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The ionic liquid isopropylammonium formate as a mobile phase modifier to improve protein stability during reversed phase liquid chromatography

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Abstract

The room temperature ionic liquid isopropylammonium formate (IPAF) is studied as a reversed phase HPLC mobile phase modifier for separation of native proteins using a polymeric column and this protein stability is compared to that using acetonitrile (MeCN) as the standard organic mobile phase modifier. A variety of important proteins with different numbers of subunits is investigated, including non-subunit proteins: albumin, and amyloglucosidase (AMY); a two subunit protein: thyroglobulin (THY); and four subunit proteins: glutamate dehydrogenase (GDH) and lactate dehydrogenase (LDH). A significant enhancement in protein stability is observed in the chromatograms upon using IPAF as a mobile phase modifier. The first sharper peak at about 2 min represented protein in primarily the native form and a second broader peak more retained at about 5–6 min represented substantially denatured or possibly aggregated protein. The investigated proteins (except LDH) could maintain the native form within up to 50% IPAF, while a mobile phase, with as low as 10% MeCN, induced protein denaturation. The assay for pyruvate using LDH has further shown that enzymatic activity can be maintained up to 30% IPAF in water in contrast to no activity using 30% MeCN.

Keywords

ionic liquid; high performance liquid chromatography; protein stability; mobile phase modifier

1. Introduction

Room temperature ionic liquids (RTILs) are normally composed of organic cations (i.e. alkyl-imidazolium, -pyridinium, -ammonium, -phosphonium) and inorganic (ie. Cl^- , PF_6^- , BF_4^-) or organic anions (i.e. HCOO^- , $(\text{CF}_3\text{SO})_2\text{N}^-$). In contrast to conventional organic solvents, ionic liquids are considered environmentally benign “green” solvents, in part because they are usually nonvolatile, nonflammable, and generally less toxic. Due to their tunable structure, ionic liquids, often with the 1-alkyl-3-methylimidazolium cation and an

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inorganic anion such as BF_4^- have been widely used in separation science and this topic has been reviewed [1–8]. Reviews of the use of ionic liquids in specific areas such as capillary electrophoresis [9], solid phase extraction [10], and gas chromatography [11] are also available.

The application of RTILs has been widely reported for reversed phase high liquid chromatography (RPLC) [12,13]. Imidazolium RTILs as mM level mobile phase additives could block organic amine-silanol interactions on the silica surface, decreasing band tailing, and improving resolution [14–16]. Alkylammonium nitrates and alkylammonium formates have been used as organic solvent replacements in RPLC mobile phases at high percentage levels [17–20]. It has been reported that 40% n-propylammonium nitrate/ 60% water showed enhanced elution for nitroanilines but reduced elution for 2,4-dinitrophenol compared with 40% methanol/ 60% water [21]. Methylammonium formate (MAF) has also been used as a mobile phase modifier and shown to be better for the separation of nitrofurans than methanol, probably due to the silanol blocking effect [17]. Similarly, ethylammonium formate (EAF) showed better resolution of some nitroaromatic and aromatic carboxylic acid peak pairs, such as o-nitrophenol/ p-nitrophenol and p-aminobenzoate/ benzoate in contrast to methanol [20]. In part because the amines are available as dissolved in solution, the shortest chain alkylammonium formates are somewhat difficult to synthesize and purify as well as prone to amide formation. Recently, isopropylammonium formate (IPAF), a branched alkylammonium formate RTIL, has been synthesized from the high purity neat amine as well as formic acid and characterized chromatographically as a suitable organic solvent replacement in the mobile phase for RPLC [22]. IPAF seems to have enhanced stability over other alkylammonium formates and can be stored at room temperature for several months without any apparent color change.

