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Recent Advances in the Applications of Ionic Liquids in Protein Stability and Activity: A Review

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Abstract Room temperatures ionic liquids are considered as miraculous solvents for biological system. Due to their inimitable properties and large variety of applications, they have been widely used in enzyme catalysis and protein stability and separation. The related information present in the current review is helpful to the researchers working in the field of biotechnology and biochemistry to design or choose an ionic liquid that can serve as a noble and selective solvent for any particular enzymatic reaction, protein preservation and other protein based applications. We have extensively analyzed the methods used for studying the protein–IL interaction which is useful in providing information about structural and conformational dynamics of protein. This can be helpful to develop and understanding about the effect of ionic liquids on stability and activity of proteins. In addition, the affect of physico-chemical properties of ionic liquids, viz. hydrogen bond capacity and hydrophobicity on protein stability are discussed.

Keywords Ionic liquid · Enzymes · Protein stability · Proteins solubility · Self-aggregation

Introduction

Protein is one of the most important biomacromolecule of living beings having clinical, biochemical, biotechnological and genetic features. As protein exhibits admirable biological properties, the production and applications of proteins have rapidly grown up in different fields such as biochemical research, chemical, food and pharmaceutical industries. Due to wide application, it is necessary to maintain the three-dimensional structure of proteins through some weak interactions including hydrogen bonds, hydrophobic and ionic interactions. A change in microenvironment of proteins disrupts these interactions, causing denaturation of proteins which leads to protein unfolding and inactivation [1–3]. Although there are many factors that cause denaturation of proteins, thermal denaturation is a major hurdle in storage of

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proteins and also during its reaction process. To stabilize proteins outside their native conditions, several strategies like chemical modification, immobilization, genetic modification, and addition of stabilizing agents have been developed. These strategies however, do not prevent irreversible thermal denaturation of proteins [4–6]. The self-lives of many proteins, including therapeutic proteins can be increased by lyophilization (freeze-drying). In order to increase the solubility and to prevent denaturation and aggregation of protein therapeutics, some excipients like sugars, salts and amino acids have been used. These excipients also fail to give reasonable long-term stability to some protein therapeutics [7].

Lately, ionic liquids (ILs) have successfully emerged as a great solvent media for enzymatic reactions [8, 9] and other protein based applications [10–12]. These ILs are the salts consisting of poorly coordinated ions, due to which they remain in liquid form at temperatures lower than 100°C or even at room temperature. Due to their inimitable properties such as low melting point (<100 °C), low vapour pressure, high polarity, high chemical as well as thermal stability, low toxicity and less hazardous effect on environment, they have been considered as 'green solvents' [13–16]. The properties of ILs can be changed according to the requirement by modifying their constituents (cation and anion) [17]. Although they stabilize the protein over a wide range of temperature, the thermal stability of proteins depends on the correct choice of ILs. This is because proteins are not homogeneously stable in all type of ILs. For example, *Candida antarctica* Lipase B (CaLB) becomes aggregated in [emim][$EtOSO_3$], [emim][NO_3] and [emim][N $(CN)_2$] ILs and its functional activity is lost [18]. However, a high thermal stability has been observed for cytochrome *c* in dhp anions containing ILs [19]. The high thermal stability for monellin in [bmpy][Tf_2N] IL [20] and for α-chymotrypsin enzymes in [emim][Tf_2N] and [btma][Tf_2N] have also been reported [19, 21].

The stability and activity of proteins is sturdily affected by many factors, like polarity, hydrophobicity and hydrogen-bond capacity of ILs, excipients and impurities. Zhao [22] has found that like inorganic salts, enzyme stability in aqueous solutions of hydrophilic ILs also follows the Hofmeister series, but it is not applicable for hydrophobic or anhydrous hydrophilic ILs. Furthermore, the stability and solubility of the proteins in aqueous solution depends on the cation and anion of the hydrophilic ILs. ILs containing chaotropic (large-sized and low charged, weakly hydrated ions that decrease the structure of water) cations and kosmotropic (small-sized and high charged, strongly hydrated ions that increase the structure of water) anions are found to optimally stabilize the biological macromolecules [23–25]. The kosmotropicity order of anions and cations can be determined by using viscosity β-coefficients and other parameters such as hydration entropies, hydration volumes, heat capacity, NMR β-coefficients and ion mobility [26].

Important Physico-chemical Properties of Ionic Liquids

As ILs have a broad area of applications, it is necessary to investigate their physical and chemical characteristics so that the research workers can have an idea about the characteristics of ILs. Also, these properties of ILs have an obvious effect on stability, solubility and activity of enzyme. This knowledge provides an idea to opt for an ideal IL as reaction media for enzymatic reactions. Some important physical and chemical properties of ILs along with their affect on enzymatic reaction and protein stability are summarized below

Hydrogen-Bond Capacity

An IL network is formed due to hydrogen bond formation between the cations and anions of ILs. For example, in imidazolium-based ILs, each cation interacts with three anions and each

anion interacts with three cations. This network is found to be similar to that of the three-dimensional network of water. When a macromolecule is inserted in this network, polar and non-polar regions are formed which results in interaction of IL with the macromolecule [27, 28]. When protein molecule interacts with IL, this hydrogen-bonded structure prevents the protein from unfolding [21]. The nucleophilicity of ILs is also determined by its hydrogen bond forming capacity. The greater the hydrogen bond capacity, the greater is the basicity and hence, the stronger is the nucleophilicity [29, 30].

