl, 1-butyl-3-methylimidazolium bromide-[C_4mim]Br, 1-butyl-3-methylimidazolium trifluoracetate-[C_4mim][CF_3COO], 1-butyl-3-methylimidazolium hydrogenosulphate-[C_4mim][HSO_4], and 1-butyl-3-methylimidazolium dicyanamide-[C_4mim][$N(CN)_2$]. Their molecular structures are shown in Fig. 1, with their respective names and abbreviations. All ILs were acquired at IoLiTec (Ionic Liquid Technologies, Germany) with mass fraction purities higher than 99 %, confirmed by us using ^{1}H-NMR, and ^{13}C-NMR. The enzyme *C. antarctica* lipase B (EC 3.1.1.3), here abbreviated by CaLB, was produced in submerged fermentation of a genetically modified *Asperghillus oryzae* microorganism and was supplied by the Novozymes® company. For the determination of the enzymatic activity by the spectrophotometric method, *p*-nitrophenyl laurate (*p*-NFL) from Fluka (purity ≥98.0 % by GC), dimethyl sulfoxide (DMSO) from LabScan and *p*-nitrophenol (*p*NF) from Sigma-Aldrich were used.

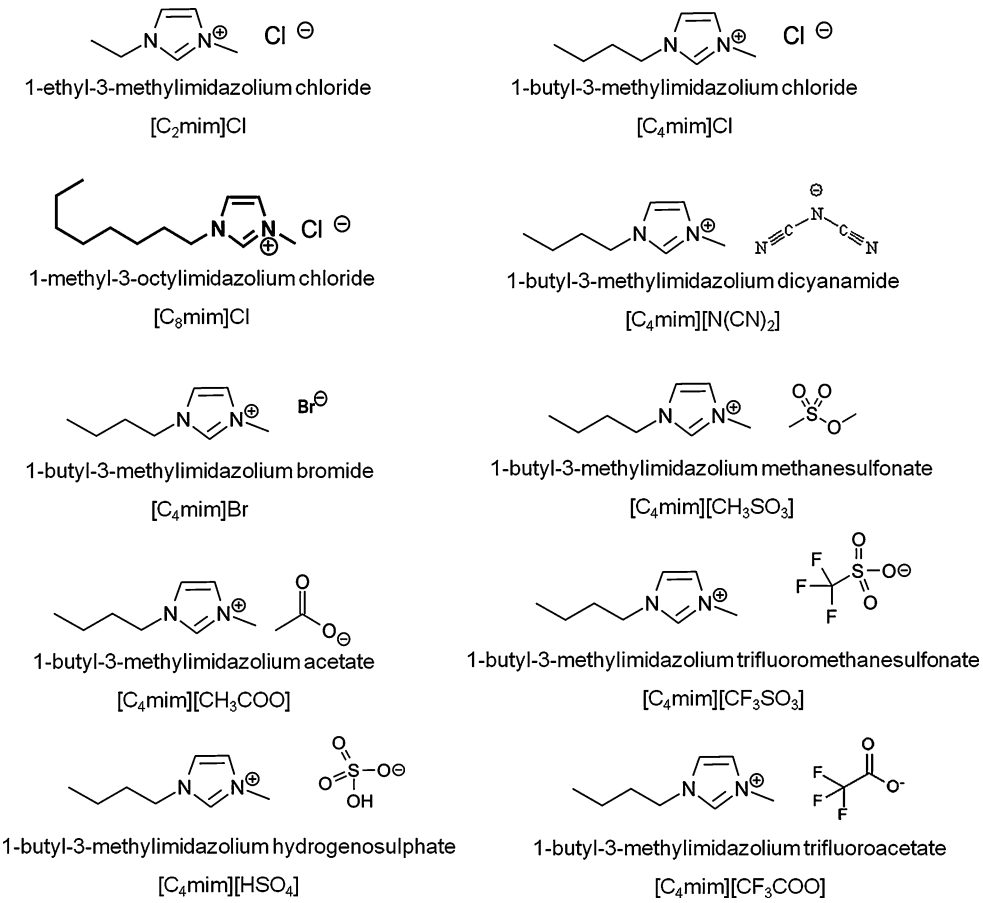

Fig. 1 Chemical structure of the ILs used

Experimental methods

Enzymatic activity

The lipase activity was assayed spectrophotometrically using a SHIMADZU UV-1700, Pharma-Spec Spectrometer. The substrate solutions composed of *p*-NFL were prepared 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 adding 25 μL of CaLB to 300 μL of the phosphate buffer solution (50 mM and pH 7.0). To measure the lipase activity, 200 μL of the enzymatic solution (containing different amounts of ILs, depending of the concentration tested, or phosphate buffer solution) was added to 1.8 mL of substrate solution. Before the addition, both the substrate and enzyme solutions were acclimatized at 37 °C during 15 and 20 min, respectively. The reaction was left to proceed also at 37 °C. To quantify the enzymatic activity, the absorbance variation at 410 nm was registered during 150 s ($\Delta Abs.min^{-1}$), over the linear range of absorbance variation with time. The enzymatic activity was calculated in U L^{-1}, being one unit defined as the amount of enzyme that oxidized 1 μmol of *p*-NFL per min (the amount of *p*-NFL oxidized was quantified based on a calibration curve previously determined). The results are reported as the ratio between the activity of the enzyme with and without the presence of IL (A/A_0).

Results and discussion

In this work, the capacity of CaLB to maintain its activity after exposure of the enzyme to several hydrophilic ILs was tested. The ILs concentration are reported as molar concentrations in all cases, to allow comparisons and discussion of results, based on the number of moles of IL in the reaction system together with the enzyme. Due to the crucial importance of the systems' pH in the lipase activity, this parameter was carefully controlled and kept constant for all the tests, using a potassium phosphate buffer solution (50 mM pH 7.0) in the preparation of all the solutions used (enzyme and substrate). Together with the pH, also the incubation and reaction temperatures play an important role in the enzyme performance. Thus, the temperature condition was also maintained at 37 °C during the preparation of all the solutions and also during the reaction catalyzed by the lipase.

Effect of the cation alkyl chain length

