Separation and Purification Technology 233 (2020) 116019

Contents lists available at ScienceDirect

Separation and Purification Technology

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

Performance of tetraalkylammonium-based ionic liquids as constituents of aqueous biphasic systems in the extraction of ovalbumin and lysozyme

Diana C.V. Belchior, Maria V. Quental, Matheus M. Pereira, Carlos M.N. Mendonça, Iola F. Duarte, Mara G. Freire*

CICECO – Aveiro Institute of Materials, Chemistry Department, University of Aveiro, 3810-193 Aveiro, Portugal

ARTICLE INFO

Keywords:
Aqueous two-phase systems
Ionic liquids
Proteins
Extraction
Recovery
Molecular docking

ABSTRACT

Ionic-liquid-based aqueous biphasic systems (IL-based ABS) have been described as promising platforms for the extraction and separation of proteins. However, imidazolium-based ILs have been the preferred choice, which may raise some biocompatibility and biodegradability concerns. In this work, novel ABS composed of tetraalkylammonium-based ILs and potassium phosphate solutions at different pH values (pH = 7, 8, 9 and 13, using K_2HPO_4/KH_2PO_4 or K_3PO_4) were investigated in terms of extraction efficiency and recovery yield for two proteins, namely ovalbumin and lysozyme. These proteins were selected due to their wide application in several sectors, being present in egg white. At pH 7, the complete extraction and recovery of lysozyme to the IL-rich phase are achieved in all systems; however, low recovery yields of ovalbumin are obtained with ABS formed by ILs with longer alkyl side chains. Furthermore, an increase in the pH above the proteins isoelectric point is deleterious for their recovery in the IL-rich phase. In order to characterize the molecular-level mechanisms that could maximize the proteins recovery, molecular docking studies were carried out, showing that ILs that preferentially establish hydrophobic interactions with these proteins are those that lead to their aggregation and lower recovery yields. Finally, it is shown the proteins recovery from the IL-rich phase by ice cold ethanol precipitation, where up to 99% of lysozyme can be recovered. These results support the viability of adequate IL-based ABS to extract ovalbumin and lysozyme and the possibility of recovering stable proteins from the IL-rich phase into an adequate buffered aqueous solution, thus contributing to the design of effective separation processes.

1. Introduction

Proteins play a crucial role in biological processes and are of fundamental value in biotechnological, therapeutic and diagnostic applications [1]. Their function and biological activity is related to their native structure, which is however delicate since their three-dimensional structure can be disturbed by changes in the medium composition, pH and/or temperature [2]. Therefore, current purification methods for proteins are not only costly, but may also lead to their loss of stability [3].

A raw material rich in high-value proteins is egg white, which contains 88% of water and 11% of proteins, including ovalbumin, ovotransferrin, lysozyme and ovomucin [4]. Ovalbumin reprents ca. 54% of the total protein content in egg white, being a phosphoglycoprotein with 385 amino acids, with a molecular weight of 45 kDa, and an isoelectric point of 4.5–4.6 [5–7]. Ovalbumin is widely used as a nutrient supplement and as an allergen to establish animal models of asthma, food and dermal allergies [8,9]. Lysozyme constitutes 3.4–3.5% of the total protein content in egg white, has a low molecular weight (14.3 kDa) and a high isoelectric point (10.7) [5,10]. Given its multiple functions (antiviral, antitumor and immune modulatory activities), lysozyme is often used as a model protein, ranging from enzymatic activity to proteins aggregation and crystallization studies [11–13]. Furthermore, lysozyme is an enzyme with bactericidal and bacteriostatic properties, used as a natural preservative by the food industry [14]. The potential of lysozyme as an anticancer drug and in the treatment of HIV has also been discussed [15]. Both lysozyme and ovalbumin are present in a low cost raw material, namely egg white, but still requiring the development of cost-effective separation and fractionation processes in order to obtain highly pure and stable/biologically active proteins at low cost.

Amongst the various separation and purification methods applied to proteins, aqueous biphasic systems (ABS) have been largely investigated. ABS are formed by combining two polymers, one polymer and one salt, or two salts above given concentrations in aqueous solution [16]. These systems are mainly composed of water, and thus represent a favorable environment for the purification of biologically active biomolecules [16]. Accordingly, ABS have been investigated for the recovery of proteins,

* Corresponding author.
E-mail address: maragfreire@ua.pt (M.G. Freire).

https://doi.org/10.1016/j.seppur.2019.116019
Received 13 April 2019; Received in revised form 26 August 2019; Accepted 2 September 2019
Available online 03 September 2019

enzymes and antibodies from their biological-containing media [17]. In addition to the widely investigated polymer-based ABS, Rogers and co-workers [18] reported the possibility of creating ABS by mixing ionic liquids (ILs) and inorganic salts in aqueous solutions, leading to a plethora of ABS with remarkable advantages, such as low viscosity, fast phase separation, and tunable polarities [19]. Based on these advantages, a large number of ABS composed of ILs + water + organic/inorganic salts, amino acids, polymers or carbohydrates has been investigated [20]. ILs are salts composed of large organic cations and organic or inorganic anions. Although they display a negligible vapor pressure at ambient conditions, with inherent benefits from an environmental point of view, the main advantage of ILs as phase-forming components of ABS conveys on the possibility of tailoring the polarities of the coexisting phases by the manipulation of their ions chemical structures, therefore allowing the design of improved ABS to target applications [19,21].

IL-based ABS have been investigated in the extraction of several proteins, such as bovine serum albumin, lysozyme, trypsin, myoglobin, peroxidase, cytochrome c, hemoglobin, ovalbumin, among others [1,22–24]. In these studies, imidazolium-based ILs and inorganic salts have been the preferred choice as ABS phase-forming components [22]. However, these ILs may raise some biocompatibilty and biodegradability concerns [25]. Accordingly, the synthesis and use of ILs featuring enhanced biocompatibility and a low environmental footprint have been proposed in more recent years [26–30]. Among these, tetraalkylammonium-based fluids have been described as ILs of lower environmental impact and of lower cost [31]. However, few studies have considered tetraalkylammonium-based ILs as phase-forming constituents of ABS for the extraction of proteins [32–34]. Furthermore, in most IL-based ABS studied hitherto the pH was not comprehensively investigated, which is a crucial factor regarding the separation of proteins and enzymes, either because of their susceptible nature or differences in partition due to their isoelectric point.

In this work, a series of ABS composed of tetraalkylammonium-based ILs and potassium phosphate buffer solutions at different pH values (pH = 7, 8, 9 and 13, achieved by applying different K_2HPO_4:KH_2PO_4 mole ratios or K_3PO_4) were evaluated for the extraction of ovalbumin and lysozyme, by determining both the extraction efficiencies and recovery yields. The goal of this work is to better understand the IL chemical structure of less investigated ILs and pH effects on the extraction and recovery yield of ovalbumin and lysozyme, while foresseing the design of effective separation processes based on ILs for proteins.

2. Experimental section

2.1. Materials

Eight ILs were investigated towards the creation of ABS combined with buffer aqueous solutions constituted by potassium dihydrogen phosphate, KH_2PO_4 (99.5 wt% of purity; CAS: 7778-77-0), and potassium hydrogen phosphate trihydrate, $K_2HPO_4{\cdot}3H_2O$ (> 98 wt% of purity; CAS: 16788-57-1), both acquired from Sigma–Aldrich, and tribasic potassium phosphate, K_3PO_4 (98 wt% of purity; CAS: 7778-53-2), supplied from Acros Organics. All ILs, as well as the proteins studied, namely ovalbumin (> 98% of purity; CAS: 9006-59-1) and lysozyme (> 90% of purity; CAS: 12650-88-3), were purchased from Sigma–Aldrich. The chemical structures of the investigated ILs are depicted in Table 1, which additionally comprises their full name, acronym, purities and CAS numbers. The water used was double distilled, passed by a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus.

2.2. Determination of the ABS phase diagrams

Aqueous solutions containing 40 wt% of the salts K_2HPO_4 and KH_2PO_4 at different mole ratio (for pH values of 7, 9 and 10) or K_3PO_4 (for the pH value of 13), and aqueous solutions of the different ILs with concentrations ranging from 40 wt% to 80 wt%, were initially prepared and used for the determination of the binodal curves. The pH of the prepared buffer solutions was confirmed using a Mettler Toledo S47 Seven Multi™ dual meter pH equipment with an uncertainty of ± 0.02. The binodal curves of the ABS phase diagrams formed by each IL and K_2HPO_4/KH_2PO_4 or K_3PO_4 were determined through the cloud point titration method at (25 ± 3) °C and atmospheric pressure [35,36]. The cloud point titration method consists in the repetitive dropwise addition of the salt solution into the aqueous solution of each IL until the detection of a cloudy (biphasic) solution, followed by the dropwise addition of ultrapure water until the observation of a limpid solution (falling within the monophasic region). All additions were carried out under constant stirring. The ternary system compositions were determined by the weight quantification of all components added ($\pm 10^{-4}$ g). The experimental solubility curves were correlated using the following equation proposed by Merchuk et al. [37]:

$$[\mathrm{IL}] = A\exp[(B \times [\mathrm{Salt}]^{0.5}) - (C \times [\mathrm{Salt}]^3)] \quad (1)$$

where [IL] and [Salt] represent the IL and salt weight fraction percentages, respectively, and *A*, *B*, and *C* are constants obtained by the regression of the experimental binodal data.

