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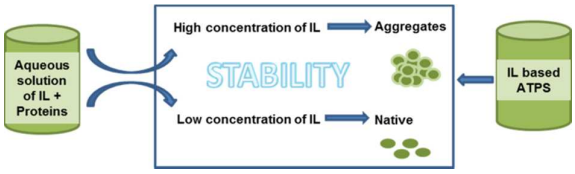
This article discusses extraction of Rubisco in ionic liquid (IL) based aqueous two phase system and stability of proteins in aqueous solutions of IL.



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ARTICLE TYPE

Extraction and stability of selected proteins in ionic liquid based aqueous two phase systems

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Ionic liquid-based aqueous two-phase extraction of a plant protein, Rubisco (Ribulose-1, 5-biphosphate carboxylase oxygenase), using Iolilyte 221 PG and sodium potassium phosphate buffer was investigated as a new alternative extraction method and compared with a conventional PEG–based two-phase system. The influence of various factors, such as concentration of phase components, pH and temperature on partitioning of Rubisco, was evaluated by design of experiments. Rubisco partitions to the ionic liquid (IL) phase and the partition coefficient for IL based two-phase system were 3-4 times higher than in a PEG-based system. Additionally, studies were done in aqueous solution of IL with varying concentrations to develop a relationship between IL concentration and protein stability. In addition to Rubisco, the stability of BSA and IgG1 was investigated in aqueous solution of two ionic liquids: Iolilyte 221PG and Cyphos 108. No fragmentation or aggregation was observed at 10% w/w concentration of the ionic liquid. However, all three proteins studied formed aggregates at 50% w/w concentration of ionic liquid. This indicates a narrow window for application of IL's in protein extraction.

Key Words: Rubisco, Ionic Liquid, ATPS, BSA, IgG1, Bio-refinery

Introduction

Aqueous two phase extraction of biomolecules has been widely studied over the past decades. First used by Albertsson [1] in the 1950's, the technique has gained attention because it combines several early processing steps such as clarification, concentration and primary purification in one step. The technique is used as a mild primary recovery step for reducing the processing volumes [2]. Aqueous two phase systems (ATPS) are formed by combining two aqueous solutions of polymer and polymer, polymer and salt or salt and salt. When these phase components are mixed beyond a certain critical concentration they separate in two distinct aqueous phases. Selective partitioning of the target molecule depends upon the affinity of the molecule for the phase component used (Polymer type, salt) and system parameters such as pH, temperature and tie line length. PEG is known to stabilise proteins and high water content (~80-90%) in the bulk of the two phases provides a gentle environment for the protein. Thus aqueous two phase extraction is a mild and the most suitable for extracting biologically active molecules[3-6]. This system has further developed by implementing a new class of extractants, "ionic liquids", to form an aqueous biphasic system. ATPS based on ionic liquids are formed by combining an ionic liquid (IL) solution with a salt solution [7], thus replacing the polymer component of the conventional polymer-salt two phase system.

Ionic liquids are salts, composed solely of ions with a melting point below 100°C. These ILs when compared to organic solvents have low vapour pressure, high solvation capacity and better thermal and chemical stability.

Ionic liquids are emerging as the new class of solvents with tuneable properties. The physical properties of ionic liquids such as polarity, hydrophobicity, viscosity can be controlled by permutation and combination of anions and cations. This high tuneability makes them a desirable class of extractants in liquid–liquid extraction. Ionic liquids are studied in many fields, ranging from inorganic synthesis [8], extraction of metals [9] to biocatalysis [10]. Despite the several interesting features, most ILs suffers the drawback of being expensive and poorly biodegradable. Nevertheless ILs could be attractive if they could be regenerated and reused.

Aqueous two phase extraction studies with ionic liquids normally involve the use of imidazolium ionic liquid as the cation. Coutinho and co-workers [11] have done some ATPS studies based on ionic liquid with phosphonium as the cation. The Kragl group [12] studied an ammonium based ionic liquid – Ammoeng 110 which is effective in forming aqueous two-phase and purifying enzymes (two different alcohol dehydrogenases); this IL can stabilize the enzymes and enhances the solubility of hydrophobic substrates. In another study extraction of proteins from biological fluid has been investigated using IL- based ATPS [13]. IL based ATPS have been mainly studied for extracting small proteins such as BSA, myoglobin, amino acids and small molecules such as caffeine, vanillin, penicillin and testosterone [14-17]. Apart from this, most of the research using ionic liquids focuses on formation and characterisation of two phase system while a few studies have been done on protein stability in ATPS [18, 19].

Leveraging on the advantages offered by ATPS and coupling the unique features of IL, such as controlled hydrophobicity, polarity and miscibility, could provide selective extraction of proteins from the biomass. Use of IL based ATPS for extracting commercially important proteins is not well explored. It is thus

important to study the extraction of commercial proteins and how it affects the stability of the protein after being extracted in the ionic liquid rich phase.

In this work, studies were done to systematically understand the relationship between IL concentration and protein stability with ATPS system. Thus, extraction of pure Rubisco (purity ~80%) in IL based ATPS (Iolilyte 221PG/Sodium-potassium phosphate ATPS) was evaluated as it would be the most interesting target for such an application. The influence of different process parameters on extraction were studied and compared with conventional two phase system (PEG/Potassium Citrate ATPS). Additionally the stability of Rubisco, together with two other model proteins BSA and IgG1 in aqueous solution of IL was studied.

The ionic liquid selected on the basis of literature study [17] are: Iolilyte 221PG-an ammonium based ionic liquid with oligopropyleneglycol unit containing side chain and Cyphos 108 (Tributyl(methyl) phosphonium methylsulfate)- an phosphonium based ionic liquid.