In addition to the chromatographic applications, RTILs have also been widely applied as solvents in biocatalysis [23] and for protein stability [24]. It is well known that organic solvents, such as acetonitrile (MeCN) and 1-propanol have appreciable effects on protein conformation [25–28]. The conformation of lysozyme begins to change within 40% MeCN/ 60% water [29]. Similarly, cytochrome c conformation begins to change in a 40% methanol (MeOH)/ 60% water solution but is stable in a 10–70% MAF or EAF range in water [30]. Using a fluorescence detection approach for tetramethylrhodamine labeled cytochrome c, ethylammonium nitrate, even to 2.5 M, was found to be the most biocompatible solvent, as compared to 1-butyl-3-methylimidazolium ionic liquids with various anions [31]. Human serum albumin and cytochrome c could retain their higher order structure up to 25% 1-butyl-3-methylimidazolium chloride but became highly denatured at 50% [32].

The effect of RTILs on enzyme activity is complicated and several reviews are available [23, 33, 34]. Zhao reported that in the presence of water, hydrophilic ionic liquids, such as 1-ethyl-3-methylimidazolium tetrafluoroborate would dissociate into ions and follow the Hofmeister series [33]. The Hofmeister series (or the kosmotropicity order) ranks both anions and cations found in salts in their ability to interact with water. Kosmotropic ions have low polarizability and stay highly hydrated promoting hydrophobic interactions of solutes in an aqueous solution. Chaotropic ions are highly polarizable and interact weakly with water tending to increase the solubility of hydrophobes. Considering the equilibrium

between less dense water and more dense water, kosmotropes interact with more dense water shifting the equilibrium to the left and chaotropes do the opposite, interacting with less dense water and shifting the equilibrium to the right. The combination of a kosmotropic anion and chaotropic cation in an aqueous solution has been found to be a general trend for the stabilization of enzymes [33]. Carboxylate anions such as CH_3COO^- and also likely HCOO^- with a high charge density tend to be kosmotropic and organic cations with a low charge/size ratio such as tetraethylammonium and likely isopropylammonium are chaotropic. Therefore IPAF appears to offer promise as a stabilizing enzyme solvent.

So far the majority of enzymes reported to be active in RTILs are lipases. In 2007, van Rantwijk and Sheldon reemphasized the importance of the Hofmeister series in explaining the effect of ionic liquids on lipase activity [34]. The activity of enzymes was also related to the polarity, network, viscosity, hydrophobicity, and surfactant effect of the RTILs [23]. Other researchers have explained that the conformational changes of proteins would be slowed down due to the high viscosity of RTILs, therefore enzymes could maintain their native structures and activities [34]. It has been suggested that enzymes might be embedded in the RTIL network, in a way that maintains their native structures [35]. Although the mechanism of protein stabilization by an ionic liquid is complicated, the Hofmeister series is the most acceptable explanation particularly for prediction of enzyme stability in RTIL-water mixtures. An investigation of alkylammonium based ionic liquids as media for enzymatic glycerolysis showed that ILs with a long alkyl chain and free hydroxyl groups in the cations could dramatically increase conversion of triglycerides [36]. Horse radish peroxidase (HRP) showed more than 10 times great activity in the presence of RTIL—tetrakis (2-hydroxyethyl) ammonium trifluoromethanesulfonate than that in the conventional organic solvent—methanol. This “specifically designed” RTIL containing multiple hydroxyethyl groups could maintain HRP activity due to its particular compatibility [37].

As early as 1984, it was demonstrated that proteins such as cytochrome C, myoglobin and lysozyme were easily denatured using a RPLC column [38]. For RPLC of papain, the early peak was found to be active and the later peak to be inactive based on enzymatic assay of the collected fractions [39]. The accepted mechanism is that the hydrophobic residues from the protein interior are exposed in the process of protein denaturation and cause an increase of retention; in comparison, the native protein with hydrophobic residues buried inside have less retention [40]. More recently, the emphasis of RPLC for the high speed separation of proteins using pellicular particles has been made [41]. Factors such as the use of the organic solvent modifier, hydrophobic stationary phase, and high temperature were all acknowledged to cause protein denaturation. Ultra high pressure LC for proteomics has been reviewed [42] and this technique has been shown to potentially improve protein recovery and apparently minimize protein denaturation in some cases [43]. The characterization of reversed phase stationary phases (C4 and C18) on fully porous sub-two micron silica particles and wide pore core-shell sub four micron particles was made for a standard protein mix and a therapeutic protein such as oxidized and reduced forms of interferon [44]. Methods for intact proteins of pharmaceutical interest have been reviewed [45]. As expected, emphasis was on size exclusion chromatography and ion exchange LC however RPLC was indicated to be a promising technique. The LC of recombinant proteins of high bioactivity has been reviewed [46] as has the technique of protein folding LC which is

intended to raise the efficiency or shorten the time for protein refolding. On-line separation of native proteins using 2-D LC with a single weak cation exchange column and a change in the mobile phase composition to permit hydrophobic interaction chromatography (HIC) has been reported [47].