Figure 2 shows that the increase in the alkyl chain length of the cation induces a decrease in the enzymatic activity at all the IL concentrations tested. The effect becomes more significant for long alkyl chain lengths. This behavior was previously observed by Attri et al. (2011) and may be explained by the increase of the ILs cation hydrophobicity with the elongation of the alkyl chains. This increase in the hydrophobicity leads to the increase in the *van der Waals* interactions between the alkyl chains and the non-polar domains of the enzyme (Klähn et al. 2011), being responsible for the spontaneous formation of aliphatic domains, comprised by these non-polar alkyl chains (Klähn et al. 2011). The formation of these domains was shown by molecular dynamics simulation and later by experimental studies (Wang and Voth 2005; Triolo et al. 2007, 2009). They tend to accumulate around the non-polar enzyme active site, leading to its partial or total obstruction (depending of the IL concentration in the system). As described in Klähn's work (Klähn et al. 2011), with the obstruction of the enzyme active site, the substrate access to the active site is hindered, due to a decreased diffusion, causing mass transfer problems, and the consequent decrease on the enzymatic activity.

Other works reporting the opposite trend on the enzymatic activity with the increase in the length of the alkyl chains (de los Rios et al. 2007a, b; De Diego et al. 2009), used hydrophobic ILs based in the water unstable [PF_6] anion (De Diego et al. 2009) and/or immobilized enzymes and they generally failed to provide an adequate explanation for the enhanced activities observed.

Effect of the ionic liquid concentration

One of the major problems using ILs as solvents for enzymatic biocatalytic processes is that their use decreases the water hydration layer around the enzyme surface. The effect of the concentration of the various ILs studied in this work on the enzymatic activity of CaLB is shown in Figs. 2 (cation effect), and 3 (anion effect). It can be observed that in all cases an increase in the IL concentration leads to a decrease in the enzymatic activity.

To take into account the availability of water for the hydration of the enzyme, since the level of hydration of an enzyme is crucial for its performance, (Eckstein et al. 2002; Basso et al. 2005; Barahona et al. 2006) the enzyme loss of activity was plotted as a function of the water activity (a_w), in Fig. 4. The highly diluted solutions used in this work made the direct measurement of the water activity impossible by conventional methods and thus, an alternative approach was used. Given the ability of COSMO-RS to describe the non ideality nature of diluted aqueous solutions of ILs (Freire et al. 2008), the a_w was estimated using the predictive method COSMO-RS, for some of the ILs studied in this work. From the results presented in Fig. 4 it can be observed that, as previously reported, (Salvador et al. 2010) the enzymatic activity decreases with the water activity decrease. Although the results clearly point for an important and direct relationship between the enzymatic activity and the water activity, the differences observed among the curves for the various ILs (different slopes), show that this parameter is by no means the sole responsible for the loss of activity and that other factors must be taken into account to explain the loss of activity of CaLB.