The commercial proteins selected are: Rubisco-a plant protein; Monoclonal antibody (IgG1)-a therapeutic protein; and Bovine Serum Albumin (BSA)-a model protein. BSA and IgG1 were selected for the study in an effort to understand the influence of ionic liquids on proteins with varying size, complexity and isoelectric point (IEP)(Table 1). Rubisco is the most abundant protein found in nature and amounts to nearly 50% of the total protein found in green parts of plants and microalgae. There has been a growing interest in this protein, as it has a high potential to be used as an ingredient in human / animal food [20]. Microalgae have a big potential to be a source for biofuel production. However in order to make this economically feasible, more products from microalgae need to be derived [21]. Thus, purification and efficient separation of microalgal proteins, namely Rubisco is important from the bio-refinery perspective [22].

Results and Discussion

In this work, partitioning of Rubisco was studied in two different ATPSs; polymer-salt and IL-salt. Influence of system components: type of polymer, salt and ionic liquid and system parameters: pH, temperature on the extraction of Rubisco was investigated using DoE. Furthermore, the stability of proteins (BSA, IgG1 and Rubisco) in different concentration of aqueous solutions of ionic liquid was investigated using Dynamic Light Scattering (DLS), size exclusion chromatography (SEC-HPLC) and gel electrophoresis (SDS-PAGE) and is discussed in this section.

Partitioning of Rubisco in PEG/salt system

Effect of system components

Different PEG molecular weights were studied to understand its effect on partitioning of Rubisco in aqueous two phase system. The protein partitioned preferentially to the PEG phase (Fig.1). However, with increase in the chain length of PEG the yield decreases due to precipitation of Rubisco at the interface. This could be attributed to the fact that an increase in the chain length of PEG decreases the free volume available in the top phase [23]. Thus there is not enough space to accommodate Rubisco, which is a large protein. Precipitation of Rubisco with increase in the molecular weight is also associated to the increase in hydrophobicity of the top phase. PEG 400 was selected for further studies since it showed good recovery and partitioning with no precipitation of Rubisco.

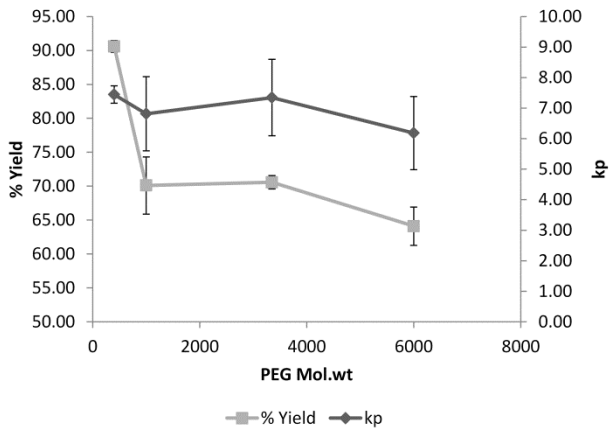

Fig.1 Effect of molecular weight on partition coefficient K_p & Yield

Different concentrations of salt and PEG were studied in order to evaluate their effect on partitioning of Rubisco. The salt concentration was increased from 24%w/w to 28%w/w and PEG concentration was increased from 24%w/w to 27%w/w. As shown in Fig. 2a & 2b there is marginal increase in the partition coefficient and recovery of Rubisco with increase in concentrations of phase components.

Effect of system parameter

The effect of pH on partitioning of Rubisco was evaluated by varying the pH from 6-8. The net charge on the protein varies with the pH of the ATPS and depending on the pI of the protein it influences the partitioning of protein between the two phases. Rubisco partitioned preferentially to the top phase at all pH studied. The partitioning to the top phase can be explained by the electrostatic interaction between the biomolecule and PEG [24]. From the previous studies by different authors[3, 24, 25], it is observed that negatively charged proteins partition to the PEG phase. Since the isoelectric point of Rubisco is between 5.5-5.7, it is negatively charged at all the pH studied and thus partitions to the PEG phase. The response contour plots (Fig. 2c and 2d) shows that an optimum extraction efficiency of ~93% and partition coefficient of ~12 is achieved at pH close to neutral ~7.

Partitioning of Rubisco in IL/salt system

Effect of system components

As observed for the PEG based ATPS, partitioning in IL-based ATPS is also influenced by several parameters such as concentration of IL and salt, pH and temperature. The effect of

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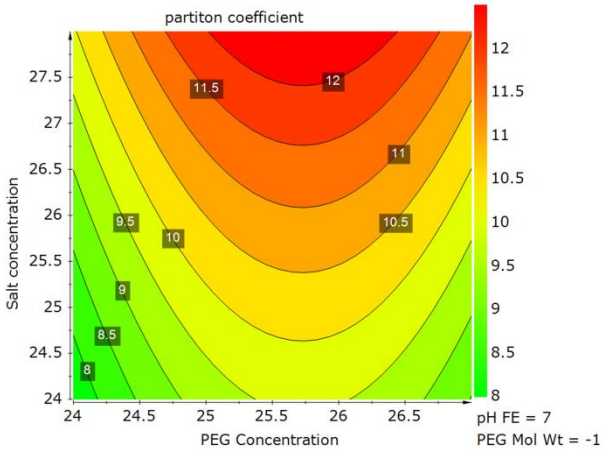

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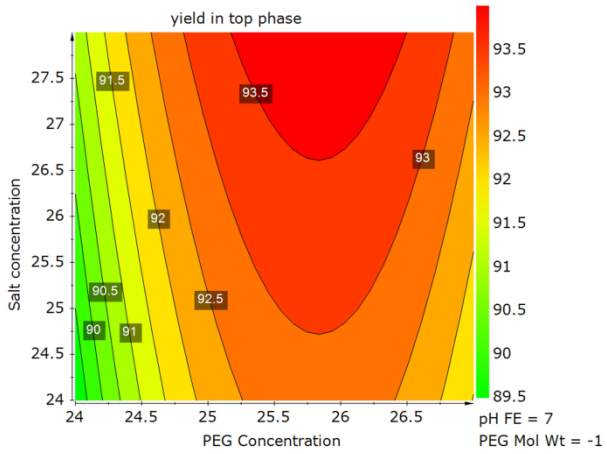