The hydrogen bond basicity and nucleophilicity of anions also affect the enzyme activity and stability in IL-based solvent media [31–33]. Anions having lower hydrogen bond basicity and nucleophilicity are found to be enzyme-compatible, because low hydrogen bond basicity and nucleophilicity minimize their interference with the internal hydrogen bonds and interaction with the positively charged sites in the enzyme and hence reduce the tendency to change the enzyme's conformation [31–35]. For example, ILs containing BF_4^-, PF_6^-, and Tf_2N^- anions are usually enzyme compatible [36–38] while those containing anions such as NO_3^-, $CH_3CO_2^-$, $CF_3CO_2^-$, and $CF_3SO_3^-$ [39] are not. It has been found that [bmim][Cl] decreases the thermal stability of green fluorescent protein (GFP) as compared to aqueous solution [40] and induces the inactivation of cellulase (from *Trichoderma reesei*) [41]. [emim][Cl] decreases the thermal stability and activity of lysozyme as compared to [emim][BF_4] and [emim][Tf_2N]. All these effects are observed because of the larger H-bond basicity of Cl ion [42]. Similarly, [omim][Cl] decreases the activity of lipase in [omim][Tf_2N] [43], [bmim][Cl] and [hmim][Cl] reduces both enzymatic stabilities and activities of α-amylase from *B. amyloliquefaciens*, *B. licheniformis* [44], [chol][Cl] and [bmim][Br] having destabilizing effect on the model protein RNase A [45]. Low activity of CaLB in [bmim][lactate] IL was found, because of change in secondary structure of protein via H-bonding interaction between lactate anions and peptide chains [46]. In another study, the following order of stability were obtained for CaLB in different anion containing ILs: [hmim][PF_6]>[hmim][Tf_2N]>[hmim][BF_4], and [bmim][PF_6]>[bmim][dca]. The stability of Penicillin G acylase was in a similar order [bmim][Tf_2N]>[bmim][PF_6]>[bmim][BF_4] [47]. Decreasing stability was, in general, consistent with the increasing order of nucleophilicity ($PF_6^- < BF_4^- < Tf_2N^- <$ dca$^-$). It was observed that [bmim][dca] is an enzyme denaturing IL because of high H-bond basicity of the anion [48–50].

There are so many reports which show that higher nucleophilicity of anions decrease the stability of enzyme [51–53]. However, in some other studies, contradictory results were obtained which may be due to the influence of other factors that have an obvious effect on enzymatic reaction in IL-based medium [54, 55].

Hydrophilicity and Hydrophobicity

The hydrophobicity of ILs can be determined by log *P*, which is the logarithm of the partition coefficient of IL in an octanol/water mixture [54, 56]. There are several reports where this scale is used to determine the activity of enzymes in IL. In most of the cases (with some exceptions [44, 57, 58]), it was found that enzymes are more stable in hydrophobic ILs than in hydrophilic ILs as hydrophobic IL do not remove the essential water from enzyme [59, 60] and also, the enzyme remains in suspended form rather than in dissolved form (inactive) in hydrophobic IL [35, 61].

Kaar et al. [39] observed that free lipase (*Candida rugosa*) was active only in hydrophobic IL [bmim][PF_6], but inactive in other hydrophilic ILs such as [bmim][CF_3COO], [bmim][CH_3COO], and [bmim][NO_3]. Similarly, Nara et al. [62] observed higher transesterification activity of lipase in hydrophobic IL [bmim][PF_6] than in hydrophilic [bmim][BF_4] IL. Similar effects were observed for CaLB catalysed reaction, where enzyme

has lower activity in water-miscible IL (such as, BF_4^-, dca^-, NO_3^-, CH_3COO^-) than in water-immiscible IL (PF_6^-, Tf_2N^-) and the activity increase with increase in cation hydrophobicity ($emim^+ < bmim^+ < hmim^+ < omim^+$) [63].

Paljevac et al. [64] reported that cellulose activity decreases with IL hydrophobicity with trend [bmim][PF_6]>[bmim][BF_4]>[bmim][Cl]. In another study, higher enantioselectivity for amino lipase has been observed in hydrophobic IL [omim][PF_6] than in hydrophilic IL [bmim][BF_4] and [bmim][Cl] [65]. Hernandez-Fernandez et al. [47] reported that the stability of CaLB and Penicillin G Acylase (PGA) increases with hydophobicity of IL (trend for CaLB is, [hmim][PF_6]>[hmim][Tf_2N]>[hmim][BF_4], [bmim][PF_6]>[bmim][dca], [omim][PF_6]>[hmim][PF_6]>[bmim][PF_6], while for PGA is, [bmim][Tf_2N]>[bmim][PF_6]>[bmim][BF_4]). PEG-modified lipase [66], *Penicillin* acylase [67] and PGA [68] showed high activity and stability in hydrophobic IL than in hydrophilic IL. Novozym 435 was more thermally stable in hydrophobic IL with an order [bmim][Tf_2N]>[bmim][PF_6]>[bmim][TfO]>[bmim][BF_4]>[bmim][SbF_6] [55]. These examples show that increasing hydrophobicity of IL increases the stability, activity and enantioselectivity of the enzyme. However, it is also observed that a large amount of increase in hydrophobicity of IL leads to a decrease in the rate of reaction by thermodynamic ground state stabilization of the substrate [69, 70].

Effect of Cations and Anions

The stabilizing and destabilizing effect of ILs on enzymes as well as proteins were studied by many researchers [71–76]. Generally, cations and anions of ILs play an equal role in stabilizing the proteins [77]. The Hofmeister ion series influences the interaction of cations and anions of ILs with proteins [71, 78, 79]. These interactions depend on the kosmotropic and chaotropic properties of ions present in ILs. Kosmotropes are considered as 'structure-makers' because they increase the protein stability in solution. On the other hand, chaotropes are the 'structure-breakers' and decrease the stability of proteins in aqueous medium [71, 72]. A combination of chaotropic (weakly hydrated) cations and kosmotropic (strongly hydrated) anions is the best solution to study the enzyme–ILs interaction [56]. The effect of anions and cations on protein stability is shown in Fig. 1 [72, 80–82].

