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Deproteinization of Modelled Polysaccharides using Selected Alkyl Chain Length Ionic Liquid

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Abstract Bovine serum albumen (BSA) at an isoelectric point is adsorbed on the surface of unmodified cellulose. This work sets out to research into the yield of modelled polysaccharides(cellulose) in a mimicked purification process (separation of modelled proteins, BSA, from modelled polysaccharides) using five different alkyl chain length of 1-alkyl-3-methylimidazolium chloride, [$C_n$mim]Cl ionic liquids and inorganic salts, K_2HPO_4 /K_2CO_3 / K_3PO_4in an aqueous two phase system. The solubility data was modelled using the Merchuck's empirical equation. Without optimization process, (38.58%) of deproteinized polysaccharide was extracted into the bottom salt-rich phase of the selected aqueous two phase system (ATPS) [C_8mim]Cl/K_2CO_3.It was confirmed that the structural conformation of separated polysaccharides and proteins in the selected ternary system was intact after extraction of proteins into the upper ionic liquid-rich phase and polysaccharides into lower salt-rich phase respectively using Fourier Transform Infrared (FT-IR) spectroscopy. In conclusion, optimization of ternary system, [C_8mim]Cl + K_2CO_3,using designed conditions of a food application technology in the near future, can provide a promising extraction efficiency of deproteinized and structurally conformed polysaccharides.

Keywords 1-octyl-3-methylimidazolium chloride; Aqueous two phase system; Spectral analysis; Extraction efficiency; Phase diagrams; Phenol-sulfuric analysis

1. Introduction

Polysaccharides are extensively utilized in the pharmaceutical industry. It is sometimes referred to as "texture modifier" due to its ability to give products the needed texture. Considering its non-toxicity nature, it is the choicest for the pharmaceutical world [1]. In search of dry binders for tablets, cellulose and starch are the most appropriate. Most importantly in drug formulations, these polysaccharides maintain drug concentration in the blood system since it has the ability to slowly release the active compound [2].

After extraction of crude polysaccharide, ion-exchange chromatography, ethanol precipitation [3] and gel permeation chromatography [4] are some few traditional processes used in the purification of polysaccharides. These techniques can be time consuming, not easily scalable and expensive. With the rising and urgent need of polysaccharide, aqueous two phase system (ATPS) can provide a short processing time and an easy scale-up for the separation scientist [5].

Pei, Li, Liu, Wang, & Wang, [6] successfully separated BSA from aqueous saccharide solutions using the ionic liquid, [C_4mim]N$(CN)_2$], together with the salt, K_2HPO_4, and observed increase in extraction efficiency with increasing number of hydroxyl groups of the saccharide (mono-, di-, poly- saccharide). Aside the kind of saccharide that directly influences the extraction efficiency, the alkyl length of the ionic liquid used in extraction has also been found paramount. Pei, Wang, Wu, Xuan, & Lu, [7] established higher extraction efficiency of BSA with increase

alkyl length. But the effect of increased alkyl length on the extraction yield of polysaccharides in a purification process (deproteinization) has not yet been revealed by research.

Nevertheless, alinear relationship has been established between chain length and hydrophobicity [8]. This report established that there is much higher ability of longer-chain ionic liquids to be incorporated into cell membrane. Hence, increase in alkyl chain length results in increased toxicity [9-11]. Wells & Coombe [12], Carson et al., [13] further confirmed this observation by reporting on low toxicity with shorter (C_2-C_4) alkyl chains. With concerns on toxicity of long chain ionic liquids, research has revealed the ability of isolated marine-derived bacteria to degrade 1-alkyl-3-methylimidazolium chloride [$C_n$mim]Cl ionic liquids [14]. Influenced by this information, this research resolved to reveal the impact of alkyl chain length on needed yield of polysaccharides during purification process (deproteinization) using [$C_n$mim]Cl. The structural conformation of the proteins (BSA) and polysaccharides (cellulose) extracted into the best extraction ternary system was also determined using FT-IR spectrophotometer. The binodal curves for the solubility data of the ionic liquids, [C_4mim]Cl, [C_6mim]Cl, [C_8mim]Cl, [C_{10}mim]Cl, [C_{12}mim]Cl + K_2HPO_4 /K_2CO_3 / $K_3PO_4$was also reported in this paper. The specific objectives of this work was: (I) Construct phase diagram to ascertain the solubility of each salt in alkyl chain length ionic liquids. (II) Monitor pH of ATPSs to select the best salt for ATPS formation for this work. (III) Quantify extracted yield of polysaccharides and proteins in the different ionic liquid ATPSs formed with the selected salt. (IV) Establish the structural conformation of separated polysaccharides and proteins by the best ternary system.