HIC separates proteins on a short chain or phenyl RPLC column based on both the cations and anions promote strong interactions with water resulting in hydrophobic retention by the proteins on the stationary phase. However, proteins separated by HIC often retain their native structure. RPLC of proteins uses similar stationary phases to those for HIC but protein elution depends on increasing the % of a water miscible organic solvent such as MeCN in the mobile phase gradient. However, proteins are almost always denatured at such high organic solvent compositions. For smaller proteins less than 30 kDa, the denaturation effects may be reversible. Our long term intent is to use an ionic liquid, particularly an alkyl ammonium formate, as the strong solvent to develop a milder form of RPLC for the semi-preparative separation of proteins of both low and high molar mass without the disadvantages of the high salt containing mobile phases of HIC. Elimination of the ionic liquid from the protein fraction should be possible using a desalting and buffer exchange column or syringe.

Our goal is to include RPLC as a possible technique for the isolation of native proteins from their denatured form. A change in modifier solvent is one underutilized idea to permit RPLC of proteins, especially those with subunits, in their native form. A preliminary study has shown that IPAF provides better thermal stability for the separation of cytochrome C (12.4 kDa) from tryptophan than methanol [22]. In this paper, IPAF is used as the RPLC mobile phase modifier to improve protein stability compared with the conventional organic solvent MeCN. Five much higher kDa proteins with different subunits, including non-subunit proteins: albumin, and amyloglucosidase (AMY); a two subunit protein: thyroglobulin (THY); and four subunit proteins: glutamate dehydrogenase (GDH) and lactate dehydrogenase (LDH) are investigated and a summary of this information as well as their molar masses is provided in Table 1. The enzymatic assay for pyruvate is conducted to further assure the effect of IPAF on LDH stability. A possible mechanism of IPAF for protein stabilization is provided in the discussion.

2. Experimental

2.1. Apparatus

A Dionex (Sunnyvale, CA) Ultimate 3000 Rapid Separation Ultra High Performance Liquid Chromatography (UPLC) instrument equipped with fluorescence detector was used. Separations were carried out using a PRP-3 polymeric column (150 × 4.6mm), packed with a 10 µm (30 nm pore size) polystyrene-divinylbenzene particles, obtained from the Hamilton Co. (Reno, NV). All the chromatograms were recorded and analyzed using Chromeleon software and then plotted by OriginPro 7 software. The enzymatic assay was conducted using a HP 8453 UV-Vis photodiode array spectrophotometer from Agilent (Wilmington, DE).

2.2. Reagents

Isopropylamine of 99.5% purity and formic acid of 98% v/v grade were separately purchased from Aldrich (Milwaukee, WI) and Fluka (St. Gallen, Switzerland). The synthesis of IPAF from these previously mentioned chemicals has been described in detail in our previous study [22]. Amyloglucosidase (AMY) (from *Aspergillus niger*), thyroglobulin (THY) (from bovine thyroid), albumin (from human serum), glutamic dehydrogenase (GDH), lactate dehydrogenase (LDH), sodium pyruvate and β -nicotinamide adenine dinucleotide (reduced form) (NADH) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (MeCN) was obtained as HPLC grade from Fisher Scientific. Deionized water (DI water) from a Millipore MilliQ filtration system with resistivity of 18.2 M Ω was used throughout.