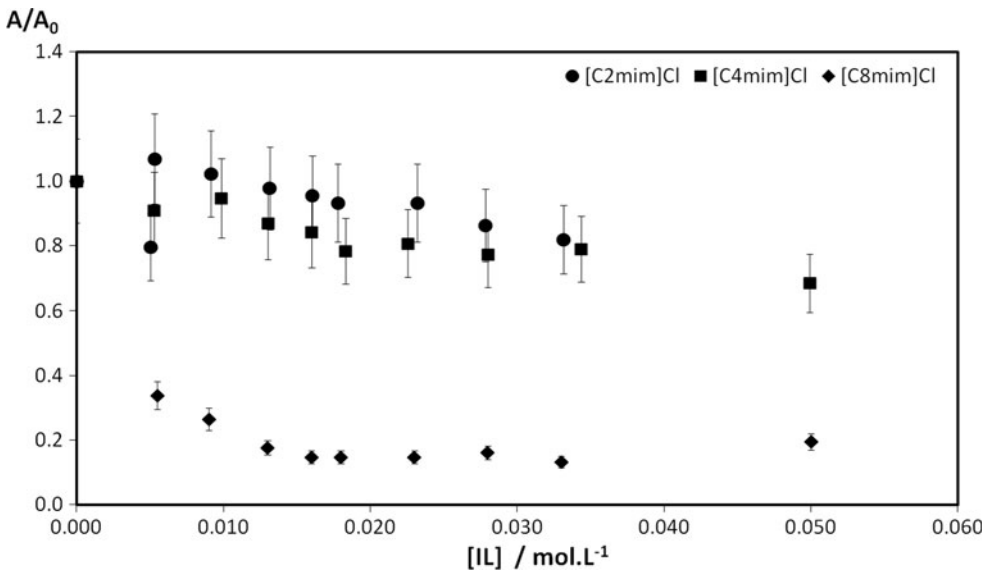

Fig. 2 Enzymatic activity of CaLB in the presence of different concentrations of [$C_n$mim]Cl-based ILs

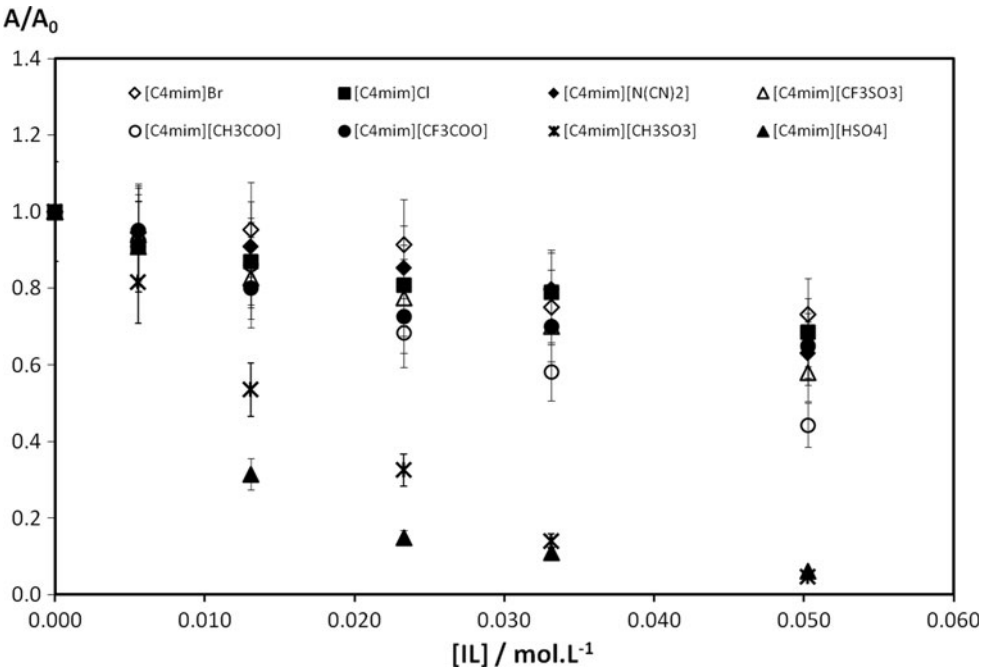

Fig. 3 Enzymatic activity of CaLB in the presence of different ILs concentrations (mol L^{-1}) for several hydrophilic ILs [C_4mim]X