Fujita et al. [83] observed that the structural activity of cytochrome *c* was maintained by dissolving in hydrated [choline][H_2PO_4] IL, which is a combination of a chaotropic cation and a kosmotropic anion. Similar results were observed by Zhao et al. [37] in their study. Constantinescu et al. [45, 84] reported that both cation and anion follow the Hofmeister series of ILs ions [85]. In another study, Lai et al. [86] observed that activity and stability of PEL and mushroom tyrosinase in aqueous solution of 14 different ILs follows the Homeister series. In

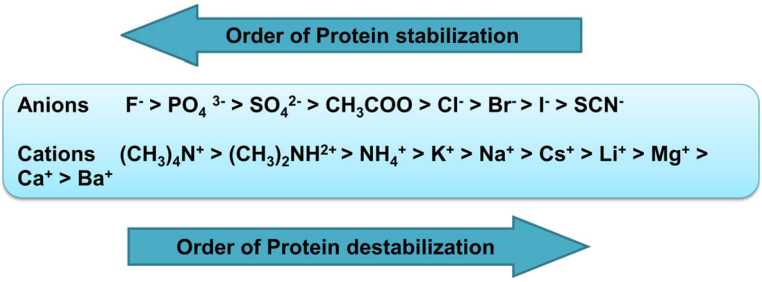


Fig. 1 The Hofmeister series as an order of the ion effect on protein stability

presence of ILs containing same anion, PEL activity decreased in order of [mmim][$MeSO_4$]>[emim][$MeSO_4$]>[bmim][$MeSO_4$], [NMe_4][ac]>[NBu_4][ac], [$NHMe_3$][$MeSO_3$]>[NBu_4][$MeSO_3$], and [$NHMe_3$][dhp]>[$NHEt_3$][dhp]>[$NHBu_3$][dhp], while for the ILs containing same cation, the order was [chol][ac]>[chol][$MeSO_3$]>[chol][NO_3], [NBu_4][ac]>[NBu_4][$MeSO_3$]. Virtually similar results were obtained for mushroom tyrosinase activity. In presence of the ILs containing same anion, the tyrosinase stability and the initial rate of reaction show a decreasing order of ILs [mmim][$MeSO_4$]>[emim][$MeSO_4$]>[bmim][$MeSO_4$], [NMe_4][ac]>[NBu_4][ac], [$NHMe_3$][$MeSO_3$]>[NBu_4][$MeSO_3$], and [$NHMe_3$][dhp]>[$NHEt_3$][dhp]. Rodrigues et al. [87] studied the effects of different ILs on the stability of lysozyme, and arranged anions according to their stabilizing effect in the following order: Tf_2N^-<hexonate<TMA^-≈OTf^-<propionate<Cl^-<lactate≈dmp^-<dhp^-. It was reported that [TMA][TfO] stabilized laccase while [bmim][TfO] and [bmpyr][TfO] destabilized laccase. This effect can be associated with the chaotropicity of the cations in Hofmeister series [88].

It was found that anions are highly polarizable and hydrated more strongly than cations [89, 90]. Hence, anions have more dominating effect and are more efficient than cations. Sedlak et al. [74] found that when a protein molecule dissolved in an aqueous solution, it has many charged groups on its surface which are responsible for interactions with the ions in solution. These charged groups were found to interact strongly with the chaotropic anions (such as SCN^-) and kosmotropic cations (such as Na^+, K^+). For example, β-glycosidase have very low activity in aqueous solutions of [bmim][BF_4], because of chaotropic nature of its anion (BF_4^-) in solutions[91, 92]. However, anion Kosmotropicity is not a single factor for controlling enzyme performance in ILs based solvent media, because behaviors of enzymes in IL-containing aqueous solution may be somewhat different from that in pure IL or ILs with trace amount of water [31, 93]. Though, it is not quite clear how IL hydrophobicity influences the kosmotropicity. The salt of PF_6^- (chaotropic anion) in combination with Na^+ or K^+ denatures enzymes in aqueous solution. Conversely, PF_6^--based ILs are hydrophobic in nature, and also known to be typically enzyme stabilizing solvents. Therefore, the Hofmeister effect (anion kosmotropicity) may not be suitable for explaining the enzymes behavior in these hydrophobic ILs or their mixtures with water [94].

Effect of Alkyl Chain Length in the Cations

The alkyl chain lengths of cation in ILs have a dramatic effect on stability and activity of enzyme. It was observed in several reports that ILs with long alkyl chains have a destabilizing effect on enzyme [95, 96]. For example, ILs with long decyl chain on imidazolium cation have more destabilizing effect on the BSA than short chain ILs [97]. Likewise, [bmim][Cl] and [hmim][Cl] having long alkyl chain reduce both enzymatic stabilities and activities of α-amylase (from *B. amyloliquefaciens* and *B. licheniformis*) [44]. Ajloo et al. [98] studied the effect of 1-allyl 3-methyl-imidazolium and 1-octhyl 3-methyl-imidazolium chlorides, on the structure and activity of adenosine deaminase and he found that the later one, due to its more hydrophobic nature exhibited more destabilizing effect than former one. Imidazolium and phosphonium cation based hydrophobic ILs, ([Bmim][Cl], [Bmim][BF4] and TBPBr) having longer alkyl chains, were found to be weak stabilizers for α-chymotrypsin, than that of small chain ILs (TEAAc and TEAP) [58]. Similarly, an increase in the alkyl chain length of *N*-alkyl pyridinium chloride and *N*-alkyl-*N*-methylpyrrolidinium chlorides caused destabilization of lysozyme [57]. It was observed that ILs having hydroxyl ethyl chain in their cations provided high activity and stability to CaLB [99]. In other study, it was found that *N*-ethyl-*N*-methylimidazolium cation-based ILs having strongly destabilizing anion with longer alkyl substitution like hexyl sulfate were unable to promote refolding while those having anion with

