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MINI-REVIEW

Study of Protein Structures under the Influence of Imidazolium Based Ionic Liquids

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Ionic liquids are nowadays extremely popular in the advanced research field of many disciplines including chemistry, chemical engineering, material science, biology and pharmaceuticals. Unique physico-chemical properties of the ionic liquids such as low vapor pressure, stability, large liquid range, broad solubility and easy modification of structures are responsible for its vast application. Imidazolium based ionic liquids are one of the most widely used ionic liquids and theses are extensively studied in the field of protein research. In this mini-review, imidazolium ionic liquid induced effect on the structure and function of protein molecules are discussed.

Keywords: Ionic liquid, Imidazolium, Protein structure, Disaggregation.

INTRODUCTION

Ionic liquid (IL) was first prepared over a century ago in the year 1914 by German chemist, Paul Walden [1]. But the ionic liquids (ILs) became widely popular among the scientific community only in the beginning of the current century and these have been extensively utilized in various hot research topic of chemistry as well as in multidisciplinary research areas including chemistry, chemical engineering, material science and biology [2-6]. Normally liquids consist of neutral molecules and various weak intermolecular attractive forces (*e.g.* hydrogen bonding, dipolar interaction, van der Waal's forces, *etc*.) operates among the molecules. On the other hand, ionic compounds have high melting point because of strong inter-ionic attraction and those are solid at room temperature. In contrast to those, there exists another type of chemicals which are ionic in nature, yet liquid at room temperature and these are called 'ionic liquids". By definition, ionic liquids are ionic compounds which melts at temperature below the boiling point of water (100 ºC) [3]. In other words, ionic liquids can be described as "low temperature molten salts". Fig. 1 represents some of the common cations and anions, which combine to form an ionic liquid. It is evident that in case of ionic liquids the cation is always an organic ion and the counterpart may be an organic or inorganic anions. Due to the large size mismatch between the bulky cation and the smaller anion, packing of lattice in these salts is not as great as in many inorganic salts and hence melting point of these salts are much lower [7]. In the growing context of "green chemistry", ionic liquids turned up as promising alternative to the traditional volatile organic solvents because of its unique physico-chemical properties such as negligible vapour pressure, non-flammability, high thermal stability, large liquid range, broad solubility, moisture and air compatibility [8]. In addition, these properties can be tuned as per requirement by modifying the constituent ions. Thus, ionic liquids emerged as a popular choice in the last decades based on its 'green' and 'designer' properties [2,4].

In chemistry, ionic liquids are vastly employed in various chemical reactions in the form of reagents, solvents or catalysts [6]. Apart from that, ionic liquids are also used in the field of analytical chemistry, electrochemistry, polymer chemistry and most importantly these are frequently reported for various biological applications [2]. Ionic liquids are extensively studied for its biocompatibility and its application in pharmaceutical chemistry, enzyme activity and protein stability [9-11].

Importance of proteins are well known and relationship of its structure with function is also well established. Each protein has unique three-dimensional quaternary/tertiary structure and