2. Materials and method

2.1. Materials

K_2HPO_4 /K_2CO_3 / K_3PO_4, bovine serum albumin (BSA) and cellulose (Mr~2000) were purchased from Sinopharm Chemical Reagent Co. Ltd, China. [C_4mim]Cl, [C_6mim]Cl, [C_8mim]Cl, [C_{10}mim]Cl, [C_{12}mim]Clwere purchased from Cheng Jie Chemical, China. Chemicals were used without any further purification and deionized water was used throughout the experiment.

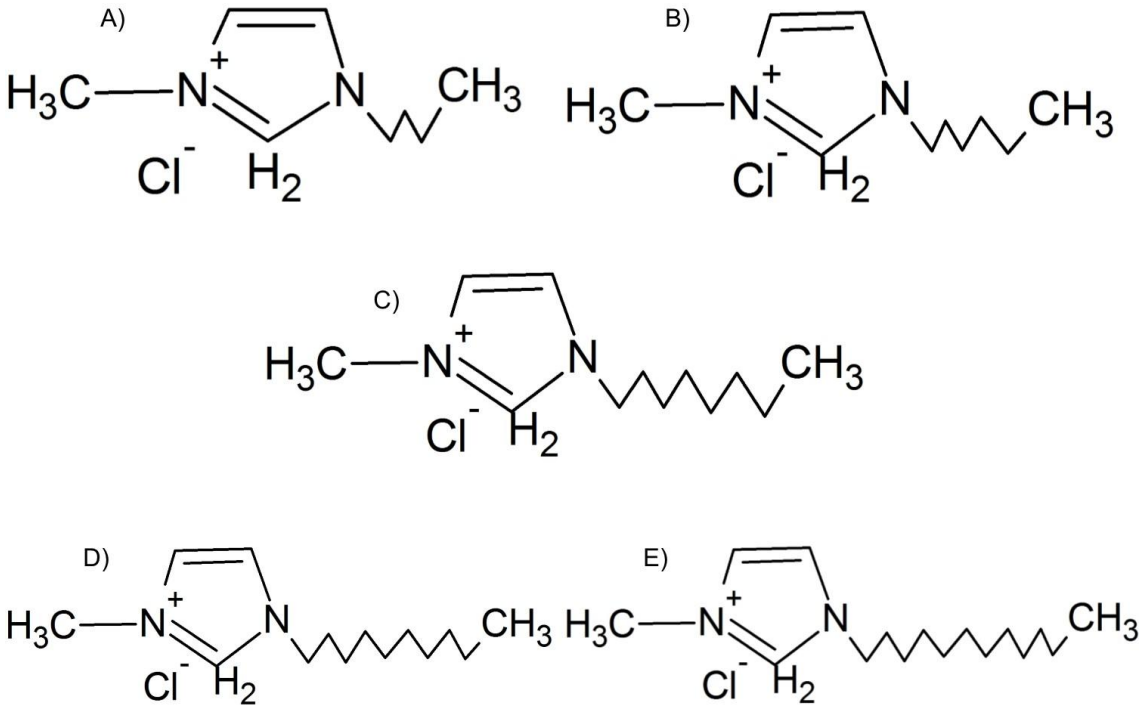

Figure 1: Chemical structures of ionic liquids used in the work:A) [C_4mim]Cl B) [C_6mim]Cl C) [C_8mim]Cl D) [C_{10}mim]Cl E) [C_{12}mim]Cl.