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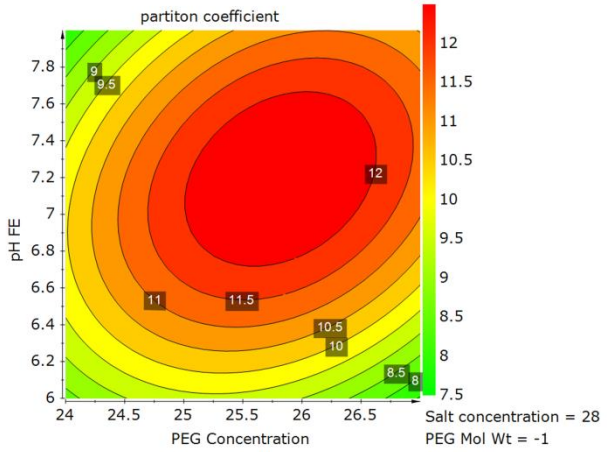

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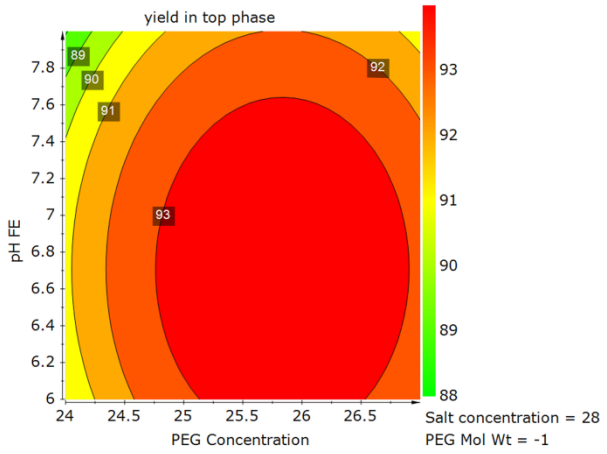


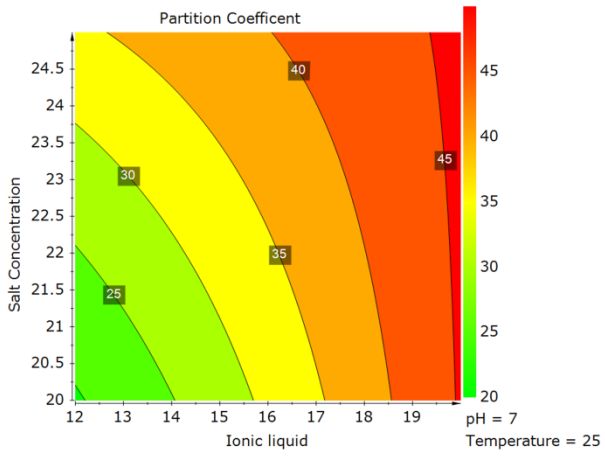
Fig.2 Contour plots of PEG and salt concentration effects on a) Partition coefficient, b) Yield of Rubisco and Effect of pH on c) Partition coefficient and d) Yield of Rubisco

varying concentration of phase components such as IL and salt on partitioning was investigated. Rubisco is partitioned preferentially to the top phase as the ionic liquid and salt concentration was increased from 12%w/w to 20% w/w and 20% w/w to 25% w/w respectively. The partition coefficient increases from 20 to 45 as the concentration of ionic liquid and salt is increased (Fig.3a). A corresponding increase in the yield of Rubisco was observed (Fig.3b). This trend of increased partition coefficient and yield is observed at pH 7 and temperature of 25°C. It is interesting to note that the partition coefficient obtained with the ionic liquid based ATPS is of the order 3-4 higher than PEG/salt system. These findings are in agreement with those done by Ruiz-Angel [26]. The authors [26] have reported that the partitioning of myoglobin, cytochrome C, haemoglobin and ovalbumin to the ionic liquid phase is of the magnitude 2-3 higher and is attributed to the vast difference in the polarity of the PEG rich phase and ionic liquid rich phase. The main driving force for partitioning of the protein to the ionic liquid phase is not well understood, though there have been some studies done in this direction[14, 17]. However, these studies suggest that the partitioning of Rubisco to the IL-rich top phase could be attributed to a combined effect of hydrophobic interaction, salting out effect and electrostatic interaction between the positively charged ammonium cation of the ionic liquid and the negatively charged amino acid residues at the surface of the protein.

Effect of system parameters

The influence of temperature and pH on formation of aqueous two phase is assessed by different authors [27,28]. The effect of pH on partitioning of Rubisco was studied in the range 6-8. The studies show that the partitioning of Rubisco increases with the increase in pH (Fig. 4a,b). Whereas, there is no significant change in the yield when the temperature is increased from 15°C to 35°C (Fig.4c,d).

a)



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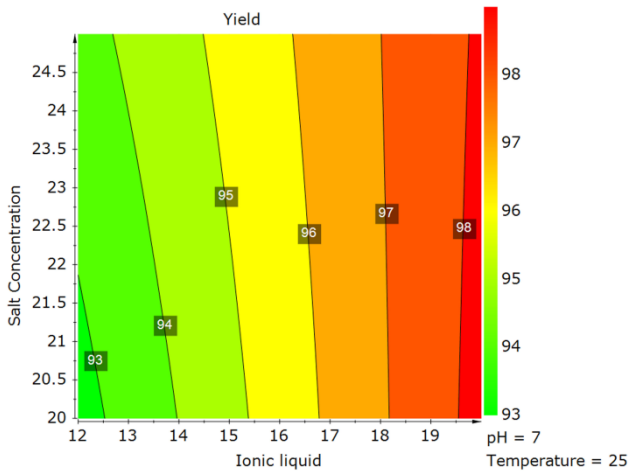