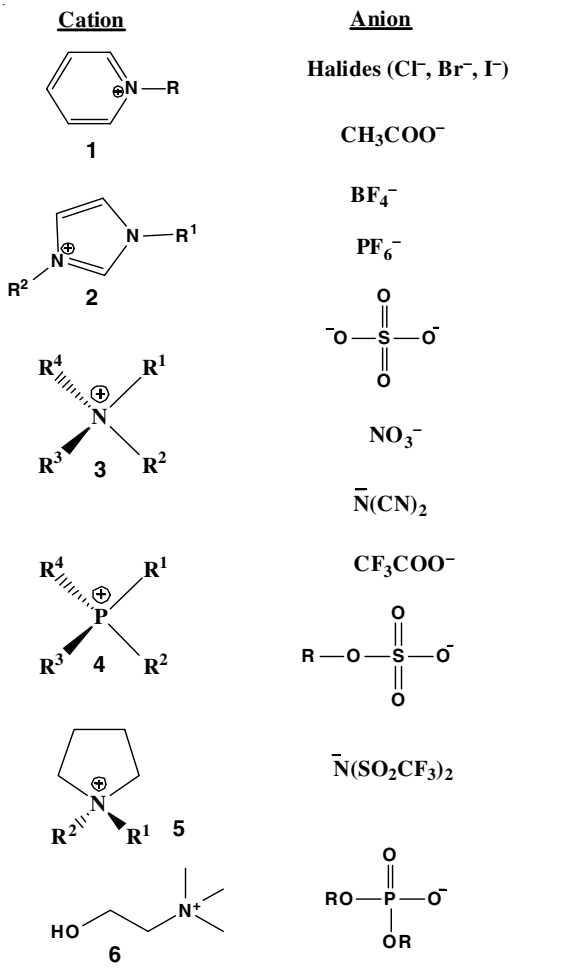

Fig. 1. Representative examples of common constituent cation and anion of ionic liquid

highly specific function of the proteins are indeed dependent on this particular structure. Any type of deformity in protein structure usually causes loss of function of the protein [12]. Now all biochemical reactions in the living systems are regulated by various protein molecules, which means they play essential role behind existence of life on the earth. Thus, stability of proteins is of utmost importance, especially in various 'stressed" conditions. Stability of a protein can be defined as the tendency of the protein to maintain its native conformation. Native proteins are marginally stable with compared to the unfolded or denatured state and the ΔG value between these two extreme states is in the range of only 20 to 65 kJ/mol [13]. Folded conformation of protein is held together by the effect of various weak interactions like hydrogen bonding, π-stacking, ionic interaction and hydrophobic effect [14,15]. Disulfide linkage formation having covalent bond, also plays crucial role in some cysteine containing proteins in maintaining the folded three dimensional structure. Stability of the proteins are very much sensitive to its environment and changes in solvent, pH, presence of chaotropic agents, heat, *etc*. cause protein structural change [13].

Nowadays, stability of protein molecules is assessed in the ionic liquid environment and number of publications describing the effect of different ionic liquids on the structure of proteins including the enzymes increases at an explosive rate [11,16-18]. In this review, we shall focus on the structural and/or functional changes by the action of a particular type of ionic liquids, which contains an imidazole ring in the cation part.

Imidazolium based ionic liquids: There exist mainly four types of organic cations as constituents of ionic liquids: (i) alkylammonium ion, (ii) alkylphosphonium ion, (iii) alkylpyridinium ion and (iv) imidazolium ion (Fig. 1). The imidazolium cations are normally dialkyl substituted (entry 2 in Fig. 1) and these are the most studied. Normally imidazolium-based ionic liquids are considered to be chemically stable, except few cases [19]. These are also synthesized by simple methods and derivatization at various position of the imidazole ring can be employed [6,21]. For example, *N*-methylimidazole are converted to suitable dialkyl derivative by reacting with corresponding haloalkanes. Imidazolium cations result in the formation of ionic liquids with large variety of counter ions, *e.g*. Cl^-, Br^-, CH_3COO^-, PF_6^-, CF_3COO^-, BF_4^-, *etc*. Few commonly used imidazolium ionic liquids are depicted in Fig. 2.

Effect of imidazolium based ionic liquid on protein structure: Different imidazolium ionic liquids were extensively utilized for study of its impact upon structures of various protein molecules. Protein structure are best described by four