2.2. Phase diagram

The binodal curve was obtained using the cloud point method [5]. Briefly, 5g of a stock solution of ionic liquid (100 %) was weighed into a 25 ml conical flask. The flask was then weighed and the stock solution of the salt was added drop-wise until the first sign of turbidity. This was carried out while the system was continuously being mixed. The weight of the salt solution (80 %) necessary to the cloud point formation was noted. A known weight of diluent was then added to below the cloud point and the procedure was repeated. The conical flask was immersed in a jacketed glass vessel, and the temperature was maintained at T=298.15 K at atmospheric pressure. Merchuck's empirical equation was used to model the solubility curves [15]. The agreement between experimental and correlated data was analyzed in terms of the correlation coefficients (R^2) and relative standard deviation (σ)

$$Y = A \exp [(BX^{0.5}) - (CX^3)] \quad (1)$$

Where Y and X, are the [$C_{4, 6,8, 10,12}$mim]Cl and salt mass fraction percentages, respectively, and A, B and C are the regression constants.

2.3. Selection of ternary system

Five different ionic liquids ([$C_{4,6,810,12}$mim]Cl), each with three different salts was used to form a series of ATPS and each combination with three different compositions (25/15/60, 35/17/48, and 45/21/34) used by (Gutowski et al., 2003) was prepared. The highest salt percentage mixture with a visible solubility was noted. The pH of top and bottom phase was then measured using a pH meter.

2.4 Separation of proteins and polysaccharides

To a 15 ml centrifuge tube, an amount of [$C_n$mim][Cl], water, salt, cellulose and BSA were added (Fig. 2). Another mixture with the same phase components without cellulose and BSA was prepared as a blank to avoid interference. The mixture was vigorously stirred to make the salt dissolve completely. Phase separation was speeded up by the centrifugation at 2000 rpm for 5 min, and two clear phases was formed. The top phase was composed of ionic liquid and protein, whiles the bottom phase was salt and cellulose. The volume of each phase was noted.