Fig.3 Contour plots of effect of IL and salt concentration on a) Partition coefficient and b) Yield of Rubisco

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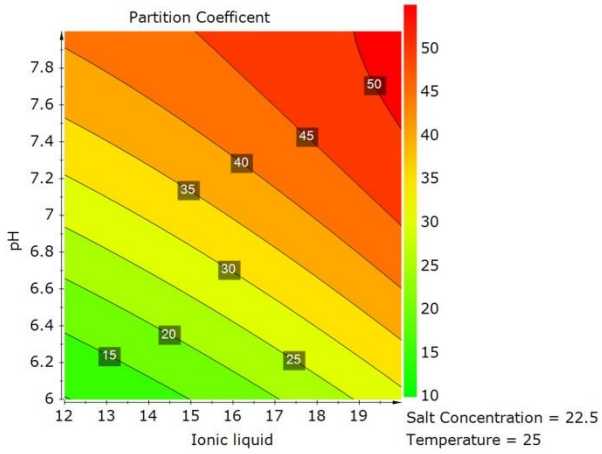

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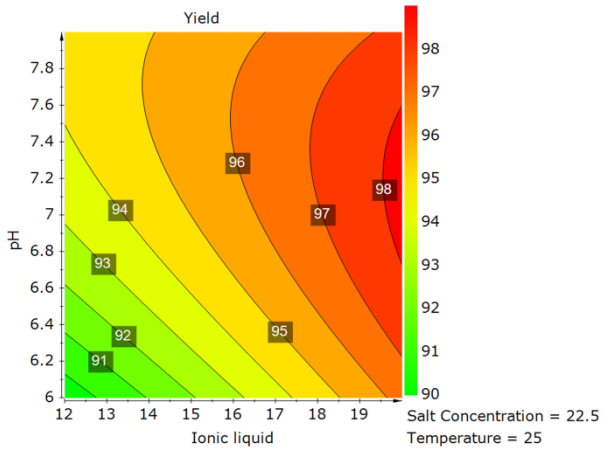

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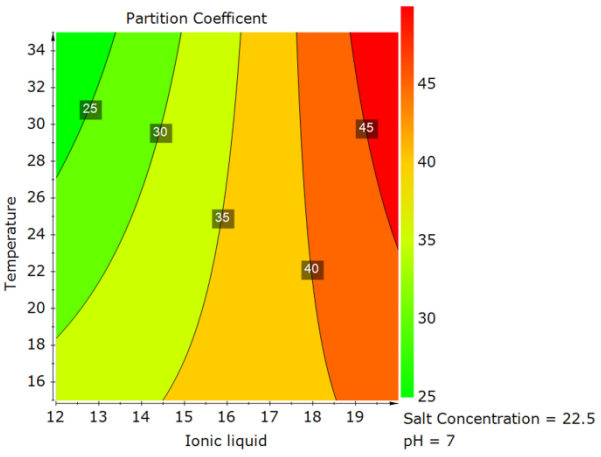

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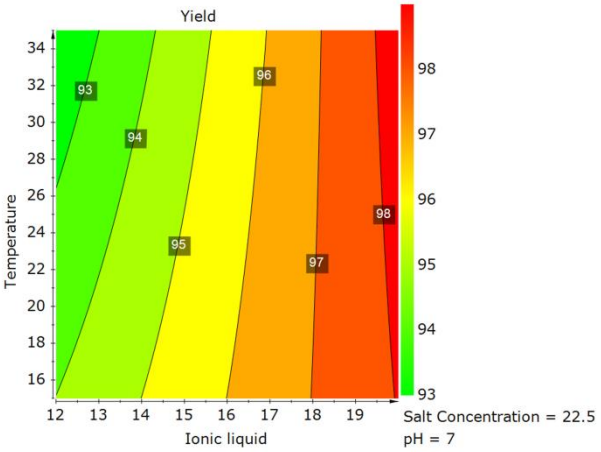

Fig.4 Contour plots of effect of pH on a) Partition coefficient b) Yield and Effect of Temperature on c) Partition coefficient d) Yield of Rubisco

Model fit and Analysis

A second order polynomial regression model was used to calculate the response contour plot for each response variable. These plots help to visualize the relationship between the variables and the responses [29]. The coefficient of the model's independent variable describes the effect of the variables on the response. The effects that had less than 95% significance were considered insignificant and discarded. The following reduced regression models were obtained for the responses; partition coefficient and yield for the two systems

PEG/Potassium citrate ATPS

$$K_p = 0.958 + 0.0315X_1 + 0.022\,X_2 + 0.0166X_3 + 0.0734X_4 + 0.046X_1X_4 - 0.040X_1X_3 + 0.0464X_2X_4 + 0.11X_1^2 - 0.0743X_2^2 - 0.096X_4^2$$

$$R^2 = 0.85$$

$$Y_e = -1.365 - 0.175X_1 + 0.053X_2 + 0.0115X_3 - 0.040X_4 - 0.022X_1X_3 - 0.044X_1X_4 - 0.046X_3X_4 + 0.356X_1^2 - 0.121X_2^2 - 0.07X_4^2$$

$$R^2 = 0.91$$

Iolilyte 221PG/Sodium Potassium phosphate system

$$K_p = -0.263 + 0.188X_1 + 0.085X_2 + 0.307X_3 - 0.028X_4 - 0.080X_1X_2 - 0.095X_1X_3 + 0.096X_1X_4 - 0.11X_2X_4 - 0.11X_3^2$$

$$R^2 = 0.92$$

$$Y_e = 1.98 + 0.011X_1 + 0.0009X_2 + 0.006X_3 - 0.001X_4 - 0.001X_1X_2 - 0.004X_1X_3 + 0.002X_1X_4 - 0.002X_2X_4 - 0.006X_3^2$$