2.3. Chromatographic conditions

Each tested protein was dissolved in reverse osmosis, deionized water. Solvent A was this same water; both IPAF and MeCN were pre-mixed separately with water (80:20, v/v) and degassed 10 min before use as solvent B. All the chromatograms were obtained at a flow rate of 0.5 mL/min with 20 μ L injection volume at 20°C. The PRP-3 column was pre-conditioned and equilibrated for 15 min using the mobile phase before each injection. Analysis time is 10 min. For detection of tryptophan (W) in the proteins, fluorescence detection at 270 nm excitation and 350 nm emission wavelengths was applied and all the measurements were in units of detector counts. All the chromatograms have been repeated at least twice with good reproducibility.

2.4. Enzymatic assay

The enzymatic assay for LDH activity using pyruvate was done by UV-Vis photodiode array spectrophotometry. LDH can accelerate the reaction between pyruvate and NADH:

Stock solutions of 6.6 mM NADH and 30 mM sodium pyruvate were prepared in 0.2 M tris-HCl buffer (pH 7.3). Different percentages of IPAF and MeCN ranging from 10% to 40% in reverse osmosis, deionized water were added to the quartz cuvette with 0.2 mL of 30 mM sodium pyruvate and 0.6 mM NADH. The total volume of the solution in the cuvette is 3 mL when filled with tris-HCl buffer. The solution was carefully mixed in the cuvette using a plastic pipette before each measurement. The initial kinetic rate is recorded at 340 nm with a 30 s run time and 1 s cycle time. The enzymatic assay has been repeated twice.

3. Results and Discussion

First, it was important to demonstrate that IPAF is a reversed phase modifier solvent, similar to MeCN, on this PRP-3 column. Figure S1 in the Supplementary Information shows a retention time comparison of tryptophan using MeCN and IPAF as modifier solvents in water. As previously shown [22], IPAF is not as strong as solvent as MeCN as indicated by the consistently shorter retention time of about 5–10%. The solvent parameter S of IPAF

was found to be 2.4 slightly lower (more polar) than that for MeCN of 2.7 [22]. The percentage ratio of *S* values of IPAF to MeCN is 89%, similar to the ratio of tryptophan peak heights of IPAF to MeCN measured from S1 as 82%. This is expected since tryptophan has a higher quantum yield in a more hydrophobic solvent environment as compared to a hydrophilic environment [48].

We also checked for the possibility of adsorption by the isopropyl cation of IPAF to the polymeric stationary phase. The column was equilibrated with IPAF, then washed with water, and the test probe nitrate injected. As compared to retention of nitrate using just a standard aqueous mobile phase, there was increase (or change) in retention due to the possibility of ion exchange.

Before studying the effect of IPAF on protein stabilization in RPLC, we first checked the pH optimum range as indicated in the literature for the proteins of interest. The apparent “pH” range of 10% IPAF/ 90% water and 50% IPAF/ 50% water is from 6.0 to 5.5 compared with 5.52 to 5.11 for MeCN in water with the same percent composition. The optimal pH range for the studied proteins are: 4.7–8 for albumin [49], 2.5–6.5 for AMY [50], 5.5–6.0 for THY [51], 7.0–8.0 for GDH [52], and 5.5–8.5 for LDH [53]. No mobile phase pH adjustment was deemed necessary before protein separation.

The numbers of tryptophan (W) in the protein structures were found from protein bank information and are listed in Table 1. However, it may too simplistic an argument to relate the intensity of the fluorescent peak in the chromatogram to the number of tryptophans in the protein. The intensity of the tryptophan fluorescence is directly dependent not only on the degree of hydrophobicity of the immediate solvent environment but the proximity of certain amino acid side chains such as lysine and tyrosine have also been shown to quench tryptophan fluorescence [54]. As shown in previous studies with papain [55] and pepsin [56], fluorescence of these proteins will increase quite linearly as the percentage of organic solvent such as MeCN increases in water. We are primarily interested in the fluorescence peak area ratio of the weakly retained (likely native protein with less hydrophobic exposed areas) to more retained peaks (protein denatured to some degree) as a function of increasing IPAF and MeCN in water. The magnitude of this peak area ratio is likely a combination of the increased tryptophan fluorescence in a less aqueous solvent creating a more hydrophobic environment but also due to more exposed hydrophobic amino acids such as tryptophan as indicated by the broad chromatographic retained peaks for both IPAF and MeCN. The broad chromatographic retained peaks could also be due to protein aggregation.