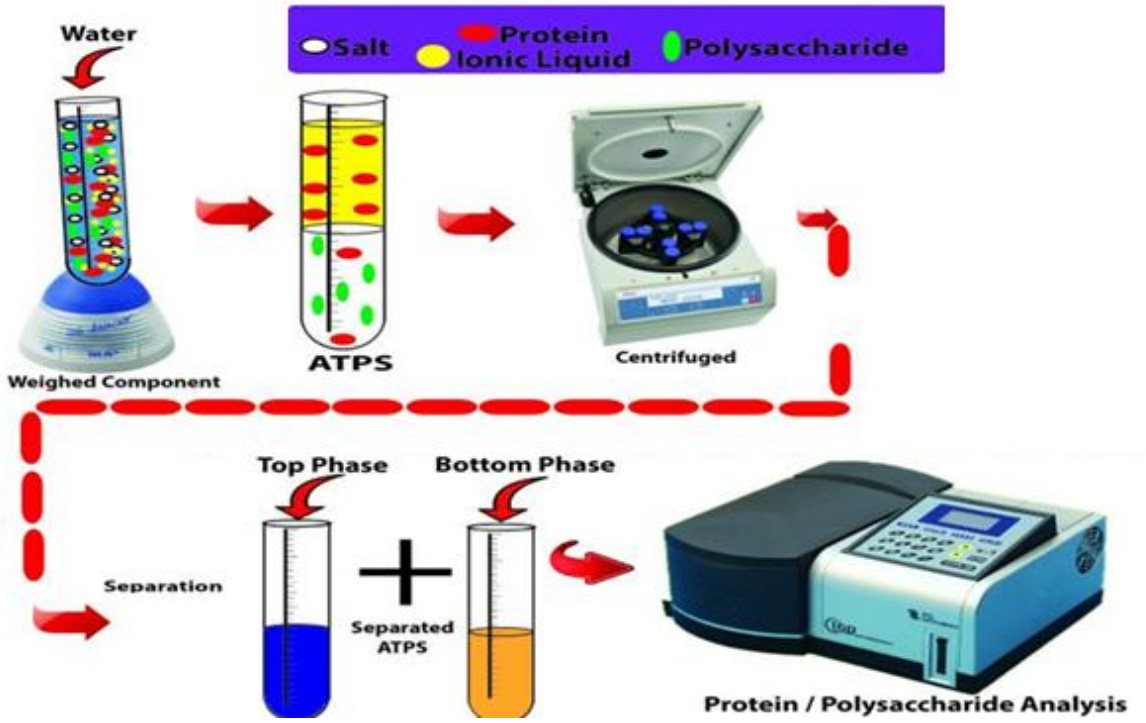

Figure 2: A schematic diagram of aqueous two phase system formation, separation and analysis of extracted polysaccharides and proteins

2.5. Analysis of extracted proteins and polysaccharides

The top and bottom phase was separated for protein concentration analysis using Bradford method (Bradford, 1976)with Coomassie Brillant Blue G250. Total protein concentration was determined using BSA as standard. Briefly, 0.1 ml of IL-rich phase was withdrawn into a 10 ml colorimeter tube and diluted to 1.0 ml, then 5.0 ml of Coomassie Brillant Blue G250 solution was added and the tube was allowed to stand for 5 mins after being well shaken. Samples were measured at 280 nm by a UV-vis spectrophotometer. The extraction efficiency of BSA into the IL-rich phase was calculated by using the equation (2):

$$Ep = C_t V_t / (C_t V_t + C_b V_b) \quad (2)$$

Where V_t and V_b represent the volume of the top and bottom phase, C_t and C_b are equilibrium concentrations of the partitioned BSA in the IL-rich top phase and the salt-rich bottom phase respectively.

The bottom phase was also analyzed for polysaccharide using phenol – H_2SO_4 method. Briefly, 0.2 ml of aqueous cellulose solution was pippeted into a colorimetric tube. Then 1 ml of 5% phenol was added. Additionally, a 5 ml concentrated sulfuric acid was added rapidly. It was well shaken and allowed to stand for 10 mins. It was then placed in a water bath for 20 min (25-30^{o}C) and samples were read at 490 nm. The extraction efficiency of polysaccharide into the salt-rich phase was calculated by using equation (3):

$$E_{ps} = C_b V_b / C_o V_o \quad (3)$$

Where C_b and V_b are equilibrium concentrations of the partitioned polysaccharide in the salt-rich bottom phase and the volume of this phase, C_O and V_O represent the concentration and volume of aqueous polysaccharide added into the test tube before the separation process.

Also noted was the phase volume ratio (R) which is defined as volume ratio of the top phase to the bottom phase.

$$R = V_t / V_b \quad (4)$$

Where V_t and V_b represents the volume of the top and bottom phase respectively.

2.6. FT-IR spectra

The spectra of cellulose and BSA in the best yield ternary system ($C_n$mim)Cl /saltwas determined to expose change in their conformation. The top IL-rich phase containing BSA and bottom salt-rich phase containing cellulose was collected to measure the FT-IR spectra. The blanks containing the same phase composition but without cellulose and BSA was used as reference solution. FT-IR spectra were recorded by using a (Thermo Nicolet IS50) FT-IR spectrophotometer with a resolution of 4 cm^{-1} in a region of 4000-400 cm^{-1}.The concentration of Cellulose and BSA used was 4.0 $mgmL^{-1}$.