short alkyl chain like di-Me, and di-Et, were able to promote refolding [11]. After study the influence of a series of *N*-alkyl, and *N*-(ω-hydroxyalkyl)-*N*-methylimidazolium chlorides on hen egg white lysozyme and an antibody fragment ScFvOx, it was observed that more hydrophobic imidazolium cations carrying long alkyl chain showed more destabilizing effect, while terminal hydroxylation of alkyl chain made them more compatible with protein stability [100]. Recently, Attri and Venkatesu [101] studied the thermal stability of α-chymotrypsin and observed that more hydrophobic ammonium cation carrying longer alkyl chains of ILs ($[DEA]^+$ or $[TEA]^+$) were found to be weak stabilizers for α-chymotrypsin, while small alkyl chain cation of trimethyl ammonium ILs were found to be strong stabilizers and therefore more biocompatible for α-chymotrypsin structure. However, there are some reports, where the stability of enzyme decreases with decrease in alkyl chain length of cation. For, stability and enantioselectivity of enzymes in imidazolium based ILs were found to decrease with decrease in alkyl chain length of imidazolium cations from $[omim]^+$ to $[bmim]^+$ [102]. These contradictory results were obtained presumably due to some other factors controlling the enzymatic activity and stability in IL-based media.

Proteins Stability and Activity in Ionic Liquids

Proteins consist of a particularly heterogeneous class of biological macromolecules. Out of their native environments they are frequently unstable. If certain buffer conditions are not maintained, extracted proteins may not function properly. Also, for their unusual structural integrity and activity, purified proteins require being stored for an extended period of time. The degree of storage 'shelf life' can fluctuate from a few days to more than a year and is dependent on the nature of the protein and the storage environment used. In recent years, ILs has become environmentally benign and a media for carrying out advanced biological reactions because of their tunable property which permits the selection of an IL of choice, simply by altering the cation and anion of ILs. An immense amount of work has been done by researchers in order to study the ability of ILs to solubilize and stabilize proteins in vitro for an extended period of time [19, 20, 83]. Broad applications of ILs in protein research are represented in Fig. 2.

Proteins Stability

ILs enhances the protein stability, thus maintaining their three-dimensional structure. Thermal stability of lysozyme has been examined in aqueous solution of ILs consisting of $[emim]^+$ cations and other anions. It was observed that ILs maintain the structural stability and activity of enzyme by preventing irreversible aggregation of unfolded lysozyme at higher temperature [42]. It was also investigated that choline dhp provide long term stability (>18 months at room temperature) and functional activity to cytochrome *c* by maintaining its native conformation [83]. Other researchers have also emphasized the importance of ILs in maintaining the structure of proteins and their long-term stabilization. For example, dhp anion-based IL provides enhanced solubility and high thermal stability to cytochrome *c* [103]. In another case, while studying the effect of imidazolium based IL on serine protease cutinase, Micaelo and Soares [104] observed that $[bmim][PF_6]$ provided significantly high thermal stability to the enzyme at high temperature (343 K). Byrne et al. [105] reported that ethylammonium nitrate (EAN) ILs increased the thermal stability of lysozyme and hence provided long-term stabilization, against aggregation and hydrolysis of lysozyme during repeated thermal unfolding/refolding cycles. Table 1 presents some examples of the effects of ILs on the proteins' stability.

Fig. 2 Applications of ILs in protein research

Though lot of work has been published in regard to the fact that ILs increase the protein stability in many respects (thermal and long term stability), an exact explanation about the fact by which ILs increase protein stability is still not clear.

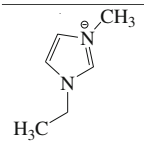
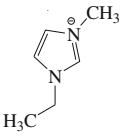
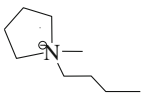
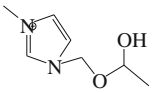
Protein Solubility

High solvating capacity of ILs enables them to solubilize the protein. Upon solubilization, aggregation is prevented which leads to increased stability and activity of the protein. However, only few reports are available in this regard. Baker et al. [20] and Heller et al. [40] found that [bmpy][Tf_2N] IL possessed the ability to dissolve the monellin protein but its ability was found to be limited to dissolve a small amount of this protein. In another study, it was found that dhp anion based ILs showed the ability to solubilize a considerable amount of cyt *c* and surprisingly, the protein was found to retain its structure and functional activity in the solution up to much higher temperature than in aqueous solution [103]. There are some other cases where ether structures have been used to stimulate protein solubilization in hydrophobic ILs [106, 107]. These characteristics of ILs have helped in preparing the liquid formulations of therapeutic proteins which is one of the major problems in the use of protein-based therapeutic drugs. These biocompatible ILs solvate and stabilize the therapeutic proteins which has opened a new vista in pharmaceutical industry [7].

Protein Crystallization

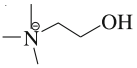
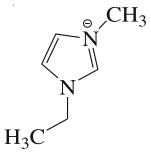
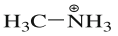
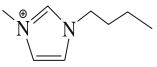
ILs have been used in protein crystallization in order to overcome the problems of slow speed and low yields as well as reproducibility. The first experimental work in protein crystallization with IL was done by Garlitz et al. [108], who used EAN as an additive in lysozyme