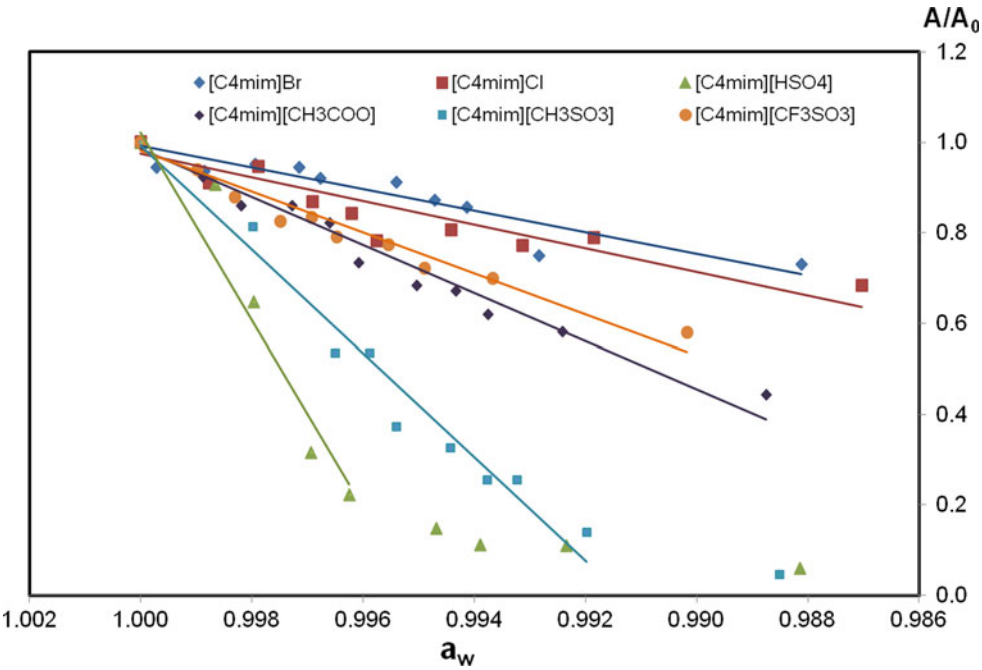

Fig. 4 Dependency of the residual enzymatic activity (A/A_0) of CaLB on the water activity (a_w)

Effect of the anions' nature

Having shown that besides the water activity other factors are also relevant to understand the activity loss of CaLB, in presence of ILs, the nature of the anion was investigated. In this context, the type of interactions between the ILanion and the enzyme were considered, aiming at understand the different impacts in the enzymatic activity observed for the different anions, and in particular the large effect of [HSO_4] and [CH_3SO_3]. The various interactions that can be established by the anions with the enzyme were studied using the Kamlet-Taft solvatochromic parameters (Lungwitz and Spange 2008; Lungwitz et al. 2010; Cláudio et al. 2011), α (hydrogen-bond donor) and β (hydrogen-bond acceptor), and π representing the polar and dispersive interactions, described in Table 1 for the ILs studied.

To remove from the analysis the influence of the water activity, previously established, the dependency of the enzymatic activity with the water activity, estimated from the slopes of the lines in Fig. 4, are used in this study. Their

relations with the solvatochromic parameters reported in Table 1 are shown in Fig. 5. The results show that α seems to have no influence in the enzymatic activity. However, there seems to be a clear relation between the activity decrease with the water activity and the β and, in a lesser extent, π solvatochromic parameters. This suggests that the anions effect on the loss of enzymatic activity might be due to their action as hydrogen-bonding acceptors for the protein (effect of β), and that the dispersive forces (effect of π) also play an important role, in agreement with what was previously discussed concerning the cation alkyl chain effect. According to Fig. 5, [CF_3SO_3] appears as an exception, but it was not possible to understand its behavior.