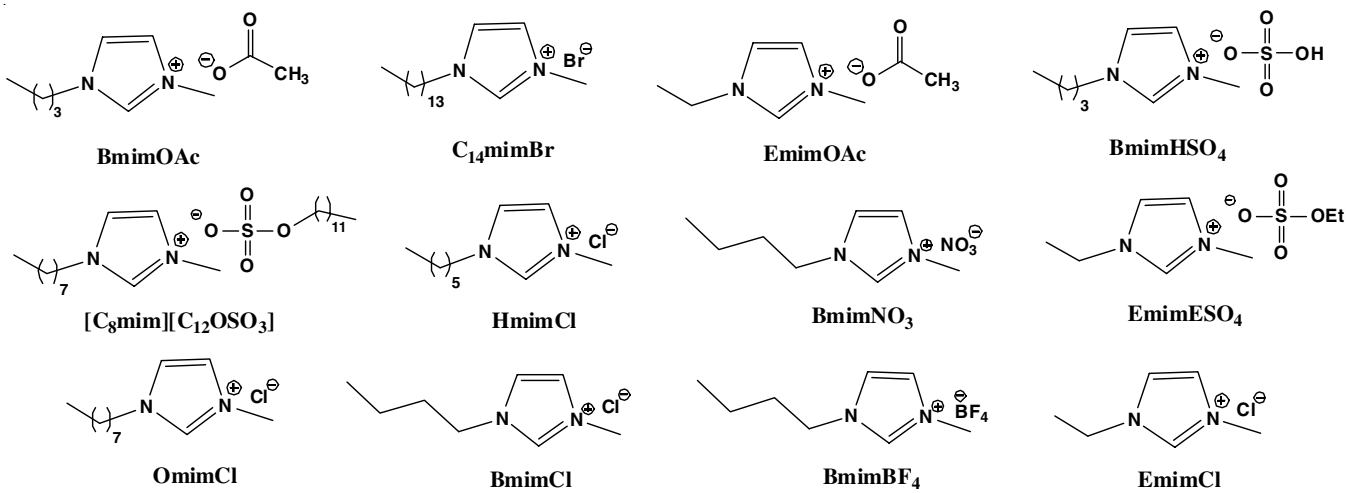

Fig. 2. Few commonly used imidazolium-based ionic liquids

hierarchy levels - primary, secondary, tertiary and quaternary. In general, primary structure of the proteins, which links the different amino acids by covalent bond, do not altered by the presence of these ionic liquids. In this review, imidazole containing ionic liquids induced fate of proteins structures are discussed and results are delineated below with the focus on generalized pattern, if any.

Change in transition temperature: Like many other denaturing factors, elevated temperature also causes disruption of the protein native structure. In this regard, melting temperature (T_m) of a particular protein is a quantitative measure of its thermal stability. Melting temperature is defined as the temperature at which half of the protein is unfolded [21]. Higher melting temperature signifies more thermal stability of the protein. Myoglobin, the oxygen binding protein in the muscle tissue, was investigated for the influence of various ionic liquids consisting of 1-butyl-3-methylimidazolium cations, $[Bmim]^+$. Results showed that the ionic liquids had negative influence on myoglobin stability [22] since the melting temperature drops from 60.3 ºC to 44 ºC (Table-1). Decrease in melting temperature varied with various counter anions and among all 0.04M BmimOAc and $BmimHSO_4$ caused maximum decrease (*i.e.* 44 ºC). Various ionic solutions with normal inorganic cation also caused lowering of T_m but the order and values were not exactly the same, which was due to the distinct structural behavior of the ionic liquids. Native helix secondary structure of myoglobin was completely disrupted with addition of the imidazolium ionic liquids.

TABLE-1
MELTING TEMPERATURE (T_m) OF DIFFERENT PROTEINS IN PRESENCE AND ABSENCE OF IMIDAZOLIUM IONIC LIQUIDS

Protein	Ionic liquid	Melting temperature (°C)
Myoglobin	–	60.3
	BmimBr (0.4 M)	47.3
	BmimCl (0.4 M)	48.3
	$BmimHSO_4$ (0.4 M)	44.0
	BmimSCN (0.4 M)	45.5
	$BmimCH_3COO$ (0.4 M)	44.0
BSA	–	70.0
	$EmimESO_4$ (0.6 M)	75.4
	EmimCl (0.6 M)	73.3
	BmimCl (0.6 M)	63.1
Lysozyme	–	74.0
	$EmimESO_4$ (0.6 M)	85.0
	EmimCl (0.6 M)	80.0
	BmimCl (0.6 M)	64.0
	OmimCl (0.6 M)	60.0