Table 1 Some examples showing the effect of ILs on proteins

S.No.	Protein	Ionic liquid used	Structure of ILs		Inference	Reference
			Cation	Anion		
1.	Lysozyme	1-ethyl-3-methylimidazolium tetrafluoroborate		BF_4^-	After heat treatment of lysozyme, remaining activity was 88%	[42]
		1-ethyl-3-methylimidazolium trifluoromethanesulfonate		$F_3CSO_3^-$	Remaining activity was 68%	
2.	Bovine Serum Albumin (BSA)	1-tetradecyl-3-methylimidazolium bromide		Br^-	Below CMC, C_{14}mimBr stabilizes protein. Above CMC, protein is destabilized.	[170]
3.	Monellin	1-butyl-1-methylpyrrolidinium bis(trifluoromethane sulfonyl)imide		$(CF_3SO_2)_2N^-$	Extreme stability against thermal inactivation	[20]
4.	Serum albumin (Bovine and Human)	1-ethoxyethanol-3-methylimidazolium chloride		Cl^-	Enhanced stability of protein in IL	[177]

crystallization. It was observed that the enzyme crystallized easily, maintained its activity and have increased stability in IL containing media than in pure aqueous media. Similarly, a large-sized crystal with reduced crystal polymorphism was obtained when water-soluble, halogen-free, alkylammonium-based ILs was used for lysozyme crystallization [109]. Pusey et al. [110] used imidazolium and pyrollidinium-based ILs as an additive for improving the crystalization of proteins (canavalin, lactoglobulin B, xylanase and glucose isomerase). It was found that these ILs were unable to induce the crystallization because they had a solubilizing effect on these proteins. A similar experiment was performed by Judge et al. [111] for some model

Table 1 (continued)

S.No.	Protein	Ionic liquid used	Structure of ILs		Inference	Reference
5.	Cytochrome c	Choline dihydrogen phosphate (choline dhp)		$H_2PO_4^-$	Cyt *c* was active after 18 months of storage in choline dhp	[83]
6.	Lysozyme, Catalase, Myoglobin, Trypsin, Glucose isomerase, and Xylanase	1-ethyl-3-methylimidazolium tetrafluoroborate		BF_4^-	Improvement in crystal size and morphology	[111]
7.	Cytochrome *c*	Methylammonium formate		$HCOO^-$	Protein separation by reverse phase liquid chromatography	[140]
8.	Human body Fluids	1-butyl-3-methylimidazolium chloride		Cl^-	Direct protein extraction using IL/aqueous two-phase system	[126]

proteins (lysozyme, catalase, myoglobin, trypsin, xylanase and glucose isomerase), and they observed that crystal size significantly improved only for lysozyme and trypsin. In another experimental work involving lysozyme crystallization, effect of ILs on thermal stability and morphology of lysozyme crystals was determined [112]. It has been recently reported that the addition of [bmim][Cl] IL improved the reproducibility of crystal growth of *Cupriavidus necator*, NapAB (a nitrate reductase enzyme) [113]. Kennedy et al. [114] found that protic ILs have a significant effect on crystallization of trypsin, lysozyme, and glucose isomerase. They enhanced the size, quality of crystals and reproducibility of crystallization process. Currently, 1,3-butylimidazolium chloride (BBimCl) was proved to be able to improve the crystallization of lysozyme. Also, it was observed that BBimCl facilitate the direct crystallization of lysozyme from a natural sample matrix (egg white) by increasing the tolerance to the coexistence of impurities in the crystallization medium [115].

Protein Extraction

ILs form biphasic system with water because of their immiscibility in water. They have the ability to dissolve a wide variety of materials including salts, fats, proteins, amino acids, surfactants, sugars and polysaccharides and a wide range of organic molecules, including crude oil, inks, plastics, and even DNA. These two properties make them suitable for liquid–liquid extraction [116–125]. An IL/aqueous two-phase system consisting of hydrophilic [mim][Cl] IL and K_2HPO_4 was used for the first time for the extraction of proteins from human body fluids. FTIR and UV–vis spectroscopy showed that there was no interaction between ILs and proteins and no alteration in the protein structure [126]. Similar work was done in extraction of protein from yeast cells by using ILs. High-resolution techniques such as SDS-PAGE and 2-DE demonstrated that there was no chemical alteration in protein structure during the extraction process. The results obtained from western blotting of the extracted protein showed that protein retained all its functional activities (Fig. 3) [127]. In another study, TOMAC IL was used for extraction of penicillin G [128], [btmsim][PF_6] IL for extraction of hemoglobin [129], [bmim][Tf_2N] IL for extraction of lactoferrin [130] and an IL/aqueous two-phase system was used for extraction of two different alcohol dehydrogenase enzymes and it was found that IL stabilizes both the enzymes and substrates and also increase rate of reaction and product yield [131]. Two methyl imidazolium-based ILs having alkylsulfate and alkylsulfonate anions have been recently used for extraction of *Candida antarctica* lipase A (CaLA) from aqueous solution. It was shown that extraction with [emim][C_4SO_4] with ammonium sulfate gave 99 % enzyme recovery[132]. It was observed that the IL hybrid were also acts as selective and biocompatible material for isolation of the proteins. The *N*-methylimidazole–polyvinyl chloride (PVC) hybrid was found to provide the efficient adsorption of basic proteins, i.e., lysozyme, cytochrome *c* and hemoglobin, with adsorption efficiencies of 97 %, 98 % and 94 % while negligible adsorption of acidic proteins, i.e., bovine serum albumin (BSA), transferring (Trf) and immunoglobulin G (IgG) [133, 134]. Similarly, *N*,*N*-bis [2-methylbutyl] imidazolium hexafluorophosphate [PPim][PF_6] IL templated porous nano-TiO_2 particles provide selective adsorption and isolation of cyt-c with an adsorption efficiency