TLs, which give the composition of each phase for a given initial mixture composition, were determined by a gravimetric method originally proposed by Merchuk et al. [37], which is based on the relationship between the weight of both phases and the total system composition by the lever-arm rule. Ternary mixtures composed of IL + K_2HPO_4/KH_2PO_4 or K_3PO_4 + water were chosen at the biphasic region, gravimetrically prepared ($\pm 10^{-4}$ g), and vigorously agitated. The ABS were then allowed to equilibrate for at least 12 h at (25 ± 3) °C. Both phases were carefully separated and individually weigthed. At the conditions used in this work, all systems have a top IL-rich phase and a salt-rich phase as the bottom layer. Further details on the determination of the TLs and respective length, i.e. tie-line lengths (TLLs), are given in the Supporting Information. It should be remarked that all the calculations considering K_2HPO_4 were carried out discounting the water present in the commercially available hydrated salt ($K_2HPO_4{\cdot}3H_2O$).

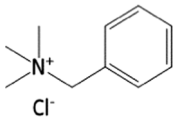
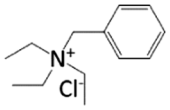
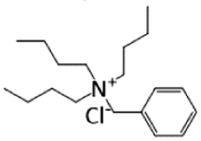
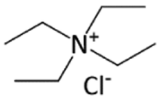
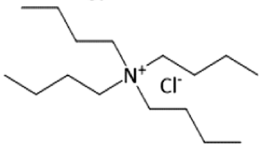
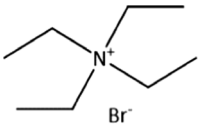
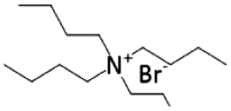
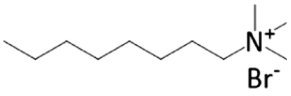
2.3. Extraction of proteins using ABS

The ternary mixture compositions used in the extraction experiments were chosen based on the phase diagrams determined in this work for each IL-salt-water ABS. Aqueous solutions of ovalbumin and lysozyme at concentrations ca. 0.5 g L^{-1} were used as the aqueous solutions added to each ternary mixture. Aqueous solutions of different molar ratio of the salts K_2HPO_4/KH_2PO_4 were used to prepare aqueous solutions with pH values ranging between 7 and 9, while aqueous solutions of K_3PO_4 were used for pH 13. Each mixture was vigorously stirred, centrifuged for 10 min, and left to equilibrate and phase separate for 10 min at (25 ± 3) °C. After, each phase was recovered and diluted at a 1:10 (v:v) ratio in a phosphate buffer aqueous solution before the injection in a size exclusion high performance liquid chromatography (SE-HPLC) for quantification. At least three independent ABS at each condition were prepared and analyzed. The equipment used was a Chromaster HPLC system (VWR Hitachi) equipped with a binary pump, column oven, temperature controlled auto-sampler, DAD detector and a Shodex Protein KW- 802.5 (8 mm × 300 mm) column. For the detection of the two proteins in each phase, different HPLC conditions were applied. The description of the HPLC conditions used for the identification and quantification of each protein is given in the Supporting Information, Table S1. The temperature of the column and auto-sampler was kept constant at 25 °C. The injection volume was of 25 μL. The wavelength was set at 280 nm, whereas the retention times of ovalbumin and lysozyme were found to be 15 min and 9 min, respectively. The quantification of proteins in each phase was carried out using the respective calibration curves.

The percentage extraction efficiency of the studied ABS for each protein to the IL-rich phase, *EE*%, was determined according to Eq. (2):

$$EE\% = \frac{w_{IL\,\mathrm{Prot}}}{w_{IL\,\mathrm{Prot}} + w_{salt\,\mathrm{Prot}}} \times 100 \quad (2)$$

Table 1
Identification, purity, chemical structure and CAS number of the studied ILs.

Name	Acronym	Purity (wt%)	Chemical structure	CAS number
Benzyltrimethylammonium chloride	$[N_{111(C7H7)}]Cl$	> 98%		56-93-9
Benzyltriethylammonium chloride	$[N_{222(C7H7)}]Cl$	> 99%		56-37-1
Benzyltributylammonium chloride	$[N_{444(C7H7)}]Cl$	> 98%		23616-79-7
Tetraethylammonium chloride	$[N_{2222}]Cl$	> 98%		56-34-8
Tetrabutylammonium chloride	$[N_{4444}]Cl$	> 96%		1112-67-0
Tetraethylammonium bromide	$[N_{2222}]Br$	> 99%		71–91-0
Tetrabutylammonium bromide	$[N_{4444}]Br$	> 97%		1643-19-2
Octyltrimethylammonium bromide	$[N_{1118}]Br$	> 98%		2083-68-3

where w_{ILProt} and $w_{SaltProt}$ are the total weight of each protein in the IL-rich and in the salt-rich aqueous phases, respectively. The *EE*% describes the relative amount of each protein in the IL-rich phase in respect to that in the coexisting phases, thus not considering precipitation effects.

The recovery yield of each protein into to IL-rich phase, *RY*%, is the percentage ratio between the amount of protein in the IL-rich aqueous phase (w_{ILProt}) to that present in the initial mixture ($w_{InitialProt}$), defined according to Eq. (3),

$$RY\% = \frac{w_{IL\,Prot}}{w_{InitialProt}} \times 100 \quad (3)$$

The *RY*% describes the relative amount of each protein in the IL-rich phase in respect to that added to each ABS, and thus addresses the proteins aggregation and precipitation.

Finally, attempts on the recovery of the proteins from the IL-rich phase were carried out. To this end, ice cold ethanol was added to the IL-rich phase containing the target protein, and the solution was kept at −80 °C for 1 h. The precipitated fraction was carefully separated by centrifugation (3 min at 500 rpm) and dried under inert atmosphere. Proteins were redissolved in PBS buffer (100 mM and pH 7.4) and quantified by SE-HPLC as described before.

2.4. Molecular docking

The interaction sites of Ova and Lys with the ILs ions were identified using the Auto-dock vina 1.1.2 program [38], which is based on algorithms and force fields of molecular dynamic simulations. Like in most docking programs, its algorithm was prepared for gas phase interactions. The following crystal structures were used: Lysozyme (PDB 4ym8) and Ova (PDB: 1ovalbumin adapted removing B, C and D chains). The Auto DockTools (ADT) [39] program was used to prepare the protein input files by merging non-polar hydrogen atoms, adding partial charges and atom types aiming at having similar net charges at the pH conditions used in the experimental assays, namely pH 7. Ligand (ILs ions) 3D atomic coordinates were computed by the Discovery Studio Visualizer [40] and ligand rigid root was generated using AutoDockTools (ADT), setting all possible rotatable bonds defined as active by torsions. The grid center at the center of mass (x-, y-, and z-axes, respectively) to cover the whole interaction surface of ovalbumin was 104 Å × 98 Å × 88 Å and of lysozyme was 100 Å × 112 Å × 126 Å. The binding model was searched out from 10 different conformers for each ligand (IL cation or anion). The molecular docking software has been studied to ascertain the binding sites of other IL ions to proteins, where additional information can be found [27,41]. The β-sheet content of each protein was analyzed using the same crystal structure of lysozyme and ovalbumin applied for molecular docking studies as input files.

3. Results and discussion

3.1. ABS phase diagrams

The experimental ABS phase diagrams determined at 25 °C and at atmospheric pressure for each IL + water + K_2HPO_4/KH_2PO_4 system at pH 7 are illustrated in Fig. 1.