$$R^2 = 0.92$$

The value of R^2 defines how well the model fits the data. In the model fit, the observed R^2 value shows a good correlation with the predicted values. The statistical significance of regression model was evaluated by the analysis of variance (ANOVA). The ANOVA analysis (SI, Table 1) performed on the two systems shows that the model is significant. The first F-test, which compares variation in regression model and residuals, is satisfied when $p < 0.05$. The second test also known as the lack of fit test, compares the model and replicate errors and is satisfied when $p > 0.05$. According to the ANOVA, all parameters, PEG mol. wt., PEG and salt concentration and pH have significant influence on the partitioning (K_p) of Rubisco in PEG based ATPS. However, the influence of PEG mol. wt. on yield is very prominent. Rubisco precipitates at high mol. wt. PEG and thus low yields. Similarly for the IL based ATPS, as seen in the response contour plots (Fig.3, 4) IL, salt and pH substantially influences the partitioning of the Rubisco to the IL phase.

Stability of the protein

Proteins are complex macromolecules and retain their structural and functional stability in native environment. Small changes in the protein environment such as temperature, pH and solvent can alter the native fold of the protein. Studies on protein interaction with ionic liquid have been performed by different authors[30-32].

The partition coefficient obtained for the IL/salt ATPS is much higher than the PEG based systems. Nevertheless the stability of the protein in the extracted phase is a prerequisite. The samples of the IL-rich top phase were analysed by gel electrophoresis and SEC-HPLC. In the SEC-chromatogram (Fig.5), the band of Rubisco in the top phase is not visible. This result is in accordance with the native gel which shows a very faint band (data not shown). This indicates that Rubisco does not retain its native structure in the IL rich phase.

Although the starting concentration of IL-based ATPS was varied from 12-20%w/w, depending on the partitioning and resulting volumes of each phase, the concentration of ionic liquid in IL-rich phase can vary considerably. Therefore, to understand the relationship between ionic liquid concentration and protein stability, pure Rubisco was dissolved in increasing amount of Iolilyte 221 PG and samples were run on SEC-HPLC.

The SEC-HPLC chromatogram (Fig.6a) shows a progressive decrease in Rubisco peak intensity with increase in ionic liquid concentration. Additionally, there is no visible fragmentation of Rubisco observed in the SEC chromatogram. This is further confirmed by gel electrophoresis (Fig.6b). However, there is some amount which does not migrate and remains in the well. These studies indicate probably, formation of high molecular weight aggregates with increasing concentration of ionic liquid. The aggregates are too large to pass through the SEC column and hence not observed in SEC chromatogram. Under denaturing conditions (use of SDS and β-mercaptoethanol) these aggregates are reduced to the Rubisco subunits (55 and 13 kDa) and migrate through the gel (Fig.6b). However, at higher concentration of the ionic liquid, some amount of protein is still observed in the well under denaturing condition. This indicates that the aggregates so formed are linked by both non-covalent and/or disulphide linkages.

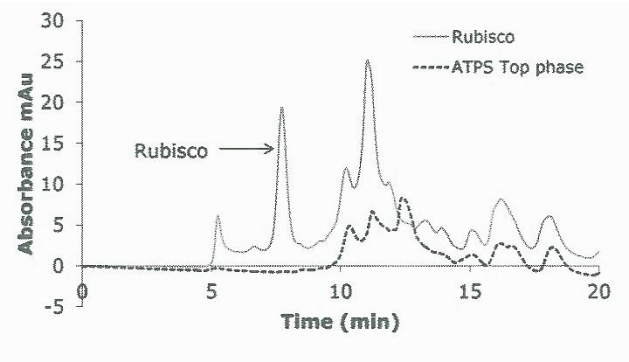

Fig.5 Size exclusion chromatography –IL/Salt ATPS top phase

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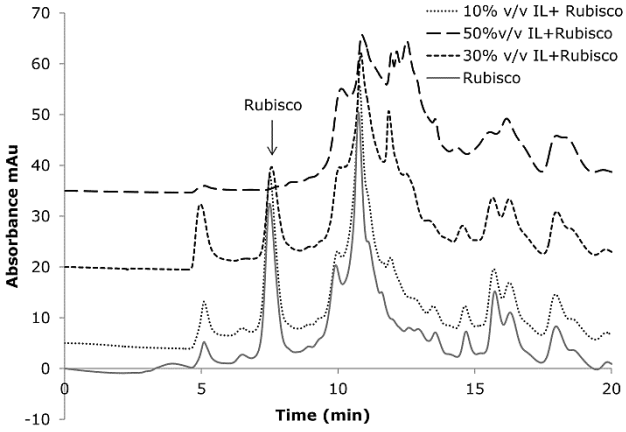

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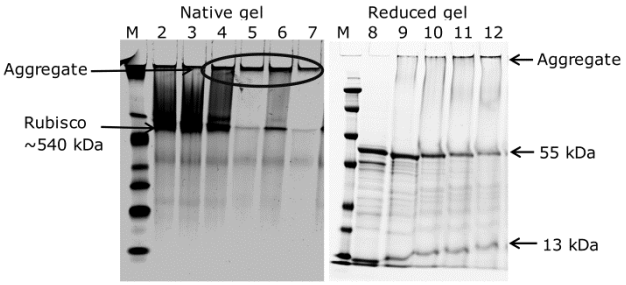

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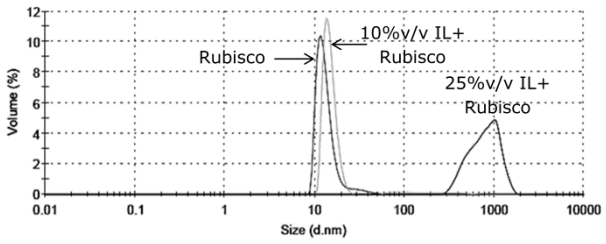