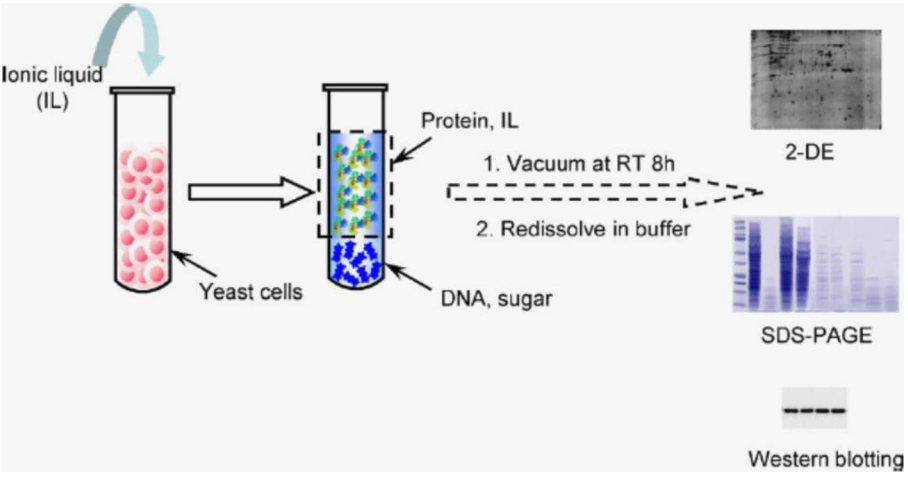


Fig. 3 Schematic representation of protein extraction from yeast cell by using ionic liquid. Reprinted from ref. [139] with permission from Elsevier

of 87 % in comparison to 30 % obtained by the non-templated nano-TiO_2 [135]. Likewise, $PPimPF_6/TiO_2$ and polymeric IL-SiO_2 nanocomposites were found to provide a promising potential for the selective adsorption of neutral proteins (e.g., hemoglobin) and acidic proteins (e.g., ovalbumin), respectively. Also, it was found that they exhibit favorable biocompatibility for protein during the adsorption and desorption process [136, 137]. Chen et al. [138], in their review, also explained the role of ILs in protein extraction and isolation/purification

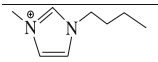
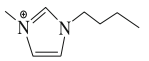
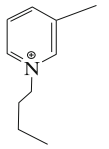
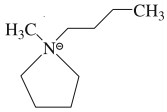
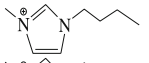
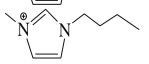
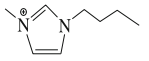
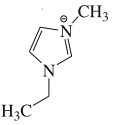
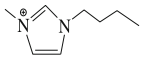
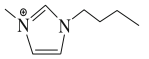
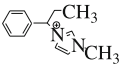
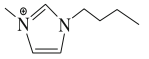
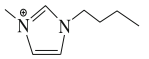
Protein Separation

ILs plays an important role in protein separation, since they have miscellaneous character and designable property. IL-assisted SDS-PAGE [139] is used for peptides or proteins separation and have the potential to improve protein separation and its efficiency. Alkyl ammonium formate (AAF), for example methyl ammonium formate (MAF) and ethyl ammonium formate (EAF) ILs has the potential to be used as mobile phase for the separation of proteins (cytochrome *c*) in their native form by reverse-phase liquid chromatography (RPLC) [140]. Other than IL-assisted SDS-PAGE and mobile phase in RPLC, they also used either as a functional group fixed on stationary phase of HPLC [141] or as running buffer additives in capillary electrophoresis for the protein separation. Methylimidazolium based ILs used as non-covalent coating agents and additives of the electrolyte solutions (BGE) for electrophoretic separation of basic proteins were found to provide fast and efficient separation of model basic protein mixture in co-electroosmotic capillary electrophoresis [142]. 1-Butyl-3-methylimidazolium tetrafluoroborate IL was used as background electrolyte for the separation and determination of amino acids [143] and as coating material for separation of basic and acidic proteins such as lysozyme, cytochrome c, RNase A, albumin, and α-lactalbumin in capillary electrophoresis. It was found that they provide fast, efficient and reproducible separation by reversing the surface charges on the capillary inner surface and preventing the adsorption of positively charged proteins onto the silica surface, as well as associated with proteins [144–146].

Biocatalysis

ILs can be used as an alternative reaction media for biocatalysis and biotransformation [147–149]. Many researchers demonstrate that ILs play an important role in increasing the activity, stability and enantioselectivity of enzymes [35, 149–152]. Moreover, ILs provides better recoverability and recyclability, when used as a reaction media or coating material for enzymes [34, 46, 153–157]. For example, [emim][Tf_2N] and [btma][Tf_2N] ILs were found to provide much higher activity and stability to the CaLB and α-chymotrypsin enzymes as compared to water and hexane [19, 21]. The observed activity and stability seem to be associated with both the maintenance of the 50 % of initial α-helix content and the enhancement of β-strands of the enzyme at 50°C, resulting in a more compact enzyme conformation able to exhibit catalytic activity [19]. Some researchers have established that lipases such as CaLB [158–161] and *Pseudomonas cepacia* lipase (PcL) [160, 162] are catalytically active in imidazolium and pyridinium based ILs, having anions such as BF_4^-, PF_6^- and Tf_2N^- [159]. The effect of ILs on enzymes activity are summarised in Table 2. Enantioselective esterification of (±)-menthol by *Candida rugosa* lipase (CRL) in [bmim][PF_6] and [bmim][BF_4] has been observed [163]. ILs are effective in increasing enzyme stability, selectivity and high enzymatic efficiency. For example, [bmim][BF_4] provide high selectivity and efficiency to hesperidinase when used as co-solvent in hesperidinase catalysed rutin biotransformation reaction. An increase in the product yield (1.67-fold and 2.33-fold) and reduction in reaction