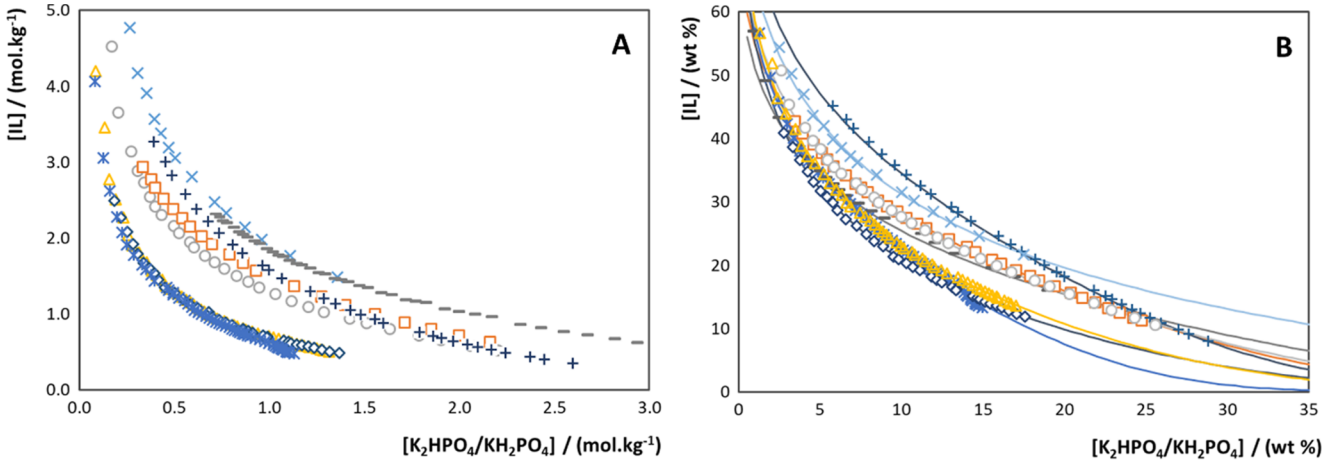


Fig. 1. Phase diagrams of ABS formed by IL + K_2HPO_4/KH_2PO_4 + water at pH 7, 25 °C and atmospheric pressure in molality units **(A)** and weigth fraction percentage **(B)**. ILs: $[N_{111(C7H7)}]Cl$ (×); $[N_{2222}]Cl$ (–); $[N_{2222}]Br$ (□); $[N_{222(C7H7)}]Cl$ (○); $[N_{444(C7H7)}]Cl$ (△); $[N_{4444}]Cl$ (◇); $[N_{4444}]Br$ (✳); $[N_{1118}]Br$ (+). Lines correspond to the fitting by Eq. (1).

The binodal curves are plotted both in weigth fraction and in molality units. The first representation is useful to directly identify the mixture compositions required to form and prepare ABS for separation purposes, whereas the second representation is valuable to remove the effects related with differences in the ILs and salts molecular weight aiming the interpretation of the molecular-level mechanisms responsible for phase separation. The experimental weight fraction data of binodal curves are provided in the Supporting Information, Tables S1–S18. Data for TLs and TLLs are given in Table S19 in the Supporting Information. The experimental binodal data were fitted using the empirical relationship described by Eq. (1), also shown in Fig. 1. The regression parameters estimated by the least-squares regression method, respective standard deviations, and correlation coefficients are provided in the Supporting Information, Table S20.

Fig. 1 depicts the phase diagrams obtained at a constant pH, namely pH 7, allowing to address both the effect of the IL cation and anion to form two-phase systems. For a given salt composition, for instance, using K_2HPO_4/KH_2PO_4 at ≈ 0.25 mol·kg^{-1}, the immisicibility region or ability of ammonium-based ILs to form ABS decreases in the following order: $[N_{4444}]Br \approx [N_{4444}]Cl \approx [N_{444(C7H7)}]Cl > [N_{222(C7H7)}]Cl > [N_{2222}]Br > [N_{1118}]Br > [N_{2222}]Cl \approx [N_{111(C7H7)}]Cl$. Diagrams with a larger area above the binodal curve (immisicibility region) have a higher ability to form two phases, i.e. the IL is more easily salted out by the phosphate-based salt due to a decrease in the IL affinity for water. In general, ILs with longer alkyl side chains are more prone to be salted-out and to form ABS, in agreement with previously published data [42,43]. Furthermore, bromide-based ILs are more able to form ABS than the chloride IL equivalents due to the bromide lower ability to accept protons, in agreement with the bromide lower hydrogen-bond basicity [44].

The pH effect (pH 7, 8, 9 and 13) on ABS formation was evaluated using three tretraalkylammonium-based ILs, namely $[N_{2222}]Cl$, $[N_{2222}]Br$ and $[N_{222(C7H7)}]Cl$. It should be stressed that the pH values of 7, 8 and 9 were achieved with mixtures of the salts K_2HPO_4 and KH_2PO_4 at different ratio, whereas pH 13 was accomplished with the salt K_3PO_4. The results depicted in Fig. 2 show that for all ILs investigated the capacity to form ABS decreases with the decrease of the pH (13 > 9 > 8 > 7). Overall, a decrease of the pH leads to the protonation of the phosphate anions from PO_4^{3-} to HPO_4^{2-} to $H_2PO_4^-$, which depending on the pH value exist at different ratio. The speciation distribution curves of these phosphate anions are given in the Supporting Information, Fig. S1. The protonation and decrease of the charge and charge density of the phosphate anions leads to a decrease of their salting-out potential, resulting thus in smaller immiscibility regions and leading to a lower ability to create ABS. These results are in agreement with previously published results with ABS formed by other ILs and salts [23,45,46]. It should be remarked that the ILs chosen are tetraalkylammonium combined with the chloride anion, and do not suffer speciation at the studied pH range.

3.2. Extraction of ovalbumin and lysozyme using ABS, molecular docking studies and recovery stategies

Ternary mixtures at pH 7 were initially selected to evaluate the ABS performance to extract lysozyme and ovalbumin. Based on the phase diagrams, mixture compositions with a similar tie-line length, i.e. (50 ± 2) wt%, were chosen to minimize the differences in the proteins partition behavior arising from differences between the two phases compositions. These ABS are composed of 16–24 wt% of K_2HPO_4/KH_2PO_4 (pH 7) and 21–35 wt% of IL. The detailed mixture compositions, extraction efficiencies and recovery yields of the ABS at pH 7 for ovalbumin and lysozyme are given in the Supporting Information, Table S21. The respective TL data are given in Table S19 in the Supporting Information. This set of studies was carried out at a fixed protein concentration (0.5 g L^{-1}) to reduce the number of possible variables when addressing the IL chemical structure effect on both proteins extraction and recovery to the IL-rich phase. Perturbed-chain statistical associating fluid theory (PC-SAFT) was recently proposed to model proteins in aqueous solutions, where different osmotic coefficients of dialyzed and non-dialyzed protein solutions with a strong dependence on pH and ionic strength have been identified [47]. Therefore, and although not considered in this work, the protein concentration is also a relevant parameter to consider in the extraction and separation of proteins by IL-based ABS.

Fig. 3 depicts the extraction efficiencies and recovery yields of the studied ABS for ovalbumin at pH 7. In all investigated systems, ovalbumin preferentially partitions to the top (IL-rich) phase in respect to the salt-rich phase, with extraction efficiencies of 100% obtained in a single-step. In general, the partitioning of proteins between the two phases of ABS is a complex phenomenon, guided either by differences in the hydrophobicity of the phases or by preferential interactions established between the protein being partitioned and the phase-forming components, being the latter possibility particularly relevant when dealing with ILs. Proteins can interact with ILs through hydrogen-bonding, electrostatic interactions and dispersive forces. Given that the isoelectric point of ovalbumin is 4.5–4.6 [5–7], in all systems and results shown in Fig. 3 at pH 7, ovalbumin is negatively charged and prefers the phase of lower ionic strength (IL-rich). This trend reinforces the salting-out effect exerted by the salt used [48], and possible preferential hydrogen-bonding and dispersive interactions occurring between the protein and the IL.