Unlike myoglobin, lysozyme was reported for both increase and decrease in melting temperature in the presence of various imidazolium based ionic liquids [23]. 1-Ethyl-3-methylimidazolium ethyl sulphate ($EmimESO_4$) and 1-ethyl-3-methylimidazolium chloride (EmimCl) were reported for rapid increment of the melting temperature of lysozyme, whereas two other ionic liquids *viz*. 1-butyl-3-methylimidazolium chloride (BmimCl) and 1-octyl-3-methylimidazolium (OmimCl) caused lowering of the melting temperature (Table-1). These effects were enhanced with the rise in the concentration of ionic liquid. $EmimESO_4$ exhibited highest stabilizing effect upon lysozyme and this effect was attributed to the interaction of both the cation and anion with the oppositely charged amino acid side chains *via* electrostatic as well as hydrophobic forces. EmimCl with the same cation also has the stabilizing effect, but chloride ion lacks the stabilizing power like ethyl sulphate ion. Again, increased hydrophobic effect in the cationic moiety with larger alkyl group as in BmimCl and OmimCl destabilized the native lysozyme structure. Based on these observations, it is concluded that $EmimESO_4$ was the most promising and biocompatible ionic liquid for Lysozyme at a wide range of concentrations.

From the above two case studies, it is clear that thermal stability of proteins decreases with higher hydrophobic character of the imidazolium cation. Thermal denaturation study with bovine serum albumin (BSA) in presence of various ionic liquids exhibited similar trend [24] and order of the value of T_m was as follows: $EmimESO_4$ > EmimCl > BmimCl (Table-1). Thus, hydrophobicity plays an important role in protein's thermal stability.

Unfolding of protein molecules: Imidazolium ionic liquids are also reported for denaturation of protein native structure just like the chaotropic agents. Protein structure were examined with increasing concentration of the ionic liquid in aqueous solution. Water-miscible ionic liquid, 1-butyl-3-methylimidazolium chloride (BmimCl), was reported for transforming the native cytochrome c and human serum albumin (HSA) into completely unfolded state at 50 vol% of the ionic liquid [25]. At lower concentration of the ionic liquid, the structure of both the proteins remained intact. Behaviour of BmimCl was similar to that of chaotropic agents such as urea or guanidine hydrochloride except the fact that guanidine hydrochloride or urea induced denatured state was always monomer, whereas BmimCl-induced denatured state of HSA was in dimeric form, although cytochrome c yielded monomeric unfolded state. Study of the chicken egg white lysozyme under the influence of 1-butyl-3-methylimidazolium nitrate ($BmimNO_3$) revealed that the protein lost its native tertiary as well as the secondary structure in presence of 6 M ionic liquid [26]. But according to the published report, at higher concentration of the ionic liquid, $BmimNO_3$ (>10 M), lysozyme adopts a partially globular structure. The authors highlighted strong nano-heterogeneity of the medium consisting of the imidazolium ionic liquid and water as the reason behind this 'reorganization' of the polypeptide chain. Another experimental study by Fiebig *et al*. [27] on myoglobin, included the effect of two imidazolium ionic liquid on the unfolding process of protein caused by the chaotropic agent, guanidine hydrochloride. Guanidine hydrochloride induced denaturation is associated with the loss of heme absorptivity. When ethylmethylimidazolium acetate (EmimOAc) was used as a cosolvent, the unfolding pattern remained unaffected whereas butylmethylimidazolium boron tetrafluoride ($BmimBF_4$) did affect the unfolding process. Though $BmimBF_4$ itself did not denature the protein myoglobin, but at moderate concentration (150 mM), this ionic liquid initiates the protein unfolding with lower concentration of guanidine hydrochloride which implied destabilization of the protein structure in presence of $BmimBF_4$. Control studies with NaOAc

and $LiBF_4$ confirmed that the imidazolium part is not responsible for the dissimilar observation and repulsive interaction of tetrafluoroborate anion (BF_4^-) caused the destabilization. Based on these observations, it is concluded that since EmimOAc did not affect protein structure, it may be chosen as cosolvent additive whereas $BmimBF_4$ may be used as additive in the folding study specially for the proteins which are normally difficult to denature.