3.1. Non-subunit proteins

The ability of IPAF to improve protein stability was first tested by the non-subunit protein albumin. In Figures 1A and Figure 1B (as in all the analogous following figures), the chromatograms of albumin are shown in a waterfall style from 10% to 50% IPAF and MeCN as the mobile phase modifier in RPLC. Figure 1B shows similar chromatograms with a large native albumin peak followed by a small denatured peak up to 30% MeCN; at 40% MeCN or above, the denatured albumin peak increases dramatically. This corresponds well with what has been reported by Sen and co-workers who concluded that 10%–20% MeCN would not largely denature albumin, while at a higher MeCN content of 40%, the tertiary

structure of albumin started to change and aggregation was considered likely [26]. The secondary structure could remain intact up to 70% MeCN [26]. As a comparison, only native albumin peaks show up within up to 50% IPAF. It is worth noting here that the fluorescence peak is much smaller at 10% IPAF compared with others since 10% IPAF is not strong enough to elute albumin off the column.

Based on protein data bank information, albumin (M.W. 66.5 kDa) only has one W buried inside. With the increase of MeCN percent, the buried hydrophobic group with W is thought to become more exposed and this form of albumin will have a longer retention time. Therefore, the peaks with longer retention times, at around 5.8 min, were the denatured albumin peaks and the peak with a retention time at 2.0 min was the more native one. In Figure 1C, the ratios of denatured peak area versus native peak area of albumin at different percentages of IPAF and MeCN were compared. The peak area ratios remain low from 10% to 50% IPAF, at around 0.1. In comparison, the ratio for MeCN increases quickly from 0.07 at 20% to 0.28 at 30% and then rises up to 3.45 at 50%. It is noteworthy that the peak area ratios between replicate runs are more consistent for IPAF with MeCN which may be related in part to the greater extent of protein denaturation (retained irregular peak area) by MeCN than IPAF.

More pronounced results were observed in the case of AMY (M.W. 97.0 kDa) due to the larger structure and the more hydrophobic amino acids incorporated in the protein structure. As shown in Figures 2A and 2B, AMY maintains the native conformation within up to 50% IPAF, but it is markedly denatured at even a low percent of MeCN. The native peaks also decrease dramatically in area with increasing % MeCN. The large denatured broad peak at around 6.0 min likely results from the W groups that become more exposed and solvated by MeCN. Long or irreversible retention of denatured AMY causing a baseline shift but not a discrete peak may also be a factor. The denatured versus native AMY peak area ratios using IPAF and MeCN as mobile phases are plotted as shown in Figure 2C. The ratios are around 0.1 from 10% to 50% IPAF, compared with 0.64 at 30% MeCN, and then a marked increase to 4.9 at 50% MeCN.

3.2. Two subunit protein

In order to further study the IPAF effect on protein stability, RPLC experiments on the two-subunit protein THY were conducted and the results are shown in Figures 3A and 3B. Small denatured peaks begin to appear at 20% MeCN. At 40% MeCN or above, the conformation change of THY is more dramatic as evidenced by the fluorescence signal increase. As with AMY, the native peaks also decrease dramatically in area with increasing % MeCN. As with THY, the ratios of denatured versus native THY peaks in Figure 3C increase from 0.06 to 0.92 at 20% to 50% MeCN. Using IPAF, this ratio did not exceed 0.09 indicating it can be a suitable replacement for MeCN as a mobile phase modifier in the RPLC of this protein.

3.3. Four subunit proteins

To understand the effects of IPAF and MeOH solvents on more labile proteins, two dehydrogenase enzymes, both with four subunits were investigated.