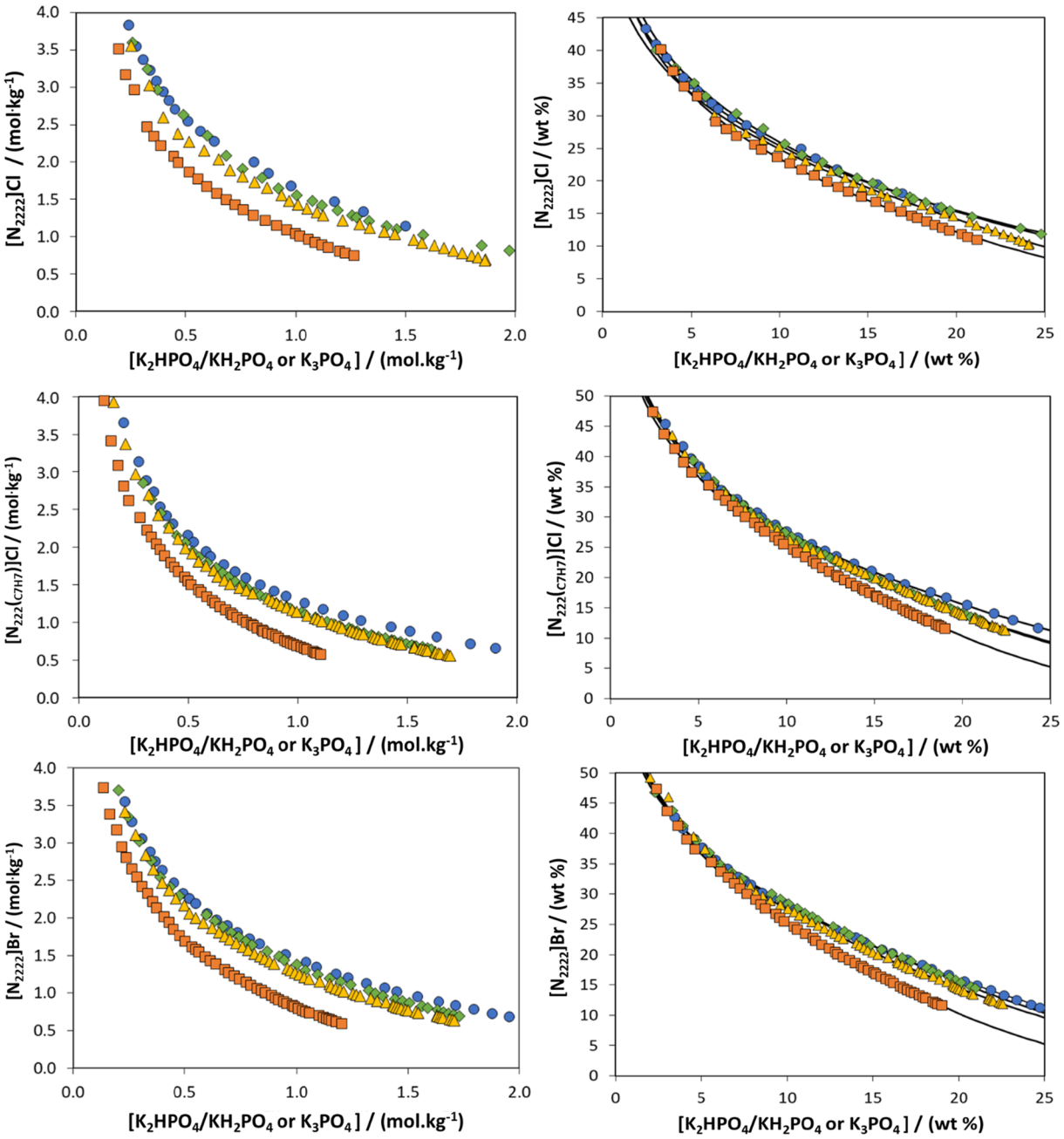


Fig. 2. Phase diagrams in molality units and weigth fraction units for ABS formed by IL ([N_{2222}]Cl, [$N_{222(C7H7)}$] and [N_{2222}]Br) + K_2HPO_4/KH_2PO_4 or K_3PO_4 + H_2O at 25 °C and atmospheric pressure: pH 7 (●), pH 8 (◆), pH 9 (▲) and pH 13 (■). Lines correspond to the fitting by Eq. (1).

Although no differences are observed in terms of extraction efficiency with the IL chemical structure, which reflects the preferential partition of a given protein between the coexisting phases, there is a different scenario when evaluating the recovery yield, which reflects the amount of stable and non-aggregated or non-precipitated protein at the IL-rich phase in respect to the amount of protein initially added to the system. By using SE-HPLC as quantification technique, with examples of chromatograms shown in Fig. 3, it is possible to identify ovalbumin aggregates that appear at lower retention times in some ABS, being the formation of these proteins aggregates the main responsible for the lower recovery yields observed. It should be however remarked that the presence of aggregates, even though in a lower extent, is also seen in the protein aqueous solution prepared as standard.

Recovery yields ranging between 33 and 100% were obtained, and decrease in ABS composed of the following ILs in the following trend: [N_{2222}]Cl ≈ [N_{2222}]Br > [$N_{111(C7H7)}$]Cl > [$N_{222(C7H7)}$]Cl > [N_{4444}]Cl > [N_{4444}]Br > [N_{1118}]Br > [$N_{444(C7H7)}$]Cl. The lower the recovery yield, the higher is the amount of ovalbumin aggregates identified by SE-HPLC. In general, ovalbumin preferentially partitions to the top phase (IL-rich phase) over the salt-rich phase. This trend reinforces the salting-out effect exerted by the salt used, since the same salt and different ILs are used in this set of assays. However, the relative hydrophobicity of the IL cation changes, with a more significant impact on the protein recovery yield. The recovery yield decreases due to the formation of protein aggregates, which are favored in ABS comprising ILs with longer alkyl side chains or higher hydrophobicity. Moreover, the presence of an aromatic ring at the IL cation leads to a decrease on the recovery yield of ovalbumin, addressed by comparing the pairs of ILs: [N_{2222}]Cl vs. [$N_{222(C7H7)}$]Cl and [N_{4444}]Cl vs. [$N_{444(C7H7)}$]Cl. This trend reflects that π···π stacking between the IL aromatic groups and the aromatic amino acids

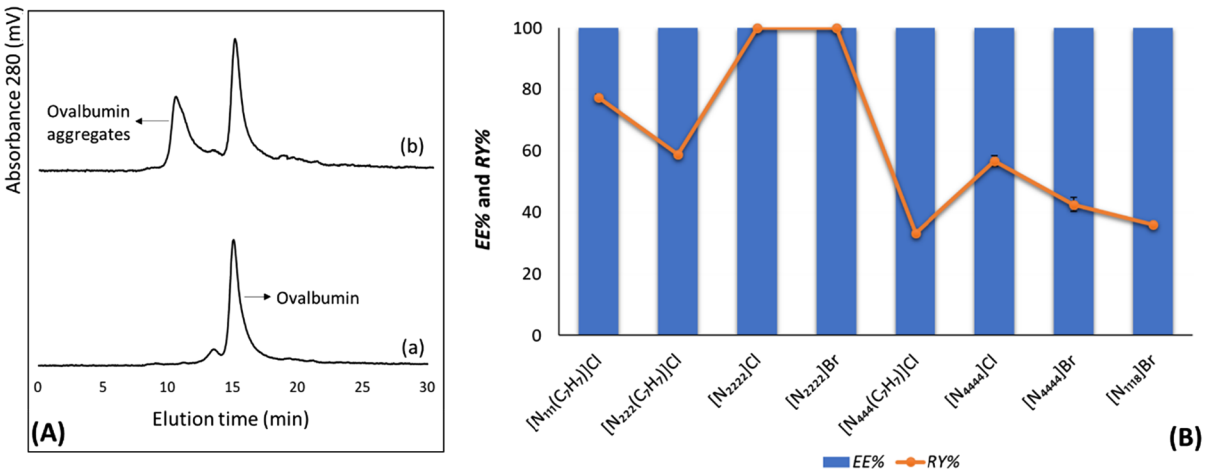


Fig. 3. (A) Size exclusion chromatograms of (a) ovalbumin solution in PBS (b) IL–rich phase of the ABS composed of $[N_{222(C7H7)}]Cl$ + K_2HPO_4/KH_2PO_4 + ovalbumin aqueous solution at pH 7. (B) Extraction efficiencies (*EE*%, bars) and recovery yield (*RY*%, symbols and line) of the ABS composed of ILs + K_2HPO_4/KH_2PO_4 + H_2O at pH 7 for ovalbumin.

residues of ovalbumin are relavant, although do not positively contribute to the protein recovery in the IL-rich phase. Furthermore, at the mixture compositions studied and according to the TL data given in the Supporting Information (Table S19), the water content difference at the IL-rich between each $[N_{4444}]Cl/[N_{444(C7H7)}]Cl$ and $[N_{2222}]Cl/[N_{222(C7H7)}]Cl$ pair is in average < 2.4 wt%, demonstrating that the lower recovery yields obtained with aromatic ILs are not due to a decrease in the hydrophobicity of the IL-rich phase, but yet to favorable π···π interactions established between the IL cation and the proteins which lead to their aggregation and decrease in the recovery yields. Overall, although ovalbumin preferentially migrates to the IL-rich phase in respect to the salt-rich phase, as the hydrophobicity or aromatic character of the IL-rich phase increases there is an increase on the formation of protein aggregates. This trend is better explained along with the molecular docking results discussed below, where it is demonstrated that ILs that can establish hydrophobic interactions with proteins are those that better induce their aggregation.