3. Results and Discussion

3.1. Phase Diagram

The addition of alkyl chains into some chemical compounds (salt) can increase its lipophilicity (Mallavadhani, Sahoo, Kumar, & Murty, 2014). The Figure 3a) showed that the ability of the salts for phase separation with the shortest chain length, [C_4mim]Cl followed the order: K_3PO_4>K_2HPO_4>K_2CO_3. This agree with the trend established for [C_4mim]Cl +K_3PO_4/K_2HPO_4/K_2CO_3(Li, Pei, Wang, Fan, & Wang, 2010)(Pei, Wang, Liu, Wu, & Zhao, 2007).However, with an increasedchain length,[C_6mim]Cl, the lipophilicity of $K_3PO_4$was thus increased hence the order: K_2HPO_4>K_3PO_4>K_2CO_3(Fig 3b). The order observed in Fig 3c):K_2HPO_4>K_2CO_3>K_3PO_4, depicted further increase in lipophilicity, reducing its ability to dissolve easily for phase separation.The salt K_2CO_3being a strong alkaline and hydrophilic but not lipophilic in nature was not affected by the alkyl chains.

However, $K_2HPO_4$is highly water soluble when compared to thesolubility of K_2CO_3. This may be attributed to the variations in solubility with reference to the solubility table. At a standard atmospheric pressure and a temperature of 20^{O}C, the solubility of K_2HPO_4 recorded150g/100g with that of K_2CO_3being, 111g/100g. At a higher temperature of

30^{O}C, the solubility of K_2CO_3has shown to be 114g/100g, obviously much lower. This explains the phase separation formation order in Fig 3c) K_2HPO_4>K_2CO_3>K_3PO_4.

K_2HPO_4could not form immiscibility with [C_{10}mim]Cl and [C_{12}mim]Cl, hence Fig 3d) and e) did not show its binodal curve. As stated by Overton rule, the more lipophilic a substance, the faster the predicted permeation rate. With a much lengthier alkyl chain [C_{10}mim]Cl and [C_{12}mim]Cl, K_3PO_4 is be expected to be much more lipophilic. Hence, per the Overton rule, K_3PO_4 can dissolve faster than K_2CO_3 which is not lipophilic in nature. Fig 3d) and e) revealed the order of phase separation ability: K_3PO_4> K_2CO_3, which confirmed the Overton rule. The solubility data was modelled using the Merchuck's empirical equation (Table 1) and R^2 obtained ranged from (82.40-100).