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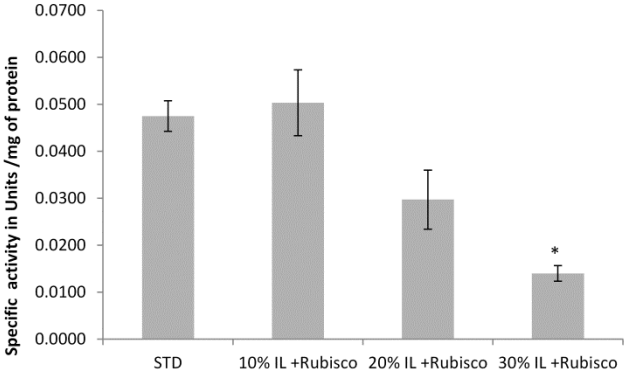


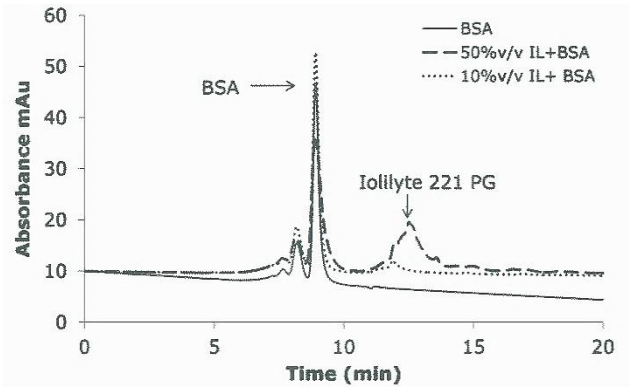
Fig. 6 Effect of increasing concentration of Iolilyte 221PG on Rubisco(a) Size exclusion chromatography (b) Native gel electrophoresis: Standard Rubisco Lane 2,8; Rubisco+10%IL in lane 3,9; Rubisco +30%IL in lane 4,10; Rubisco+40%IL in lane 6,11; Rubisco+50% IL in lane 5,12; Rubisco +60% IL in lane 7 (c) Dynamic light scattering (d) Rubisco Activity in presence of ionic liquid

*t-test -significantly different from the standard

The hypothesis that high molecular weight aggregates were formed was further confirmed by DLS studies (Fig. 6c). The DLS study shows that the hydrodynamic radius of standard Rubisco and Rubisco in 10% v/v Iolilyte 221PG is similar. As the concentration is increased to 25% v/v, Rubisco formed an aggregate which is shown by an increase in the hydrodynamic radius. Rubisco activity in presence of different concentrations of ionic liquid was also monitored (Fig. 6d). The study showed a decrease in enzyme activity with increase in ionic liquid concentration. All these results illustrate that the absence of Rubisco peak from the top phase of the aqueous two phase system is associated with the formation of high molecular weight aggregates. The study done by Dreyer [12] on Iolilyte 221PG / Potassium phosphate aqueous two phase system shows that the concentration of Iolilyte 221 PG in the top phase can be up to 40% w/w or higher depending on the initial ionic liquid concentration in the system. This confirms that high concentration of ionic liquid in the top phase leads to aggregation.

We also studied the behaviour of BSA and IgG1 in aqueous solution containing increasing concentration of the Iolilyte 221PG. Aqueous two phase extraction of BSA in Iolilyte 221PG/ Phosphate was done by Dreyer [17]. The study showed that BSA was extracted with an efficiency of 85-100% and has improved thermal stability in presence of ionic liquid. Our studies reveal that at high concentration of Iolilyte 221PG (50%v/v) there is marginal decrease in peak intensity (Fig.7a). The SEC results together with DLS study shows aggregation of BSA at 50%v/v of Iolilyte 221PG (Fig.7b). Baker and Heller [33] reported aggregation of human serum albumin at 50%v/v concentration of ionic liquid, 1-butyl-3-methylimidazolium chloride. IgG1 on the other hand too showed aggregate formation at higher concentration (Fig.7c). The SEC-HPLC chromatogram shows no aggregation or fragmentation of IgG1 and BSA at 10%v/v. The higher aggregation susceptibility of Rubisco (at 25% IL concentration) compared to BSA (50% IL concentration) could be related to the complexity of the structure, as BSA consist of a single globular subunit and Rubisco consist of 8 large and 8 small subunits linked by non-covalent bonds. So Rubisco is more sensitive for aggregation as different subunit might dissociate from each other and tend to form aggregates. Thus, we hypothesize that aggregation is also influenced by size/complexity (Table 1) of the protein.

a)



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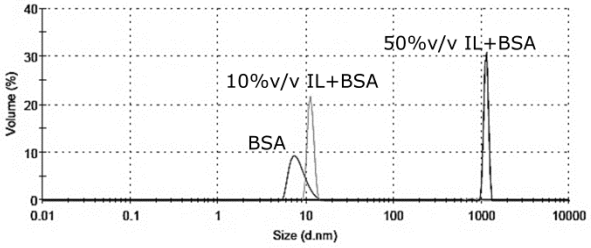

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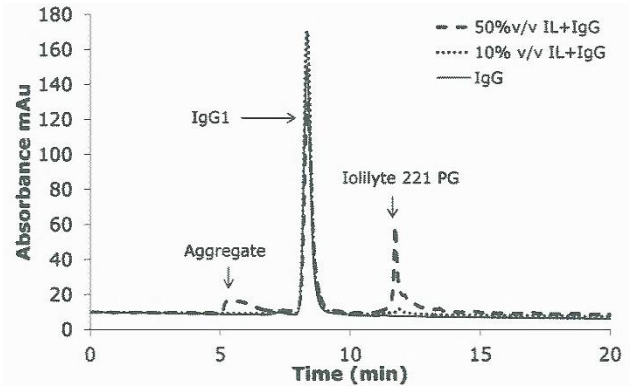