Fig. 4 depicts the *EE*% of the same ABS and at the same mixture compositions at pH 7 for lysozyme; in all systems there is the preferential partition of the protein to the IL-rich aqueous phase over the salt-rich phase, with extraction efficiencies of 100% to the IL-rich phase achieved in one-step (as observed before with ovalbumin). Lysozyme has a low molecular weight (14.3 kDa) and a high isoelectric point (10.7) [5,10], being majorly positively charged at pH 7. As verified with ovalbumin, lysozyme prefers the phase of lower ionic strength (IL-rich), reinforcing the possible salting-out effect exerted by K_2HPO_4/KH_2PO_4 and preferential interactions established with ILs, such as hydrogen-bonding, π···π and dispersive interactions. Overall, based on the proteins pI and on the pH of these ABS that was kept at 7, ovalbumin is majorly negatively charged whereas lysozyme is mainly positively charged. Although with ovalbumin some losses on the protein yield were observed due the protein aggregation, with lysozyme the recovery yield with the investigated ABS is of 100%, meaning that there is not the precipitation of lysozyme or the formation of protein aggregates during the extraction step with none of the systems investigated (Fig. 4A). This set of results suggest that the investigated IL-based ABS lead to higher recovery yields when dealing with smaller proteins and when working at pH values below the proteins pI.

Ovalbumin and lysozyme are usually studied as model proteins to investigate the driving forces of protein partition in IL-based ABS. Chen et al. [32] evaluated the effect of several parameters to identify the optimal conditions in the extraction of ovalbumin, bovine serum albumin (BSA) and bovine hemoglobin (BHb) in ABS formed by hydroxyl ammonium-based ILs. The authors showed that under the optimum conditions, the extraction efficiency of ovalbumin could reach 68%. Comparing these results with ours, particularly when addressing the recovery yield values obtained with ovalbumin, it is clear that the chemical structure of the IL plays a relevant role in the ovalbumin recovery. Desai et al. [49] revealed that the size and complexity of the protein influences protein aggregation and its stability in ILs. In some works, it has been reported that larger proteins are less prone to migrate to the IL-rich phase compared to smaller ones [50,51], in agreement with the results obtained in this work since there are no losses of the smaller (lysozyme) protein using IL-based ABS. The use of ABS in the extraction of ovalbumin, cytochrome C, myoglobin, and hemoglobin was investigated and further compared with traditional PEG-based ABS by Ruiz-Angel et al. [52]. Higher partition coefficients in IL-based ABS were reported [52], reinforcing the effectiveness of systems comprising ILs for proteins extraction and recovery.

When dealing with proteins or enzymes, the pH is one relevant parameter to take into account. Some studies showed that the closer the pH of the system is to the pI of each protein, the easier it is to manipulate the migration of proteins between the phases [49]. Aiming at better understanding the pH effect in the ABS extraction and recovery of proteins, additional ABS were prepared at pH values of 8, 9 and 13. The systems at pH 8 and 9 were prepared with the salts K_2HPO_4/KH_2PO_4, whereas ABS at pH 13 were prepared with K_3PO_4.

Fig. 5 shows the extraction efficiency and recovery yield of the studied ABS at the pH values 7, 8, 9 and 13 for lysozyme. It should be remarked that the minimum pH that can be investigated is 7.0 since it is not possible to create ABS with the studied ILs at lower pH values, as experimentally verified by us. In this sense, it was not possible to study the pH effect over ovalbumin at pH values below its pI. Lysozyme was the protein studied in this set of assays since it has an isoelectric point of 10.7 (higher than 7), and as such it was possible to address changes in pH below and above its isoelectric point. The mixture compositions, extraction efficiencies and recovery yieds of ABS at different pH values are given in the Supporting Information, Table S22. Up to a pH of 9, all ABS investigated, composed of 16–24 wt% of K_2HPO_4/KH_2PO_4 and 21–35 wt% of IL to obtain a similar TLL, i.e. (50 ± 2) wt%, provide 100% of extraction efficiency and 100% of recovery yield for lysozyme. However, at pH 13, and although extraction efficiencies are still kept at 100%, there is the loss of the protein, with recovery yields ranging from 31 to 78% (depending on the IL chemical structure). This trend is in agreement with the trend observed before with ovalbumin that was negatively charged at pH 7. Lysozyme is predominantly negatively charged at pH 13, and as observed before with ovalbumin, there is a decrease in the recovery yield. Accordingly, the IL chemical structure seems to play a more significant role when evaluating their application to extract negatively charged proteins. Overall, and as observed with ovalbumin, ILs with shorter alkyl side chains at the cation and

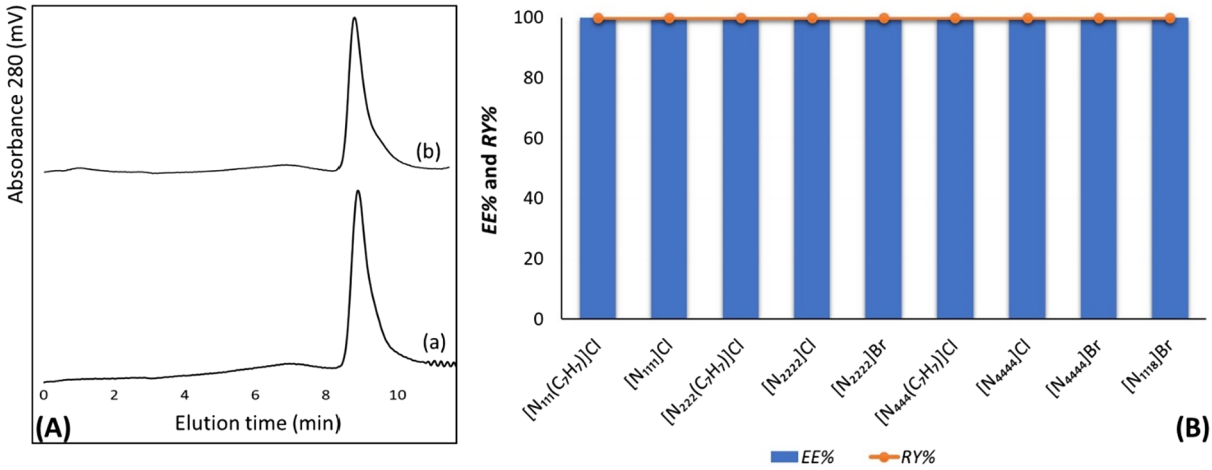


Fig. 4. (A) Size exclusion chromatograms of (a) lysozyme solution in PBS, (b) IL–rich phase of the ABS composed of $[N_{222(C7H7)}]Cl$ + K_2HPO_4/KH_2PO_4 + lysozyme aqueous solution at pH 7. (B) Extraction efficiencies (*EE*%, bars) and recovery yield (*RY*%, symbols and line) of the ABS composed of ILs + K_2HPO_4/KH_2PO_4 + H_2O at pH 7 for lysozyme.

comprising chloride anions are more favorable to recover lysozyme in the IL-rich phase. Furthermore, the increase in the pH leads to an increase in the relative amount of a negatively charged protein, which in addition to electrostatic interactions that may be established with the IL cation also lead to charge–charge repulsion of the proteins, and to the exposure of hydrophobic, SH and SS groups, as described in the literature [53–54]. These exposed groups may lead to aggregation by SH oxidation, SH-SS exchange and, to in a lesser extent, by hydrophobic interactions [53–54], being in agreement with our results. Besides these assumptions, it should not be discarded that an extreme pH value is being used in this case (pH = 13) to extract lysozyme, which may be the main responsible for the protein loss of stability.

Molecular docking was used to better understand the obtained extraction efficiencies and recovery yields for both proteins according to the different IL chemical structures. In particular, docking interactions of all ILs anions and cations with the two proteins were determined. The results obtained show that IL cations display distinct interactions according to the respective chemical structure and the protein surface. The docking bind pose for the IL cations with both proteins are displayed in Figs. 6 and 7. The docking bind pose for the IL anions with both proteins are displayed in the Supporting Information, Fig. S2. The best binding pose and docking affinities, interacting amino acids residues, type of interaction and geometry distance (Å) of each IL ion are provided in the Supporting Information, Tables S23–S24.