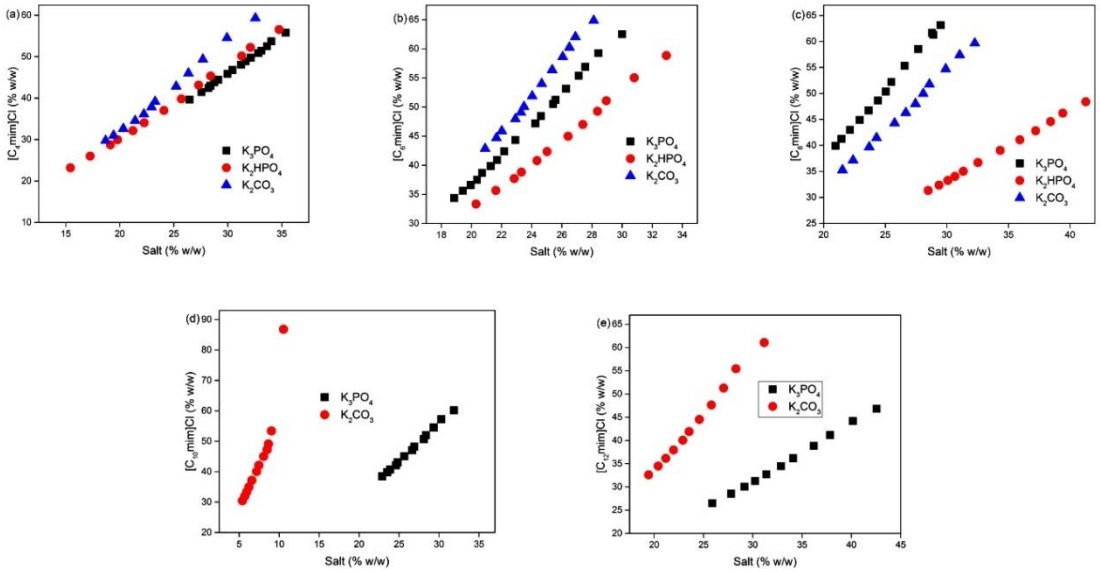

Figure 3: Phase diagram of (a) [C_4mim]Cl, (b) [C_6mim]Cl, (c) [C_8mim]Cl, (d) [C_{10}mim]Cl, and (e) [C_{12}mim]Cl and salts, K_3PO_4, K_2HPO_4, and K_2CO_3.

Table 1: Fitting parameters of eq. 1, correlation coefficients (R^2) and relative standard deviation (σ)

Ternary system	A	B	R^2	σ
[C_4mim]Cl+K_3P0_4	-3.913	0.7420	99.80	0.0909
[C_4mim]Cl+K_2HPO_4	-1.898	0.7027	99.80	0.1804
[C_4mim]Cl+K_2CO_3	-4.935	0.8841	99.70	0.2162
[C_6mim]Cl+K_3PO_4	-6.125	1.041	99.90	0.1293
[C_6mim]Cl+K_2HPO_4	-3.717	0.8277	99.80	0.1541
[C_6mim]Cl+K_2CO_3	-9.842	1.277	99.60	0.1843
[C_8mim]Cl+K_3PO_4	-2.913	0.5400	100.00	0.0514
[C_8mim]Cl+K_2HPO_4	-7.300	1.104	99.80	0.1338
[C_8mim]Cl+K_2CO_3	-6.032	0.9284	99.80	0.1656
[C_{10}mim]Cl+K_3PO_4	-7.559	0.997	99.80	0.139
[C_{10}mim]Cl+K_2CO_3	-8.410	3.494	82.40	2.6482
[C_{12}mim]Cl+K_3PO_4	-2.511	0.4995	99.9	0.0977
[C_{12}mim]Cl+K_2CO_3	-6.891	1.010	99.6	0.2318

3.2. Choice of salt

Glutelin, an alkaline soluble protein in rice was observed to have obtaineda 98% extraction efficiency for glutelin at a pH of 11.8 [16]. Meanwhile, Abdel-Aal, Youssef, Adel-Shehata, & El-Mahdy, [17], revealed a drastic reduction in the solubility of glutelin at pH below 10. Furthermore, a maximum percentage of corn protein was extracted at a pH of 11.6 [18]. Increasing alkalinity leads to higher extraction efficiency, but, research also showed that, cotton seed protein was found prone to degradation, denaturation and difficult to be kept in strong alkaline condition and therefore pH of 12.5 was the recommended cut off point [19]. Critical study of (Table 2) showed that ionic liquid,

[$C_n$mim] Cl + K_2CO_3 provided the most appropriate top phase with pH that ranged from (12.45-13.00) for the solubility and thus separation of proteins from polysaccharides.

Yam polysaccharide which is alkaline in nature also recorded a rise in the level of extraction from pH of 8-10 and a decrease after exceeding a pH of 11, which can be attributed to damage of certain structures [20]. It is therefore obvious that but for an excellent separation of proteins from the needed polysaccharides (deproteinization), K_2HPO_4, was most likely to have provided the most appropriate bottom phase pH (9.68 – 10.12) for polysaccharide extraction. It is also worth noting that, K_2HPO_4, could not form ATPSs with [C_{10} mim]Cl and [C_{12} mim]Cl. Thus K_2CO_3 was the choicest for ATPS construction for this work.

Table 2: pH values of the top (T) and bottom (B) phases for the extraction systems composed of [$C_n$mim]Cl + inorganic salt + water, at 298K and atmospheric pressure.