Table 2 Some examples showing the effect of ILs on enzymes

S.No.	Enzyme	IL Used	Structure of IL		Inference	Reference
			Cation	Anion		
1.	*Candida rugosa* enzyme	1-butyl-3-methylimidazolium hexafluorophosphate		PF_6^-	Longer half-life, (12.3 h) and as compared to organic solvent	[46]
		1-octyl-3-methylimidazolium hexafluorophosphate		PF_6^-	Longer half-life (10.6 h)	
2.	Horseradish Peroxidase (HRP)	1-butyl-3-methylimidazolium tetrafluoroborate		BF_4^-	Improved thermal stability	[178]
3.	Glucose Oxidase	1-butyl-3-methylpyridinium tetrafluoroborate		BF_4^-	Decrease in electrocatalytic activity of glucose oxidase	[152]
		1-butyl-1-methylpyrrolidinium tetrafluoroborate		BF_4^-		
		1-butyl-3-methylimidazolium tetrafluoroborate		BF_4^-		
4.	*Candida antarctica* lipase B (CaLB)	1-butyl-3-methylimidazolium hexafluorophosphate		PF_6^-	Higher rate of transesterification	[163]
5.	β-galactosidase from *Bacillus circulans*	1-butyl-3-methylimidazolium methylsulfate		$MeSO_3^-$	Yield was doubled and enzyme could be reused several times	[91]
6.	Trypsin	1-ethyl-3-methylimidazolium Bromide		Br^-	Improved activity of trypsin (~30-300%) in reverse micelles of CTAB	[172]
7.	Lipase from *Candida rugosa, Thermomyces lanuginosa*	1-butyl-3-methylimidazolium hexafluorophosphate		PF_6^-	Higher catalytic performance and operational stability	[179]
8.	Thermolysin	1-butyl-3-methylimidazolium hexafluorophosphate		PF_6^-	Synthesis of Z-aspartame at 40% turnover rate in ethyl acetate	[61]
9.	*Pseudomonas cepacia* lipase (PcL)	1-(3'-phenylpropyl)-3-methylimidazolium Hexafluorophosphate		PF_6^-	Transesterification	[180]
10.	Esterase from *Geobacillus stearothermophilus*	1-butyl-3-methylimidazolium hexafluorophosphate		PF_6^-	Increase in stability and half-life of esterase.	[10]
		1-butyl-3-methylimidazolium Tetrafluoroborate		BF_4^-		

time (0.33-fold) was observed in the presence of [bmim][BF_4] IL [164]. Alkanol group containing ILs, namely, [C_2OHmim][BF_4] and [C_2OHmim][TfO], and the choline amino acid ILs [Ch][Arg] and [Ch][Pro], were found to acts as good co-solvent for the asymmetric hydrolysis of styrene oxide to (*R*)-1-phenyl-1,2-ethanediol catalyzed by mung bean epoxide hydrolases in *n*-hexane/buffer biphasic system. ILs increase the thermal stability of enzymes and provide greater reaction rate with shorter reaction time at higher substrate concentration [165]. Ammonium-based ILs, DEAP and TEAP act as refolding additives for thermally denatured S Con A and stabilize its native structure [166]. Figure 4 shows the refolding of thermally denatured S Con A.

Conformational Studies of Proteins in Ionic Liquids

The interactions of imidazolium ILs, [bbim][Cl], [bmim[[Cl] and [bmim][NO_3] with BSA were studied by using UV–vis, fluorescence and circular dichroism (CD) spectroscopic techniques. The fluorescence quenching was investigated at excitation wavelength (λ_{ex}) 230 nm, and it was observed that the side chains of imidazolium ring have no effect on the interaction between ILs and BSA but the anionic groups play an important role in affecting these interactions [167]. Subsequently, the results from UV–vis and ATR-FTIR spectroscopic techniques showed that cytochrome *c* was found to be more active and stable in choline dhp, even after 18 months of storage at room temperature [83, 103]. Ge et al. [127] used UV–vis and FTIR spectroscopic techniques in order to confirm the use of ILs in purification, separation and quantification of proteins. Concentration-dependent effect of ILs on proteins was observed in BSA quantification experiments through UV–vis spectroscopy which led to biased protein quantification [168]. In order to investigate the structural changes of BSA induced by [C_{14}mim][Br], isothermal titration microcalorimetry was used [169]. A change in enthalpy was noticed during interaction process between [C_{14}mim][Br] and BSA which showed alteration in BSA structure. The fluorescence quenching of BSA in the IL [C_{14}mim][Br] at

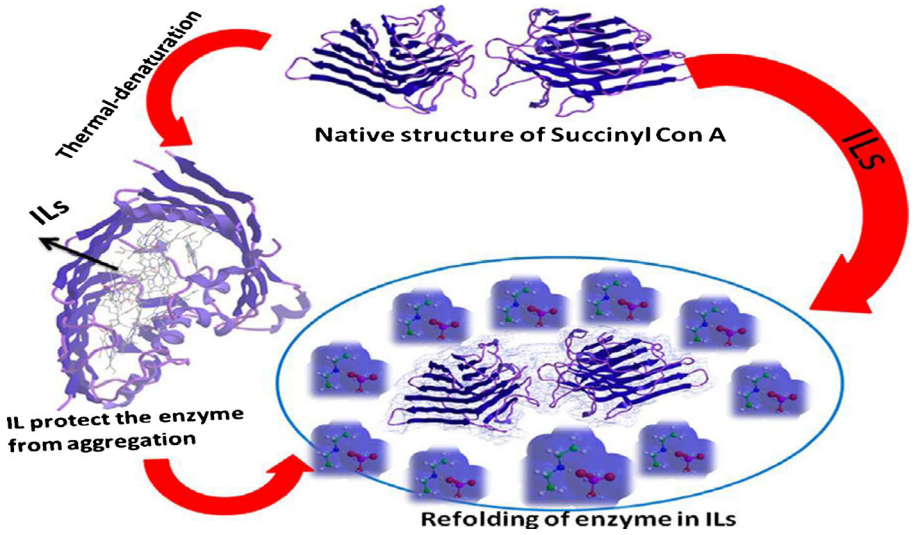


Fig. 4 Schematic depiction of folding and refolding of thermally denatured S Con A in the presence of ILs. Reprinted from ref. [169] with permission from Elsevier