For both proteins, IL anions preferentially interact by hydrogen-bonding; e.g. the docking values of affinity for ovalbumin and Cl^- and Br^- anions are similar, namely −1.3 kcal/mol and −1.2 kcal/mol, justifying the less significant differences observed in the recovery yields of ovalbumin by changing the anion when compared to the IL cation effect. Furthermore, and for both proteins, Cl^- and Br^- display a smaller distance to the protein when compared to the distance shown by the IL cations, meaning that they have a high probability to be present in both proteins first solvation layer. On the other hand, IL cations with higher absolute values of docking affinity energies with the interacting amino acids residues of ovalbumin are those that lead to lower recovery yields. The tetraalkylammonium-based cations with shorter alkyl side chains ($[N_{2222}]^+$ and $[N_{4444}]^+$) display mainly hydrogen-bonding and electrostatic interactions with both proteins. However, an increase in the size of the aliphatic moities ($[N_{1118}]^+$) or the presence of aromatic rings ($[N_{111(C7H7)}]^+$, $[N_{222(C7H7)}]^+$ and $[N_{444(C7H7)}]^+$) lead to the establishment of hydrophobic interactions with both proteins. Therefore, ILs comprising cations that preferentially

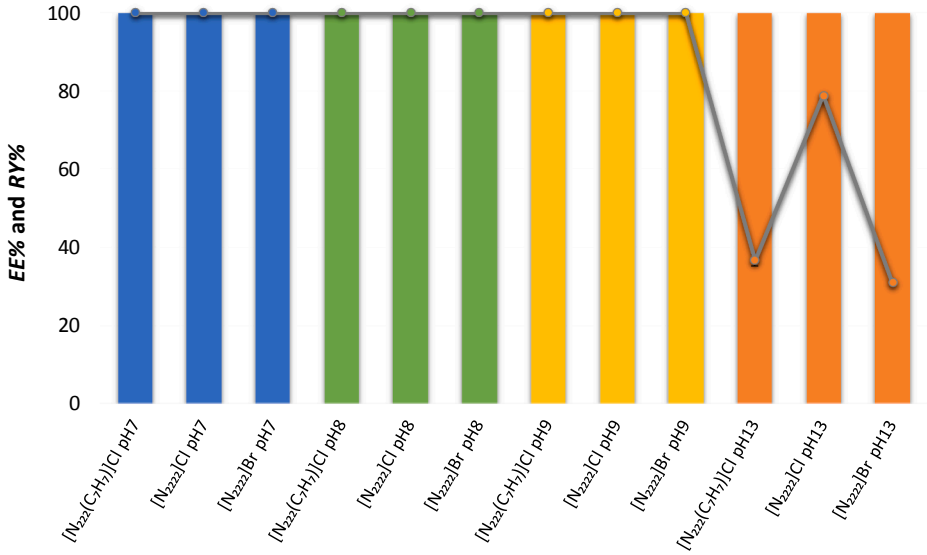


Fig. 5. Extraction efficiencies (*EE*%, bars) and recovery yield (*RY*%, symbols and line) of the ABS composed of ILs + K_2HPO_4/KH_2PO_4 (pH 7, 8 and 9) or K_3PO_4 (pH 13) + H_2O for lysozyme.

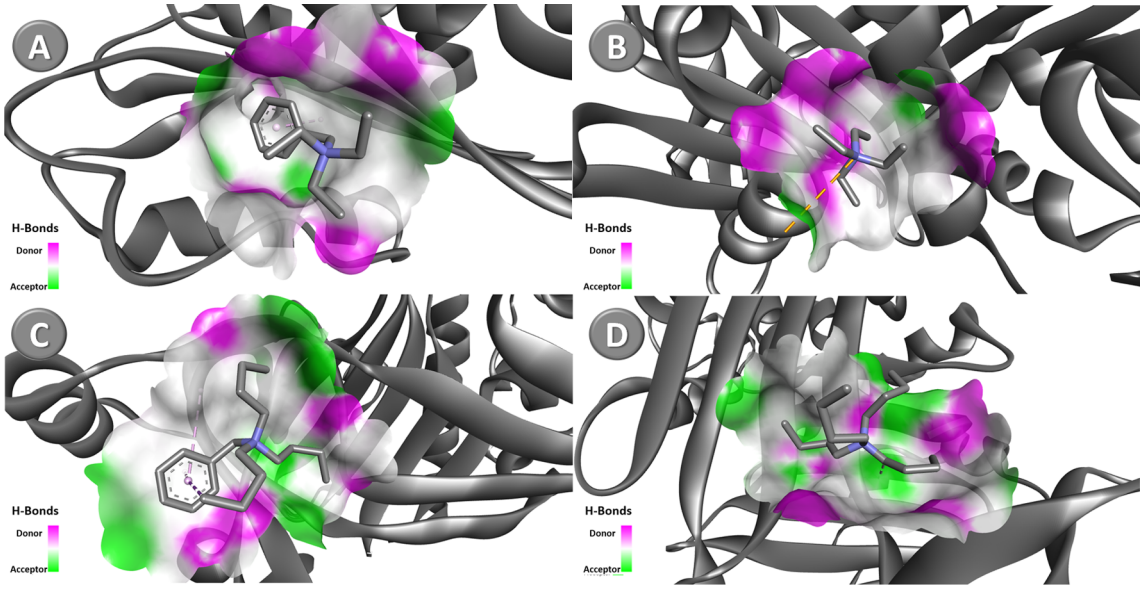


Fig. 6. Docking pose with the lowest absolute value of affinity for ovalbumin with: (A) $[N_{222(C7H7)}]^+$, (B) $[N_{2222}]^+$, (C) $[N_{444(C7H7)}]^+$ and (D) $[N_{4444}]^+$.

interact by hydrophobic interactions are those that lead to the formation of protein aggregrates and lower recovery yields, which is particularly seen with ovalbumin.

Overall, it has been found that the formation of protein aggregates is more significant in ovalbumin than in lysozyme. In addition to their different pI values, ovalbumin has a higher size and a higher content of β-sheets, being both factors favorable to induce the proteins aggregation [55–56]. The β-sheet content of each protein addressed by molecular docking studies is visually depicted in the Supporting Information, Fig. S3, being in agreement with the obtained results.

In addition to the complete extraction and recovery yield of proteins using IL-based ABS it is highly relevant to develop methodologies to further recover the target proteins from the IL-rich phase. In this work, lysozyme was precipitated from the IL-rich phase of the ABS composed of $[N_{2222}]Cl$, $[N_{2222}]Br$ and $[N_{222(C7H7)}]Cl + K_2HPO_4/KH_2PO_4$ (pH 7 and 8) or K_3PO_4 (pH 13) using ice cold ethanol. Lysozyme was resuspended in phosphate buffer (100 mM) aqueous solutions at pH 7.4 and analyzed and quantificated by SE-HPLC (cf. the Supporting Information, Table S25). From the SE-HPLC results, in all systems at pH 7 and 8 it is shown that lysozyme is stable after the induced precipitation and resuspension steps. Upon increasing the pH up to 13, significative changes in the chromatograms are observed, in agreement with previous studies demonstrating conformational changes of lysozyme at extreme alkaline pH values [53]. At pH 7 and 8, with ABS formed by $[N_{222(C7H7)}]Cl$ ca. 81% of lsozyme can be recovered, whereas with the ABS formed by $[N_{2222}]Br$ and $[N_{2222}]Cl$ the recovery of lysozyme increases up to 95 and 99%, respectively. In summary, the obtained results indicate no structural changes in the protein by the formation of aggregates after the recovery step at pH 7 and 8 (Fig. S4 in the Supporting Information), confirming the viability of using the proposed ABS for the extraction of lysozyme and of the proposed recovery strategy of proteins from the IL-rich phase.

4. Conclusions

In this work, novel ABS composed of tetraalkylammonium-based ILs

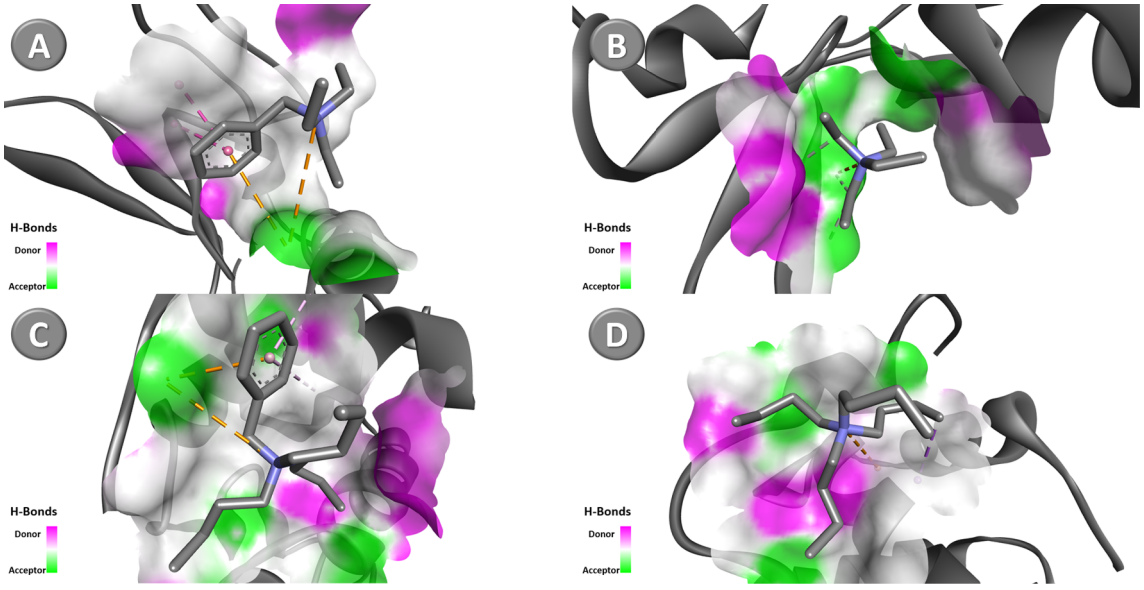


Fig. 7. Docking pose with the lowest absolute value of affinity for lysozyme with: (A) $[N_{222(C7H7)}]^+$, (B) $[N_{2222}]^+$, (C) $[N_{444(C7H7)}]^+$ and (D) $[N_{4444}]^+$.