Several long-chain imidazolium ionic liquids behave as surfactant [28] and their interaction with protein structures were also studied. Interaction of bovine serum albumin (BSA) with 1-tetradecyl-3-methylimidazolium bromide (C_{14}mimBr) was investigated by fluorescence spectra and surface tension measurement. C_{14}mimBr lead to unfolding of BSA structure above critical micelle concentration (CMC) and nature of the interaction were mainly hydrophobic [29]. Again 3-methyl-1-octylimidazolium dodecylsulfate, $[C_8mim][C_{12}OSO_3]$, also induced concentration dependent significant alterations in the structure of BSA at physiological pH [30]. This ionic liquid promoted a small unfolding of BSA in monomeric regime at low concentration, followed by a refolding up to critical aggregation concentration (CAC). Above CAC, again a small unfolding of BSA was noticed up to critical vesicular concentration (CVC) and when ionic liquid concentration reached more than CVC, structure of BSA became stable.

Sheet to helix transformation: While interacting with the ionic liquids, secondary structure of the protein molecules also changes sometimes. There are reports of transformation of a particular type of regular secondary structure to another type of regular secondary structure. Takekiyo *et al*. [31] studied the effect of imidazolium ionic liquids on β-lactoglobulin. It is a predominantly β-sheet protein at physiological pH (Fig. 3) which turned into a nonnative α-helical structure under the influence of two imidazolium based ionic liquids. Both 1-butyl-3-methylimidazolium nitrate ($BmimNO_3$) and ethyl ammonium nitrate ($EANNO_3$) disrupt the native tertiary structure and in this transition native β-sheet structure is also lost, instead a new α-helix conformation is adopted at high ionic liquid concentration (> 10 mol%). At moderate concentration of $EANNO_3$, a disordered structure was obtained. Nature of this α-helical structure was similar to that of the alcohol-induced helix. Though both the ionic liquids prompted helix formation, they showed different behavior towards the protein aggregation. $BmimNO_3$ was noticed for promoting aggregation while $EANNO_3$ inhibited it. Another ionic liquid, 1-ethyl-3-methyl-

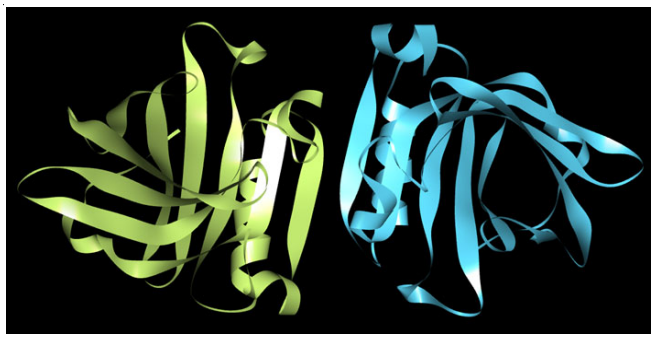

Fig. 3. Structure of bovine β-lactoglobulin (pdb id: 1beb)

imidazolium ethyl sulfate also induced a sheet to helix transition of bovine β-lactoglobulin at pH 7.5 [32]. In this case, the effect was just reverse at pH 4.0, the pH at which the protein β-lactoglobulin existed in mainly helical form [32]. At this pH with increasing concentration of the ionic liquid, helix dominant structure converted to sheet dominant structure. Again, 1-alkyl-3-methylimidazoliumbromide caused significant changes in the secondary structure of BSA, though the effect varied with the chain length of the alkyl group [33].