λ_{ex} 280 nm was investigated and the result showed that at low concentrations, [C_{14}mim][Br] stabilizes BSA, but at higher concentration BSA gets denatured [170]. Similarly, CD and fluorescence spectroscopic techniques were used to correlate the changes in the secondary structure of CaLB, through interactions with two water-immiscible ILs, [btma][NTf_2] and [emim][NTf_2] [19]. The effect of water-soluble imidazolium-bromide IL as an activator for trypsin in reverse micelles of CTAB was investigated by fluorescence, far-UV CD and dynamic light scattering (DLS) techniques. A correlation between the trypsin activity and the size of the aggregates was found in the presence of IL [171]. Baker et al. [20] studied the conformational behaviour and thermal stability of monellin protein in [bmpy][Tf_2N] using

Table 3 Techniques used to study the consequences of ILs on protein confirmation

Technique	Property	Observations	References
UV–vis	Protein native and unfolded state; complex formation between protein and IL	Change in polypeptide backbone structure and changes in degree of immobilization of aromatic amino acid residues	[58, 167]
Far-UV CD	Protein secondary structure	Changes in polypeptide backbone structure	[181, 182]
FT-IR	Protein secondary structure	Amide I band analysis (change in backbone conformation and the hydrogen bonding pattern)	[107, 183–185]
Raman spectroscopy	Protein secondary structure	Change in position of Amide I and III band of protein on binding of IL	[186–188]
	Protein tertiary structure	Skeletal bending and C–C–N stretching frequencies of the peptide backbone, and S–S and the C–S stretching frequencies of the disulphide bonds	
Near-UV CD	Protein tertiary structure	Changes in degree of immobilization of aromatic amino acid residues	[189, 190]
Trp fluorescence	Protein tertiary structure and Mechanism of complex formation between protein and IL	Changes in Trp microenvironment due to changes in protein conformation or binding of IL	[166, 182, 191]
DLS	Size and structure of protein aggregate in Ionic liquids	Change in hydrodynamic radii of protein as a result of protein–IL interaction	[192]
SANS	Shape, Size and structure of protein aggregate in IL	Use deuterated IL or deuterated proteins to get information about either component. Major contrast between solvent D_2O and micellar hydrocarbon core. Requires access to neutron source, e.g., Swiss Pallation Source	[18, 169]
Tensiometry	Stoichiometry of binding	Change in surface tension on adding IL (determine CAC and CMC in order to determine protein–IL interaction)	[193, 194]
Microcalorimetry	Stoichiometry of binding	Binding of IL to proteins is associated with enthalpy change which is exothermic for electrostatic interactions and endothermic for unfolding	[57, 170, 195]
NMR	Protein conformation in presence of IL	Spectral variation	[166]

fluorescence spectroscopy and the protein was found to be extremely stable. The structural stability of cytochrome *c* in AAF ILs such as MAF and EAF were investigated by CD spectroscopy. It was demonstrated that AAF ILs have the potential to be used as mobile phase for the separation of proteins in their native form by RPLC [140].

A combination of standard continuous wave (CW) electron paramagnetic resonance (EPR) spectroscopy and nanoscale distance measurements through double electron–electron resonance spectroscopy (DEER) was used to characterize the conformational changes in human serum albumin (HSA) induced by imidazolium based IL [emim][BF_4] in buffer solution [172]. Furthermore, IR spectroscopy was used for the conformational study of lipases in water/IL microemulsions in order to demonstrate the efficiently of water/IL as reaction media for various biocatalytic reactions [145, 171]. Sate et al. [18] studied the activity of CaLB in three ILs, [emim][$EtOSO_3$], [emim][NO_3] and [emim][$N(CN)_2$], through DLS and small angle neutron scattering (SANS) techniques. A correlation between the activity of enzyme and nature of their interaction with IL was observed, and it was found that aggregation is associated with the loss of enzymatic activity. Subsequently, the denaturation effect of water-miscible [bmim][Cl] on cytochrome *c* and HSA was studied by using SANS spectroscopic technique [173]. The interaction of gelatin with room temperature ionic liquids (RTILs), [omim][Cl] and [bmim][C_8OSO_3] was investigated through electrical conductivity and tensiometric analysis. The results indicated that, [bmim][C_8OSO_3] micellize at lower concentration as compared to [omim][Cl] and is therefore more effective in populating the interface as compared to [omim][Cl] in the presence of gelatin [174]. Similarly, the interaction of [C_{14}mim][Br] IL with BSA was studied through electrical conductivity and tensiometric analysis. The value of CMC was measured and it was found that [C_{14}mim][Br] binds to BSA through electrostatic interaction at low concentrations (below CMC) and hydrophobic interaction at high concentrations (above CMC) [169, 170]. Differential scanning microcalorimeter (DSC) was used to evaluate the structural stability of lysozyme stored in hydrated choline dhp [7] and EAN [175]. On the basis of thermodynamic parameters (the unfolding temperature and the enthalpy of unfolding), it was found that both hydrated choline dhp and 5 % EAN have the potential of stabilizing the lysozyme. By using Raman spectroscopy, Mangialardo et al. [176] found that out of four ammonium-based ILs used in the study, EAN was the most promising additive for protein refolding (lysozyme). Techniques that have been commonly used to study the effect of IL on protein conformation are summarized in Table 3.

Conclusion

ILs has both stabilizing and destabilizing effect on protein, depending on its physico-chemical properties. Less viscous, hydrophobic ILs containing chaotropic cations and kosmotropic anions stabilize the protein optimally up to a wide range of temperature and for an extended period of time. However, the thermal stability of proteins depends on the correct choice of ILs. ILs enhances the stability and activity of proteins/enzymes by solubilizing them (preventing their self-aggregation). Based upon requirement ILs can be chosen and tailored to serve as a noble solvent for enzymatic reactions, protein preservation, and other protein based applications. Various methods used for studying the protein–IL interaction provide information about structural and conformational dynamics of proteins which in turn may be helpful for understanding the effects of ILs on the stability and activity of proteins.

Conflict of interests The authors declare no competing financial interests.

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