and potassium phosphate solutions at different pH values (pH = 7, 8, 9 and 13 using K_2HPO_4:KH_2PO_4 at different mole ratios or K_3PO_4) were investigated in the extraction of ovalbumin and lysozyme. The respective ABS ternary phase diagrams, as well as the tie-lines and tie-line lengths, were also determined at 25 °C, where an increase in the pH and in the IL hydrophobicity by the increase of the IL cation alkyl side chain length or by using anions with lower hydrogen-bond basicity is favorable for two-phase separation.

At pH 7 the complete extraction and recovery of lysozyme was achieved in a single-step with all IL-based ABS. However, low recovery yields and the formation of aggregates of ovalbumin occur with ABS formed by ILs with longer alkyl side chains. Furthermore, pH plays a crucial role in two proteins recovery yield. pH values higher than the proteins isoelectric point lead to lower recovery yields, being the respective magnitude dependent on the IL chemical structure. Overall, the increase in the pH above the two proteins isoelectric point is not beneficial for their extraction using the investigated IL-based ABS. Finally, it is shown the proteins recovery from the IL-rich phase, namely by ice cold ethanol induced precipitation, where up to 99% of lysozyme can be recovered with no changes in their structure. These results support the viability of adequate IL-based ABS to extract proteins and the possibility of recovering stable proteins from the IL-rich phase into an adequate buffered aqueous solution.

The selected ABS allowed to better identify the molecular-level mechanisms and conditions that maximize ovalbumin and lysozyme extraction efficiencies and recovery yields, while contributing to the design of effective separation processes for proteins from complex matrices.

Acknowledgments

The authors acknowledge to the project CICECO-Aveiro Institute of Materials, FCT Ref. UID/CTM/50011/2019, financed by national funds through the FCT/MCTES. The authors acknowledge the financial support from the national funds through the FCT/MEC and when appropriate co-financed by FEDER under the PT2020 Partnership Agreement. D.C.V. Belchior acknowledges the financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq for the PhD grant (202337/2015-4). M. V. Quental acknowledges the FCT PhD grant SFRH/BD/109765/2015. I. F. Duarte acknowledges the FCT/MCTES for a research contract under the Program 'Investigador FCT'. M. G. Freire acknowledges the European Research Council (ERC) for the Starting Grant ERC-2013-StG-337753.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2019.116019.

References

[1] Y. Pei, J. Wang, K. Wu, X. Xuan, X. Lu, Ionic liquid-based aqueous two-phase extraction of selected proteins, Sep. Purif. Technol. 64 (2009) 288–295.
[2] R. Patel, M. Kumari, A.B. Khan, Recent advances in the applications of ionic liquids in protein stability and activity: a review, Appl. Biochem. Biotechnol. 172 (2014) 3701–3720.
[3] X. Lin, Y. Wang, Q. Zeng, X. Ding, J. Chen, Extraction and separation of proteins by ionic liquid aqueous two-phase system, Analyst 138 (2013) 6445–6453.
[4] D.A. Omana, J. Wang, J. Wu, Co-extraction of egg white proteins using ion-exchange chromatography from ovomucin-removed egg whites, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 878 (2010) 1771–1776.
[5] H. Hatta, M.P. Kapoor, L.R. Juneja, Bioactive Components in Egg Yolk, in: Y. Mine (Ed.), Egg Biosci. Biotechnol., 2008, pp. 185–237.
[6] S. Savadkoohi, A. Bannikova, N. Mantri, S. Kasapis, Structural properties of condensed ovalbumin systems following application of high pressure, Food Hydrocoll. 53 (2014) 104–114.
[7] Y. Wang, Y. Zhou, J. Sokolov, B. Rigas, K. Levon, M. Rafailovich, A potentiometric protein sensor built with surface molecular imprinting method, Biosens. Bioelectron. 24 (2008) 162–166.
[8] L.Z. Sun, S. Elsayed, T.B. Aasen, T. Van Do, N.P. Aardal, E. Florvaag, K. Vaali, Comparison between ovalbumin and ovalbumin peptide 323–339 responses in allergic mice: Humoral and cellular aspects, Scand. J. Immunol. 71 (2010) 329–335.
[9] W.H. Yang, Z.C. Tu, H. Wang, X. Li, M. Tian, High-intensity ultrasound enhances the immunoglobulin (Ig)G and IgE binding of ovalbumin, J. Sci. Food Agric. 97 (2017) 2714–2720.
[10] K. Iwashita, A. Handa, K. Shiraki, Co-aggregation of ovalbumin and lysozyme, Food Hydrocoll. 67 (2017) 206–215.
[11] S. Mine, S. Tate, T. Ueda, M. Kainosho, T. Imoto, Analysis of the relationship between enzyme activity and its internal motion using nuclear magnetic resonance: 15 N relaxation studies of wild-type and mutant lysozyme, J. Mol. Biol. 286 (1999) 1547–1565.
[12] S. Singh, J. Singh, Effect of polyols on the conformational stability and biological activity of a model protein lysozyme, AAPS Pharm. Sci. Tech. 4 (2003) 101–109.
[13] S. Maosoongnern, C. Flood, A.E. Flood, J. Ulrich, Crystallization of lysozyme from lysozyme – ovalbumin mixtures: separation potential and crystal growth kinetics, J. Cryst. Growth. 469 (2017) 2–7.
[14] Ç. Mecitoğlu, A. Yemenicioğlu, A. Arslanoğlu, Z.S. Elmaci, F. Korel, A.E. Çetin, Incorporation of partially purified hen egg white lysozyme into zein films for antimicrobial food packaging, Food Res Int. 39 (2006) 12–21.
[15] M.Y. Arica, G. Bayramŏlu, Purification of lysozyme from egg white by Reactive Blue 4 and Reactive Red 120 dye-ligands immobilised composite membranes, Process Biochem. 40 (2005) 1433–1442.
[16] A. P-A, Partition of cell particles and macromolecules, Acta Biotechnol., third ed., 1986.
[17] M. Rito-Palomares, Practical application of aqueous two-phase partition to process development for the recovery of biological products, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 807 (2004) 3–11.
[18] K.E. Gutowski, G.A. Broker, H.D. Willauer, J.G. Huddleston, R.P. Swatloski, J.D. Holbrey, R.D. Rogers, Controlling the aqueous miscibility of ionic liquids: aqueous biphasic systems of water-miscible ionic liquids and water-structuring salts for recycle, metathesis, and separations, J. Am. Chem. Soc. 125 (2003) 6632–6633.
[19] J.F.B. Pereira, L.P.N. Rebelo, R.D. Rogers, J.A.P. Coutinho, M.G. Freire, Combining ionic liquids and polyethylene glycols to boost the hydrophobic-hydrophilic range of aqueous biphasic systems, Phys. Chem. Chem. Phys. 15 (2013) 19580–19583.
[20] M.G. Freire, J.F.B. Pereira, M. Francisco, H. Rodríguez, L.P.N. Rebelo, R.D. Rogers, J.A.P. Coutinho, Insight into the interactions that control the phase behaviour of new aqueous biphasic systems composed of polyethylene glycol polymers and ionic liquids, Chemi. Eur. J. 18 (2012) 1831–1839.
[21] A.F.M. Cláudio, M.G. Freire, C.S.R. Freire, A.J.D. Silvestre, J.A.P. Coutinho, Extraction of vanillin using ionic-liquid-based aqueous two-phase systems, Sep. Purif. Technol. 75 (2010) 39–47.
[22] M.G. Freire, A.F.M. Cláudio, J.M.M. Araújo, J.A.P. Coutinho, I.M. Marrucho, J.N.C. Lopes, L.P.N. Rebelo, Aqueous biphasic systems: a boost brought about by using ionic liquids, Chem. Soc. Rev. 41 (2012) 4966–4995.
[23] P. Selvakumar, T.C. Ling, S. Walker, A. Lyddiatt, Partitioning of haemoglobin and bovine serum albumin from whole bovine blood using aqueous two-phase systems, Sep. Purif. Technol. 90 (2012) 182–188.
[24] J.K. Yan, H. Le Ma, J.J. Pei, Z. Bin Wang, J.Y. Wu, Facile and effective separation of polysaccharides and proteins from Cordyceps sinensis mycelia by ionic liquid aqueous two-phase system, Sep. Purif. Technol. 135 (2014) 278–284.
[25] T.P. Thuy Pham, C.W. Cho, Y.S. Yun, Environmental fate and toxicity of ionic liquids: a review, Water Res. 44 (2010) 352–372.
[26] M.V. Quental, M. Caban, M.M. Pereira, P. Stepnowski, A.P. Joao, M.G. Freire Coutinho, Enhanced extraction of proteins using cholinium-based ionic liquids as phase-forming components of aqueous biphasic systems, Biotechnol. J. 10 (2015) 1457–1466.
[27] M. Taha, M.V. Quental, I. Correia, M.G. Freire, J.A.P. Coutinho, Extraction and stability of bovine serum albumin (BSA) using cholinium-based Good's buffers ionic liquids, Process Biochem. 50 (2015) 1158–1166.
[28] S. Shahriari, L.C. Tomé, J.M.M. Araújo, L.P.N. Rebelo, J.A.P. Coutinho, I.M. Marrucho, M.G. Freire, Aqueous biphasic systems: a benign route using cholinium-based ionic liquids, RSC Adv. 3 (2013) 1835–1843.
[29] R.L. Souza, R.A. Lima, J.A.P. Coutinho, C.M.F. Soares, A.S. Lima, Aqueous two-phase systems based on cholinium salts and tetrahydrofuran and their use for lipase purification, Sep. Purif. Technol. 155 (2014) 118–126.
[30] M.T. Zafarani-Moattar, H. Shekaari, P. Jafari, Aqueous two-phase system based on cholinium chloride and polyethylene glycol di-methyl ether 250 and it use for acetaminophen separation, J. Chem. Thermodyn. 107 (2017) 85–94.
[31] S.P.M. Ventura, C.S. Marques, A.A. Rosatella, C.A.M. Afonso, F. Gonçalves, J.A.P. Coutinho, Toxicity assessment of various ionic liquid families towards Vibrio fischeri marine bacteria, Ecotoxicol. Environ. Saf. 76 (2012) 162–168.
[32] J. Chen, Y. Wang, Q. Zeng, X. Ding, Y. Huang, Partition of proteins with extraction in aqueous two-phase system by hydroxyl ammonium-based ionic liquid, Anal. Methods. 6 (2014) 4067–4076.
[33] M.M. Pereira, S.N. Pedro, M.V. Quental, Á.S. Lima, J.A.P. Coutinho, M.G. Freire, Enhanced extraction of bovine serum albumin with aqueous biphasic systems of phosphonium- and ammonium-based ionic liquids, J. Biotechnol. 206 (2015) 17–25.
[34] C.X. Zeng, R.P. Xin, S.J. Qi, B. Yang, Y.H. Wang, Aqueous two-phase system based on natural quaternary ammonium compounds for the extraction of proteins, J Sep Sci. 39 (2016) 648–654.
[35] S.P.M. Ventura, S.G. Sousa, L.S. Serafim, A.S. Lima, M.G. Freire, J.A.P. Coutinho, Ionic liquid based aqueous biphasic systems with controlled pH: the ionic liquid cation effect, J. Chem. Eng. Data. 56 (2011) 4253–4260.
[36] C.M.S.S. Neves, P.M. Ventura, M.G. Freire, I.M. Marrucho, Evaluation of cation influence on the formation and extraction capability of ionic-liquid-based aqueous