Inhibition of aggregation and disaggregation: Protein molecules exist in a specific monomeric/oligomeric structure representing a particular three-dimensional structure and proteins are functional only in that state. But sometimes, in various stress conditions, many protein molecules associate to produce an aggregated state [34]. Aggregated proteins not only loss their functional ability, they do much more harm than that. Aggregation of various proteins are responsible behind many neurodegenerative diseases like Alzheimer's Disease, Parkinson's disease, *etc*. [35]. Rawat & Bohidar [36] studied the effect of imidazolium ionic liquids on the aggregation tendency of few proteins (BSA, HSA, immunoglobulin, β-lactoglobulin and gelatin-B) with same isoelectric point (pI ≈ 5) [36]. The ionic liquids employed in the study were 1-methyl-3-hexyl imidazolium chloride (HmimCl) and 1-methyl-3-octyl imidazolium chloride (OmimCl). It is very common for the proteins to assemble forming a non-aggregating and an aggregating fraction at pH near to the isoelectric point. Thus, the protein stability is very much dependent on pH of the medium. Here the study reported about pH-independent stability in presence of ionic liquids. Association of the protein molecules is significantly hindered in ionic liquid solutions. The authors concluded that the ionic liquid molecules form a double layer on the surface of the protein displacing the first layer of water molecules provided stability to the non-aggregating fraction. On the other hand, aggregating fraction is stabilized through the overlap of hydrophobic parts of ionic liquid molecules forming a stacked pH-insensitive cluster of protein molecules.

Imidazolium based ionic liquids are not only reported for inhibiting the aggregation of proteins, but several of them were also found to be capable of dissociation of protein aggregates. The same group also observed through dynamic light scattering (DLS) study that four 1-alkyl-3-methyl imidazolium chloride reduced the size of the aggregates of BSA, immunoglobulin (IgG) and β-lactoglobulin in an effective manner [37]. The effect of ionic liquids in this issue was similar or even better to that of the common anticoagulant, heparin. In case of BSA, 1-octyl-3-methyl imidazolium chloride (OmimCl) was the most effective aggregation reversal agent whereas 1-ethyl-3-methyl imidazolium chloride (EmimCl) gave the best results for immunoglobulin and β-lactoglobulin. Release of chloride ions to the solution by these ionic liquids increased the entropy of the solution resulting extra stability and this was considered as the explanation for the better efficacy than the heparin. Again, $BmimNO_3$ denatures lysozyme at ~6 M concentration, but inhibits aggregation of lysozyme at >10 M $BmimNO_3$ [26].

Enzyme activity enhancement: Enzymes are protein molecules which act as catalysts in biological systems. Activity

of several enzymes were also checked under the influence of ionic liquids. Lipases are the enzymes, which catalyzes the hydrolysis of fats. This activity of lipase was found to be enhanced in presence of imidazolium chlorides with compared to conventional organic solvents like methanol or isopropanol [38]. Thermal stability of the lipases was also considerably increased in imidazolium ionic liquid solutions. The authors could not investigate the possible reason for this because of fluorescence quenching by the ionic liquids. Thus, this effect of ionic liquids can be applied in other biotechnological processes. Various 1-alkyl-3-methyl imidazolium bromides were also reported for enhancement of activity (up to 4-fold) of enzyme trypsin in cationic reverse micelle of CTAB [39]. The report suggested that imidazolium bromides increased the nucleophilicity of water by forming a hydrogen bond and thereby rate of ester hydrolysis speeded up.

But enzyme action is not always speeded up (Table-2). Bromide salt of same imidazolium cations inhibits the activity of glucose oxidase [40]. Imidazolium cation with higher alkyl chain causes more decrease in the activity. Based on MD simulation the authors came to conclusion that there were conformational changes in the active site of the enzyme, which caused its declined activity [40]. Again, the ionic liquid, BmimCl, caused significant decline in the enzyme cellulase activity from *Aspergillus niger* [41], whereas three 1-alkyl-3-methyl imidazolium methyl sulfate also studied for reduced activity of two model enzymes *viz*. *Penicillium expansum* lipase and mushroom tyrosinase [42]. Here, also the decrease in enzyme activity was proportional with the alkyl chain length.