In Figure 4A, the chromatograms of GDH using different percentages of MeCN as the mobile phase modifier are provided. As what shown in the chromatograms, a large native GDH peak appears at 10% MeCN; then only denatured GDH peaks show up at 30% MeCN or above. The results coincide with the study by Kujo and co-workers which indicated that 15% MeCN could maintain GDH activity, but the activity would be completely lost at 20% MeCN or above [28]. It is highly possible that the GDH (M.W. 310 kDa) is unfolded at 30% MeCN or above; the 24 W groups of GDH are exposed more outside and solvated by MeCN to cause evidence of large denatured peaks at around 5.5 min. < Figure 4B shows only native GDH peaks within up to 50% IPAF; however 10% IPAF is not strong enough to elute GDH off the column. In Figure 4C, the ratios of denatured/ native GDH peak area rise from 1.3 at 10% MeCN to 10.1 at 20% MeCN. The area ratios of 30, 40, and 50% MeCN are not listed because there are not native GDH peaks at high MeCN content. In comparison, the area ratios hold constant near 0.04 from 10% to 50% IPAF.

As shown in Figure 5A, 20% IPAF is the optimum mobile phase modifier for LDH since 10% IPAF is not strong enough to elute LDH and 30% IPAF can denature LDH significantly. In Figure 5B, only denatured peaks show up because apparently the hydrophobic interaction of MeCN, even at a low percentage, exposes and solvates the LDH polypeptide chain with 8 W.

3.4. Enzymatic Assay

Corresponding enzyme activity assays for pyruvate using LDH were conducted. The Δ absorbance units/s (Au/s) of NADH with LDH as the catalyst in solution with different percentages of IPAF and MeCN are shown in Figure 5C. LDH activity decreases gradually from 10% to 30% IPAF with Δ of -0.0227 , -0.011 and -0.00514 Au/s. However, LDH is completely inactive at 20% MeCN or above. These enzymatic assay data corresponded well with the chromatograms in Figure 5A and 5B. From 10% to 50% IPAF, there was always a small native LDH peak in addition to the denatured LDH peak, while only denatured LDH peaks are shown in the chromatograms for MeCN.

Recently, Leung and Lai reported that the activity of GDH with a larger molecular mass showed less variation compared to LDH with a smaller molar mass, with changes in pH, hydrophilicity, and surfactant concentration [57]. They interpreted this activity difference of GDH and LDH as follows. The activity of an enzyme of larger molar mass (GDH) is less affected by outside condition changes; for an enzyme of smaller molar mass (LDH), sudden shifts of enzymatic activity are possible with small condition changes. The “stabilizing effect” corresponds with our observations using either MeCN or IPAF that LDH with a smaller molar mass and W group number would be more liable to become denatured as compared with GDH.

3.5. Possible mechanism of IPAF as a mobile phase modifier to stabilize proteins

Within the class of conventional organic solvents, such as MeCN, the strong interaction between the solvent and the buried hydrophobic groups will greatly change the native conformation of the protein, which causes protein denaturation. However, the Hofmeister series has been proposed as the best explanation for the effect of ionic liquids on enzyme

stabilization [23, 33, 34]. The kosmotropic or “structure-making” anion is highly hydrated and can stabilize proteins well by reducing the volume of water available to hydrate the larger surface exposed by the denatured protein. A chaotropic or “structure-breaking” cation tends to have a destabilizing effect on proteins by breaking down the hydrogen-bonded network of water allowing the macromolecule more structural freedom and therefore protein extension. However, there is evidence from a vibrational sum frequency spectroscopy study of an octadecylamine monolayer spread on different salt solutions that ions have a limited effect on the overall bonding of water in bulk solution and are more involved in moderation of the dispersion forces involving the alkyl chains [58]. The Hofmeister effect is thought to be a more interfacial phenomenon predicting how ions interact with the macromolecule/aqueous interface [59].

However, as pointed out in the Introduction, it is well known the combination of the kosmotropic anion and the chaotropic cation provides optimum stabilization of biological macromolecules by a salt. For example, the protic ionic liquid triethylammonium dihydrogen phosphate has been shown to effectively stabilize succinylated concanavalin A, a protein with four monomeric units in a folded jelly roll motif [48]. For IPAF, the formate ion should be considered to be a kosmotropic or “structure-making” anion while the isopropylammonium ion is more of a chaotropic cation.