Fig.7 Effect of increasing concentration Iolilyte 221PG on (a) BSA-SEC HPLC chromatogram (b) BSA-DLS (c) IgG1-SEC-HPLC chromatogram

Additionally, the behaviour of the proteins was studied in phosphonium based ionic liquid, Cyphos 108. IgG1 and Rubisco precipitated with increasing concentration of ionic liquid whereas BSA remained in solution at all concentrations tested. As the concentration of Cyphos 108 was increased to 20% v/v there is considerable decrease in the BSA peak intensity (Fig.8). However increasing the concentration further from 30 % v/v to 50% v/v there is increase in the BSA peak intensity. Simultaneous increase in the peak intensity at lower retention time (~5min) is also observed. This indicates the formation of high molecular weight species (aggregates). However, these results exhibit a complex behaviour and require advanced analysis for understanding the underlying phenomenon.

Among the two ionic liquids studied the phosphonium based ionic liquid, Cyphos 108 affects the protein structure more than Iolilyte 221PG. This could be attributed to the acidic nature of aqueous solution of Cyphos 108 which caused unfolding of protein and subsequent aggregation. The acidic nature of aqueous solution of Cyphos 108 is contributed by the anion methyl sulphate which is hydrolysed in water to produce HSO_4^- ion [34]. In a separate study on BSA in polymer based drug delivery system degradation in acidic microenvironment by aggregate formation and hydrolysis is reported [35]. Overall the preliminary studies on the three proteins shows that they retain the native form at low concentration of Iolilyte 221 PG ~10% v/v.

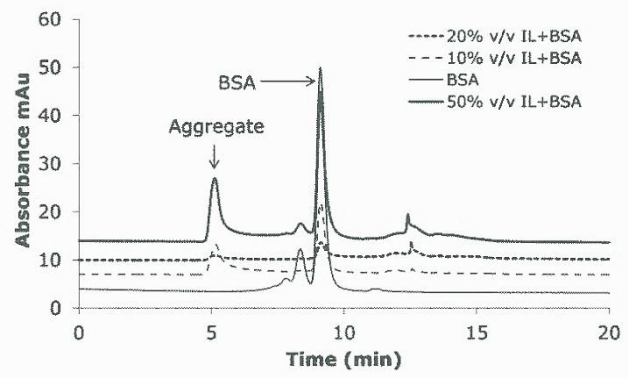


Fig 8 Size exclusion chromatography of BSA in increasing concentration Cyphos 108

Experimental

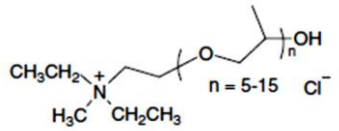
Materials

Polyethylene glycol (PEG) 400, 1000 & 3350 and potassium citrate tribasic were obtained from Sigma. Sodium phosphate, potassium phosphate and citric acid were purchased from Merck. Ionic liquids, Iolilyte 221PG and Cyphos 108 were procured from Iolitec. The structure of the ionic liquids used is shown in Fig.9. Rubisco and BSA were purchased from Sigma. IgG1 was generously provided by Synthon B.V. Netherlands. The three proteins selected (BSA, IgG1 and Rubisco) widely differ from each other in terms of source, structural complexity, isoelectric point (pI) and molecular weight (Table 1).

Table 1 Properties of Proteins

Proteins	Molecular weight, kDa	pI	No. of subunits	Mol.wt of sub units kDa	Bond between subunits
BSA	67	4.7	Monomer	-	-
IgG1	150	9.1	Four (L_2S_2)	Large - 50 Small - 25	Covalently linked subunits
Rubisco	540	5.5	Eight (L_8S_8)	Large - 55 Small - 13	Non-Covalently linked subunits

a)



b)

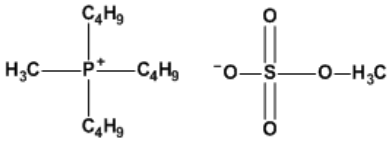


Fig.9 Structure of (a) Iolilyte 221 PG and (b) Cyphos 108.

Methods

Aqueous two phase system based on polymer (PEG-Salt)

Potassium citrate buffer was prepared by mixing appropriate quantities of 40% w/w citric acid with 40% w/w potassium citrate tribasic to attain the desired pH. Citrate salt was selected due to its biodegradability and low environmental polluting properties. Biphasic system was prepared by mixing appropriate amounts of 50%w/w stock solutions of PEG, 50% w/w stock solution of potassium citrate, water and Rubisco solution (stock solution 2% w/w) to a final weight of 5g. The final protein concentration in the system was 0.4 mg/g. The systems were mixed using a vortex mixture and incubated at 30°C for 30 min. The system was then centrifuged at 2500 rpm for 10 min to ensure complete separation of phases. The volume of top and bottom phase was measured and samples from respective phases were taken and analysed for the Rubisco content at 280nm.

Aqueous two phase system based on ionic liquid (IL-Salt)

Two phase system of ionic liquid salt was prepared by mixing appropriate amounts of Iolilyte 221PG, sodium potassium phosphate buffer, protein solution and water to a final wt. of 5 g. The buffer was prepared by mixing appropriate quantities of 40% w/w di basic potassium phosphate (K_2HPO_4) with 40% w/w mono basic sodium phosphate (NaH_2PO_4) until the desired pH was reached. K_2HPO_4 and NaH_2PO_4 were used to prepare the buffer due to the low solubility of their respective mono and dibasic salts. The system was mixed and incubated for 30 minutes at temperatures selected for the study. Samples from bottom phase were withdrawn and analysed for the Rubisco content at 280 nm. The yield in the IL-rich top phase was calculated by mass balance to avoid possible interference from ionic liquid.