TABLE-2
EFFECT OF IMIDAZOLIUM IONIC LIQUIDS ON ENZYME ACTIVITY

Enzyme	Ionic liquid	Action
Lipase (from *Pseudomonas sp*.)	BmimCl & HmimCl	Activity increases
Trypsin	BmimBr, EmimBr	Activity increases
Glucose oxidase	BmimBr, HmimBr	Activity decreases
Cellulase (from *Aspergillus niger*)	BmimCl	Activity decreases
Penicillium expansum Lipase	$MmimESO_4$, $EmimESO_4$ and $BmimESO_4$	Activity decreases
Mushroom tyrosinase	$MmimESO_4$, $EmimESO_4$ and $BmimESO_4$	Activity decreases

Function as refolding additives: There are several ionic liquids which promote refolding of some denatured proteins and few imidazole based ionic liquids are also reported [43,44]. Ethylammonium nitrate was the first ionic liquid reported for enhancing renaturation of hen egg white lysozyme [45]. Among the imidazolium ionic liquids, a series of N′-substituted-*N*-methylimidazolium chloride salts were reported for renaturation of two model proteins by Lange *et al*. [46]. Though, the efficacies were found to be variable for each salt and also dependent on a specific protein, they act by suppressing the formation of protein aggregation. Variation of substituent in the imidazole ring as well as variation of the counter anions may give optimum results and the same group studied the effect of the anions with a fixed imidazole cation on the refolding of recombinant plasminogen activator rPA. The authors found the efficacy of *N*-ethyl-*N*′-methyl imidazolium salts in the following order: $Cl^- > 2$-(2-methoxyethoxy)ethyl sulfate $> EtSO_4^- >$ acetate $>$ tosylate $> Et_2PO_4^- \approx$ hexyl sulfate. In a recent study, Sindhu *et al*. [47] found that two imidazolium ionic liquids (1-ethyl 3-methyl imidazolium ethyl sulfate, $[EmimESO_4]$ and 1-ethyl-3-methyl imidazolium chloride, [EmimCl]) functioned as very good refolding agent for the urea induced unfolded serum albumins. Based on DLS study, it is suggested that the ionic liquids narrowed down the protein aggregation and consequently enhanced the refolding process. Biocompatible nature of the ionic liquids signals about new avenue of the protein folding study, which requires further study.

Conclusion

The literature study as discussed above emphasizes the application of imidazolium based ionic liquids on the frontier field of protein research. In this mini-review, it is observed that imidazolium ionic liquids influence the protein structure and function in many ways. They both stabilize and destabilize the protein molecules, caused formation of nonnative regular secondary structure, affected protein unfolding, inhibited protein aggregation, dissolution of the aggregates, renature the proteins (Table-3). It also impacts upon the activity of various enzymes. Tuning of the structure of ionic liquids by varying the substituents of imidazole ring and by using various counter anions could be useful for optimization of the particular effect. Imidazolium ionic liquids can be utilized in many significant aspects, namely to stabilize native protein structures, dissolution of

TABLE-3
EFFECT OF IMIDAZOLIUM IONIC LIQUIDS ON DIFFERENT PROTEINS

Protein	Ionic liquid	Effect
Myoglobin	$[Bmim]^+$ salts	Decrease of thermal stability
Lysozyme	$EmimESO_4$, EmimCl, BmimCl, OmimCl	Decrease of thermal stability
Lysozyme	$BmimNO_3$	Protein denaturation
BSA	$EmimESO_4$, EmimCl	Increase of thermal stability
BSA	BmimCl	Decrease of thermal stability
Cytochrome c HSA	BmimCl	Protein denaturation
BSA	C_{14}mimBr	Protein denaturation
β-Lg	$BmimNO_3$	Change of secondary structure
BSA, HSA, IgG, β-Lg and gelatin-B	HmimCl, OmimCl	Inhibition of protein aggregation
BSA, β-Lg and IgG	OmimCl, EmimCl	Reduction of aggregation size
rPA	$[Emim]^+$ salts	Renaturation of protein

the protein aggregates, refolding of the denatured proteins and to improve the enzyme activity. But for better understanding of the effect and more generalization, more experimental data and *in silico* studies are required in this field.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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