Considering the fairly similar *S* values for IPAF and MeCN, it seems somewhat surprising that the denatured to native peak area ratios are markedly higher for MeCN as compared to IPAF for the studied proteins. One contributing reason may be that there is about twice as much MeCN as compared to IPAF based on a mole ratio instead of a % volume ratio, considering the density of MeCN is known to be 0.79 and that for IPAF was found to be 1.1 [22]. In addition, possibly, the relatively high viscosity of IPAF may reduce the collisions with the protein during the separation process, helping to maintain native structure. Previously, we have reported the viscosity diminishes markedly as the water content is raised; even traces of water can markedly lower the viscosity of AAFs. The viscosity of pure IPAF at room temperature is about 130 cP but decreases to about 25 cp at 80% IPAF-20% water, 10 for 60% IPAF- 40% water, and 2.5 for 20% IPAF-80% water [22]. The viscosity of MeCN in water follows a somewhat parabolic profile but peaks at only 1.2 cp for 20% MeCN-80% water and then declines steadily to about 0.4 for 100% MeCN [60]. Less protein aggregation in IPAF than in MECN might also be a contributing reason.

Greaves and co-workers have recently reviewed ionic liquids with respect to solvent nanostructure and how that is correlated to the amphiphile (lipids, surfactants and block polymers) self-assembly ability of ionic liquids [61]. The same research group proposed a micelle-like structure for alkylammonium protic ionic liquids such as butylammonium nitrate in the presence of water [62]. The alkyl groups are pointing inward in a circular arrangement with little inter-digitation and the polar ammonium group is on the outside. Nitrate (or formate) is interacting with both the positively charged nitrogen atom and bulk water however water-water interaction is considered more favorable than water-anion interaction. It is highly possible that the structure of IPAF with water may also (at least in part) be like a micelle structure with the polar ammonium formate groups exposed outside, and the more hydrophobic isopropyl groups gathered inside with potential inter-digitation.

Such a micelle structure with “peptide-like” polar groups and the isopropyl groups less accessible may explain in part the better protein stability in IPAF as compared to an organic solvent. Based on a study of trimethylamine-N-oxide, the alkyl groups attached to the strongly electron withdrawing quaternary nitrogen are significantly more hydrophilic than those found in a hydrocarbon and this may be a partial explanation for the protein stabilizing capability of this zwitterionic compound [63]. Based on neutron diffraction data, propylammonium nitrate was thought to self-assemble into a nanostructure resembling a L₃ sponge phase [64]. If water is present with the ionic liquid, the sponge phase likely has an interpenetrating network of channels defined by the ionic liquid forming the micelle-like channel walls, with water present inside and outside the channels.[65]. Certainly, the mechanism of protein stabilization by ionic liquids is complicated and deserves further study.

4. Conclusion

We have shown that IPAF is a better choice as a mobile phase modifier solvent for RPLC to improve protein stability as compared with a conventional solvent such as MeCN. We tested five proteins with different numbers of subunit, including albumin, AMY, THY, GDH and LDH. Based on the denatured/native peak area ratios in the chromatograms, 20% or 30% MeCN will largely denature the proteins, while for all the proteins except LDH, they are still in the native conformation within and up to 50% IPAF. The advantage of using IPAF as the RPLC mobile phase modifier is found to be more pronounced for multi-subunit proteins such as THY, GDH, and LDH. Although IPAF does more markedly denature LDH than the other proteins, the stability was still better than when MeCN was used, based on the small native peaks in the chromatograms and the enzymatic assay results. The best explanation of the IPAF effect on protein stabilization may be provided by the Hofmeister series. In the presence of water, the likely kosmotropic formate anion and the chaotropic isopropylammonium cation would interact more strongly with the native form of the protein, increasing its stability, and hindering unfolding.

Further work will concentrate on using mobile phase additives, such as polyethylene glycol mixed with IPAF, to improve the stability of labile proteins such as LDH during RPLC. This initial study is an important step for the possibility of semi-preparative RPLC of native proteins, which is the long-term goal of this project.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Isopropylammonium formate (IPAF) can replace organic solvent mobile phase modifiers for liquid chromatography of proteins.
- Proteins can more effectively be maintained in their native form using IPAF than using acetonitrile (MeCN).
- Enzyme activity of lactate dehydrogenase is evident using IPAF as the solvent as compared to MeCN.