Ternary system		**pH (T)±0.02**	**pH (B)±0.02**
K_3PO_4	[C4mim]Cl	12.83	13.25
	[C6mim]Cl	14.94	14.52
	[C8mim]Cl	13.48	13.97
	[C10mim]Cl	13.47	13.57
K_2CO_3	[C4mim]Cl	12.82	12.72
	[C6mim]Cl	12.57	12.61
	[C8mim]Cl	13.00	13.22
	[C10mim]Cl	12.69	12.82
	[C12mim]Cl	12.45	12.45
K_2HPO_4	[C4mim]Cl	9.81	9.83
	[C6mim]Cl	10.24	10.12
	[C8mim]Cl	9.66	9.68

3.3. Selection of Alkyl Length

Simultaneous separation of modelled polysaccharide (cellulose) and modelled protein (Bovine Serum Albumin) in [$C_n$mim]Cl/K_2CO_3 ternary systems (ionic liquid, salt, and water) with percentage composition (35/17/48) under room temperature was obtained. With the exception of [C_{12}mim]Cl that displayed a non-working volume bottom phase, it was observed that polysaccharides yield obtained under the four different alkyl length IL was in the order: [C_8mim]Cl > [C_{10}mim]Cl = [C_6mim]Cl = [C_4mim]Cl. Notably high amount of protein extracted (60.11%) into the top phase of the aqueous two phase system, [C_8mim] Cl + K_2CO_3, caused water molecules to be made more readily available for cellulose extraction (38.58%) in the bottom phase, making it a choice alkyl length ternary system (Fig. 4.).

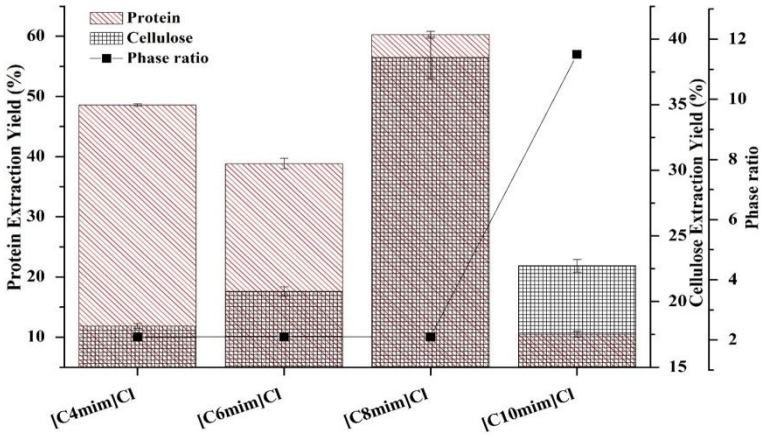

Figure 4: The extraction efficiency of BSA (top phase) and cellulose (bottom phase) in [$C_{4,6,8,10}$ mim]Cl + K_2CO_3 ATPSs at 298K and the corresponding phase ratio for each ILATPS. The test concentrations of BSA and cellulose were all at 4 mgmL^{-1}.

3.4. Before and After Conformation of Protein

In the quest to establish the conformation of protein before and after extraction, the FT-IR spectra of BSA (modelled protein) was measured. Amide linkages is core to protein molecular feature. Amide linkages in the biochemical arena are called peptide bonds when located in the main chain of a protein and isopeptide bonds in a side-chain of the protein.

Amide bond (-CONH) formation has been found to be represented by following on the FT-IR spectra: overlapping region of –C=O and N-H bands (1600-1650 cm^{-1}) and disappearance of the overtone at 3081 cm^{-1} attributed to the secondary bending of N-H at 1533 cm^{-1}(Azeredo & Waldron, 2016). As observed in Fig. 5(a), the amide band (at 1630 cm^{-1}) of the protein (BSA) was closely marked by the spectra of BSA in ionic liquid, [C_8mim]Cl. The IR band of [C_8mim]Cl was found in the same region after extraction. IR bands did not show any shift or disappearance, which signifies no change in conformation of the protein in the IL-phase after extraction.