biphasic systems, J. Phys. Chem. B. 113 (2009) 5194–5199.
[37] J.C. Merchuk, B. Andrews, J. Asenjo, Aqueous two-phase systems for protein separation, J. Chromatogr. B Biomed. Sci. Appl. 711 (1998) 285–293.
[38] O. Trott, A. Olson, AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading, J. Comput. Chem. 31 (2010) 455–461.
[39] G. Morris, R. Huey, R.K.B. William Lindstrom, Michel F. Sanner, A.J.O. David, S. Goodsell, AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility, J. Comput. Chem. 30 (2009) 2785–2791.
[40] v17. 2. 0. 1634. D.S.B. Discovery Studio Visualizer, Discovery Studio Visualizer, v17.2.0.16349, Dassault Systèmes BIOVIA, Dassault Systèmes, San Diego, 2017.
[41] M. Taha, F.A. e Silva, M.V. Quental, S.P.M. Ventura, M.G. Freire, J.A.P. Coutinho, Good's buffers as a basis for developing self-buffering and biocompatible ionic liquids for biological research, Green Chem. 16 (2014) 3149–3159.
[42] T.E. Sintra, R. Cruz, S.P.M. Ventura, J.A.P. Coutinho, Phase diagrams of ionic liquids-based aqueous biphasic systems as a platform for extraction processes, J. Chem. Thermodyn. 77 (2014) 206–213.
[43] F.A. Silva, T. Sintra, S.P.M. Ventura, J.A.P. Coutinho, Recovery of paracetamol from pharmaceutical wastes, Sep. Purif. Technol. 122 (2014) 315–322.
[44] A.F.M. Cláudio, L. Swift, J.P. Hallett, T. Welton, J.A.P. Coutinho, M.G. Freire, Extended scale for the hydrogen-bond basicity of ionic liquids, Phys. Chem. Chem. Phys. 16 (2014) 6593–6601.
[45] T. Mourão, A.F.M. Cláudio, I. Boal-Palheiros, M.G. Freire, J.A.P. Coutinho, Evaluation of the impact of phosphate salts on the formation of ionic-liquid-based aqueous biphasic systems, J. Chem. Thermodyn. 54 (2012) 398–405.
[46] M.T. Zafarani-Moattar, S. Hamzehzadeh, Effect of pH on the phase separation in the ternary aqueous system containing the hydrophilic ionic liquid 1-butyl-3-methylimidazolium bromide and the kosmotropic salt potassium citrate at T = 298.15K, Fluid Phase Equilib. 304 (2011) 110–120.
[47] M. Hübner, C. Lodziak, H.T.J. Do, C. Held, Measuring and modeling thermodynamic properties of aqueous lysozyme and BSA solutions, Fluid Phase Equilib. 472 (2018) 62–74.
[48] C.M.S.S. Neves, C. Held, S. Mohammad, M. Schleinitz, J.A.P. Coutinho, M.G. Freire, Effect of salts on the solubility of ionic liquids in water: experimental and electrolyte Perturbed-Chain Statistical Associating Fluid Theory, Phys. Chem. Chem. Phys. 17 (2015) 32044–32052.
[49] R.K. Desai, M. Streefland, R.H. Wijffels, M.H.M. Eppink, Extraction and stability of selected proteins in ionic liquid based aqueous two phase systems, Green Chem. 16 (2014) 2670–2679.
[50] Z. Li, X. Liu, Y. Pei, J. Wang, M. He, Design of environmentally friendly ionic liquid aqueous two-phase systems for the efficient and high activity extraction of proteins, Green Chem. 14 (2012) 2941.
[51] X. Ding, Y. Wang, Q. Zeng, J. Chen, Y. Huang, K. Xu, Design of functional guanidinium ionic liquid aqueous two-phase systems for the efficient purification of protein, Anal. Chim. Acta 815 (2014) 22–32.
[52] M.J. Ruiz-Angel, V. Pino, S. Carda-Broch, A. Berthod, Solvent systems for countercurrent chromatography: an aqueous two phase liquid system based on a room temperature ionic liquid, J. Chromatogr. A 1151 (2007) 65–73.
[53] M. Hameed, B. Ahmad, K.M. Fazili, K. Andrabi, R.H. Khan, Different molten globule-like folding intermediates of hen egg white lysozyme induced by high pH and tertiary butanol, J. Biochem. 141 (2007) 573–583.
[54] N. Gharbi, M. Labbafi, Effect of processing on aggregation mechanism of egg white proteins, Food Chem. 252 (2018) 126–133.
[55] A. Glyk, D. Solle, T. Scheper, S. Beutel, Evaluation of driving forces for protein partition in PEG-salt aqueous two-phase systems and optimization by design of experiments, J. Chromatogr. Sep. Tech. 8 (2017) 1000389.
[56] A.L. Fink, Protein aggregation: folding aggregates, inclusion bodies and amyloid, Fold. Des. 3 (1998) R9–R23.