The results here reported fulfill a gap on the effect of hydrophilic ILs on the CaLB enzymatic activity. They also provide an explanation for the effect of the studied ILs on the enzymatic activity based on their effect on a_w, and on the enzyme—IL interactions. It is shown that by increasing both β and π parameters, the interactions (hydrogen-bonds and dispersive interactions) between the ILs and CaLB become more important, leading to a decrease of the enzymatic activity.

Table 1 Solvatochromic parameters, α, β, and π for several hydrophilic anion-based ILs (Lungwitz and Spange 2008; Lungwitz et al. 2010; Cláudio et al. 2011

Ionic liquid	α	β	π
[C_4mim]Cl	0.32	0.95	1.13
[C_4mim]Br	0.36	0.87	1.14
[C_4mim][CF_3SO_3]	0.63	0.52	0.98
[C_4mim][CH_3COO]	0.36	0.85	1.06
[C_4mim][CH_3SO_3]	0.36	0.85	1.04
[C_4mim][HSO_4]	–	0.66	1.11

Conclusions

Since there are scarce information about their effect on enzymatic activities, this work shows the effect of various hydrophilic ionic liquids (ILs) and their concentration on the enzymatic activity of *C. antarctica* lipase B (CaLB). It is shown that the presence of hydrophilic ILs have a deleterious influence upon the enzymatic activity. The IL concentration has a negative impact on the enzymatic activity, by decreasing the water activity, a_w, but this only explains a part of the activity loss observed. A more complete explanation of the effect of the studied ILs on CaLB activity requires an understanding of the direct interactions between the enzyme and the ionic liquid and their dependence on the IL structure. The effect of the increase in the alkyl chain on the activity seems to be related with the hydrophobic nature of the alkyl side chain, which promotes the ability of the ionic liquid to obstruct the non-polar active site of the enzyme. The strength of the interactions established between the enzyme and the different ILs anions, dominated by dispersion forces and hydrogen-bonding, seems to be the major driving force behind the loss of activity observed.

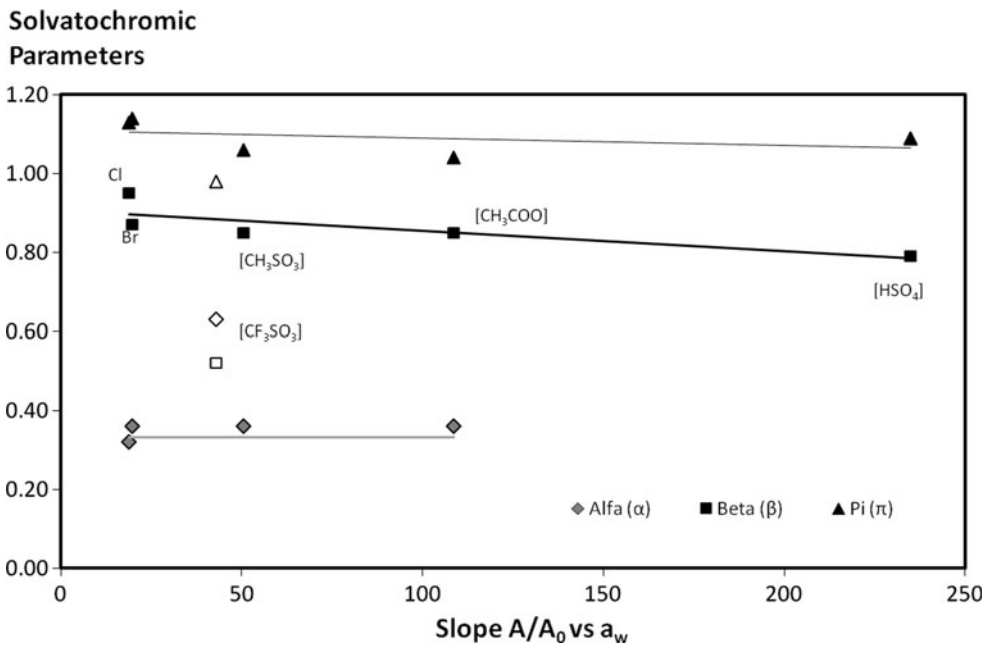

Fig. 5 Dependency of the decrease on enzymatic activity of CaLB with the water activity and the solvatochromic parameters α, β, and π

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