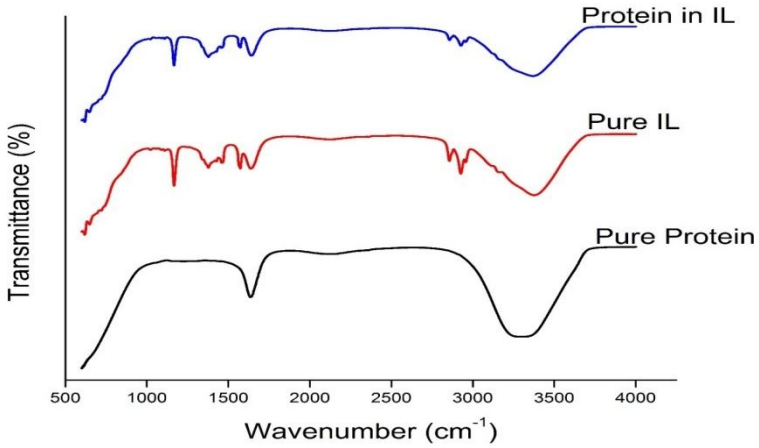

Figure 5a: FT-IR spectra of separated proteins

3.5. Before and After Conformation of Polysaccharide

Filippov [21], established that, most carbohydrate display high absorbance at (1200 – 950 cm^{-1}), allowing for polysaccharide identification. Peaks at (1630cm^{-1}) has been ascribed to the absorption of the carboxylic group (COO-deprotonated) and stretching peaks toward (1080-1150 cm^{-1}) also suggest the presence of C-O bonds [22]. In agreement with these findings, Fig. 5 (b) of this work showed stretching peaks (at 1630 cm^{-1}) of the cellulose to be closely marked by the spectra of cellulose in salt (K_2CO_3). The IR band of salt (K_2CO_3) was also found in the same region after extraction. Again, IR bands did not show any shift or disappearance, which signifies no change in conformation of the cellulose in the salt-phase after extraction.

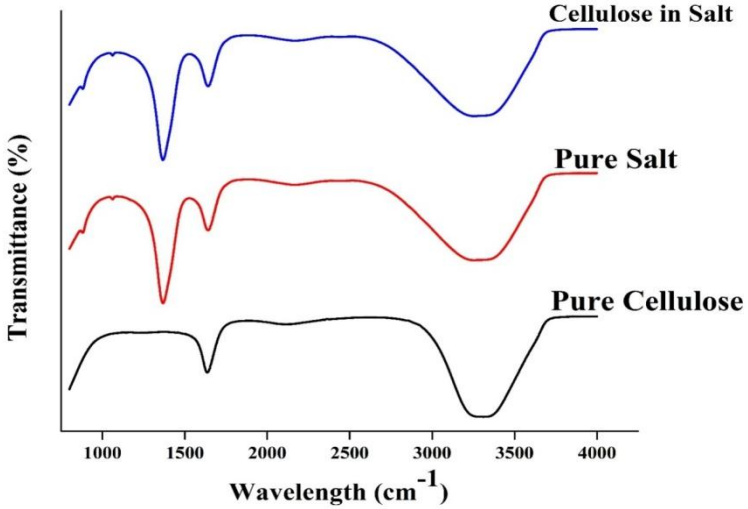

Figure 5b: FT-IR spectra of separated polysaccharides

4. Conclusion

The salt, K_2CO_3, though presented the second highest ability in easy formation of ATPS with the alkyl chain length $[C_8mim]Cl$, it was found to possess the most appropriate top phase pH (12.45-13.00) for an excellent separation of proteins from needed polysaccharides .The selected ternary system, $[C_8mim]Cl/K_2CO_3$, with ionic liquid, salt, and water percentage composition (35/17/48)extracted the highest amount of proteins (60.11%) from the needed polysaccharides (38.58%). The functional groups of polysaccharides and proteins was maintained after successful separation with the selected alkyl chain length ionic liquid ATPS.

Acknowledgments

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Declaration of Interest

None.

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