Figure 1.

A and B. The overlay chromatograms of albumin using IPAF (A) and MeCN (B) as the mobile phase modifier. The chromatograms from bottom to top are: (A) 10% IPAF, 20%

IPAF, 30% IPAf, 40% IPAf, and 50% IPAf. The insert is the enlarged chromatograms of the denatured peaks (from retention time 3 to 10 min). (B) 10% MeCN, 20% MeCN, 30% MeCN, 40% MeCN, and 50% MeCN. Other conditions: albumin concentration: 100 ppm, flow rate: 0.5 mL/min, injection volume: 20 μ L.

C. Ratio of denatured to native albumin fluorescence peak areas obtained from 10% to 50% IPAf (◆) and 10% to 50% MeCN (▲) in Figure 1(A) and (B).

Figure 2.

A and B. The overlay chromatograms of AMY using IPAF (A) and MeCN (B) as the mobile phase modifier. The chromatograms from bottom to top are: (A) 10% IPAF, 20%

IPAF, 30% IPAF, 40% IPAF, and 50% IPAF. The insert is the enlarged chromatograms of the denatured peaks (from retention time 3 to 10 min). (B) 10% MeCN, 20% MeCN, 30% MeCN, 40% MeCN, and 50% MeCN. AMY concentration: 100 ppm, other chromatographic conditions are the same as Figure 1(A) and (B).

C. Ratio of denatured to native AMY fluorescence peak areas obtained from 10% to 50% IPAF (◆) and 10% to 50% MeCN (▲) in Figure 2(A) and (B).

Figure 3.

A and B. The overlay chromatograms of THY using IPAF (A) and MeCN (B) as the mobile phase modifier. The chromatograms from bottom to top are: (A) 10% IPAF, 20% IPAF, 30% IPAF, 40% IPAF, and 50% IPAF. The insert is the enlarged chromatograms of the denatured peaks (from retention time 3 to 10 min). (B) 10% MeCN, 20% MeCN, 30% MeCN, 40% MeCN, and 50% MeCN. THY concentration: 100 ppm, other chromatographic conditions are the same as Figure 1.

C. Ratio of denatured to native THY fluorescence peak areas obtained from 10% to 50% IPAF (◆) and 10% to 50% MeCN (▲) in Figure 3(A) and (B).

Figure 4.

A and B. The overlay chromatograms of GDH using IPAF (A) and MeCN (B) as the mobile phase modifier. The chromatograms from bottom to top are: (A) 10% IPAF, 20% IPAF, 30% IPAF, 40% IPAF, and 50% IPAF. The insert is the enlarged chromatograms of the

denatured peaks (from retention time 3 to 10 min). (B) 10% MeCN, 20% MeCN, 30% MeCN, 40% MeCN, and 50% MeCN. GDH activity: 3.5 units/mL, other chromatographic conditions are the same as Figure 1.

C. Ratio of denatured to native GDH fluorescence peak areas obtained from 10% to 50% IPAF (◆) and 10% to 50% MeCN (▲) in Figure 4(A) and (B).

Figure 5.

A and B. The overlay chromatograms of LDH using IPAF (A) and MeCN (B) as the mobile phase modifier. The chromatograms from bottom to top are: (A) 10% IPAF, 20% IPAF, 30% IPAF, 40% IPAF, and 50% IPAF. (B) 10% MeCN, 20% MeCN, 30% MeCN, 40%

MeCN, and 50% MeCN. LDH activity: 145 units/mL, other chromatographic conditions are the same as Figure 1.

C. Comparison of enzyme activity of LDH under different percentages of IPAF (◆) and MeCN (▲).

Table 1

Protein information summary

	Albumin	AMY	THY	GDH	LDH
Subunit number	monomer	monomer	dimer	tetramer	tetramer
Molecular weight (kDa)	66.5	97	660	310	140
Tryptophan number	1	14	44	24	8