Design of experiments

Modde v.9.1 Design of Experiments (DOE) software (MKS Umetrics, Sweden) was used to study the effect of different factors on the partitioning of Rubisco in PEG-Salt and Ionic liquid-salt two phase systems. A central composite face centred design (CCF) was used for both systems. Four independent variables (factors) at three levels and three replicates at the centre point were studied for the two systems. The coded value for each factor studied for the two systems are shown in Table 2 and 3.

Table 2 Factors and value levels used in CCF design for PEG/Potassium citrate system in % w/w

Variables	Factors	Low value (-1)	Centre value (0)	High value (+1)
PEG molecular weight	X1	400	1000	6000
PEG concentration % w/w	X2	24	25.5	27
Buffer concentration % w/w	X3	24	26	28
pH	X4	6	7	8

Table 3 Factors and value levels used in CCF for Iolilyte 221 PG/Sodium -Potassium phosphate system

Variables	Factors	Low Value (-1)	Centre Value (0)	High Value (+1)
Iolilyte 221 PG concentration % w/w	X1	12	16	20
Buffer concentration % w/w	X2	20	22.5	25
pH	X3	6	7	8
Temperature °C	X4	15	25	35

The two response variables studied were partition coefficient (K_p) Y_1 and yield (Y_e) Y_2. The distribution of Rubisco in ATPS was determined by measuring the partition coefficient K_p which is calculated as the ratio of Rubisco concentration in the upper phase to that in the lower phase.

$$K_p = \frac{C_t}{C_b}$$

The yield (Y_e) is calculated as the percentage of the amount of Rubisco in the top phase to the initial amount for PEG based system and for IL based system it is calculated as:

$$Y_e = 100 - \frac{C_b \times V_b}{Initial\ Amount} \times 100$$

Response surface methodology was used to optimise the extraction and the responses were fitted in a quadratic (second order) polynomial regression model to understand the effect of different interactions on the responses. The model's validity and significance were evaluated using the analysis of variance (ANOVA).

Stability in ionic liquid solutions

The effect of ionic liquid on the three proteins was investigated by incubating the proteins in different concentration of aqueous ionic liquid solutions. The samples were prepared by mixing the ionic liquid with water and adding the protein at a concentration of 2 mg/ml. The concentration Iolilyte 221PG and Cyphos 108 was increased from 10-50% v/v. The stability of proteins was then analysed by gel electrophoresis and size exclusion chromatography (SEC-HPLC). The concentration of ionic liquid in the top phase of ATPS could vary from 20-60% w/w. Therefore the top phase of the ATPS was also analysed for protein stability using gel electrophoresis and size exclusion chromatography.

Analytical techniques

UV Spectroscopy

The amount of Rubisco in both PEG-Salt and IL-Salt biphasic systems was analysed by measuring the absorbance at 280 nm. All samples were analysed against blanks having the same composition but without protein to avoid interference from the phase components.

Rubisco Activity

Rubisco activity is measured spectrophotometrically using NADH-linked enzyme coupled system [36]. The final reaction mixture(3ml) contains 259 mM Tris, 5 mM magnesium chloride, 67 mM potassium bicarbonate, 0.2 mM ß-nicotinamide adenine dinucleotide, reduced form, 5 mM adenosine 5'-triphosphate, 5 mM glutathione, reduced form, 0.5 mM D-ribulose 1,5- di-phosphate, 5units alpha-glycerophosphate dehydrogenase trios phosphate isomerase, and 5units glyceraldehyde-3-phosphate dehydrogenase/3-phosphoglyceric phosphokinase. Rubisco is added and the oxidation of NADH is measured by monitoring the change in absorbance at 340nm over a period of 6minutes. The enzyme activity is then calculated from the rate of NADH oxidation using an extinction coefficient of 6.22mM^{-1}.

Size exclusion chromatography (SEC-HPLC)

The stability of the protein, in terms of fragmentation and/or aggregate formation, was analysed by SEC-HPLC (Thermo Separation Products P4000 pump and AS3000 auto sampler and Ultimate 3000 Diode array detector) using Biosep-SEC-S-3000 column (Phenomenex 300 X 7.8mm column, 5µ particle size). The mobile phase was 0.1M sodium phosphate buffer pH 7 and 0.3M sodium chloride. The samples were centrifuged at 11000 rpm for 1 min to remove any insoluble particles prior to injection (inj. volume 20 µl). The samples were run in an isocratic mode at a flow rate of 1ml/min and the protein was detected at 280 nm.

Electrophoresis

To investigate the formation of fragments and aggregates and to confirm that the protein retains its native form, the samples were further analysed by native gel electrophoresis. The samples from the top and bottom phase were diluted with native sample buffer in the ratio 1:2. The samples were then applied on 4-20% Criterion TGX, Tris glycine precast gel and run with 10X Tris glycine native buffer at 125V for 75 min. SDS- gel electrophoresis was performed using precast Criterion XT Bis-Tris gel 12%. The samples were mixed with the sample buffer, reducing agent and heated at 95 °C for 5 min. The samples were applied on the precast gels and then run using 3-(N-morpholino) propane sulfonic acid (MOPS) buffer at constant voltage of 200 V. The gel (both native and reduced gels) was stained with Coomassie Brilliant blue R250. All the reagents used for gel electrophoresis were procured from Biorad.

Dynamic light scattering (DLS)

Dynamic light scattering is a non-invasive technique which measures size and size distribution of proteins, particles and other molecules in liquid solution. It measures the fluctuation in light intensity as a function of time. Hydrodynamic radius of proteins in aqueous ionic liquid solution were analysed by Zetasizer Nano S System from Malvern Ltd. The light scattering was measured at a constant temperature of 25°C and scattering angle of 173°. The samples were measured in 40µl disposable cuvettes. The viscosity and refractive index of the dispersant, Iolilyte 221 PG, was set at

1.29 mPa.s and 1.346 for 10 % v/v; 2.49mPa.s and 1.37 for 25% v/v & 9.8478 mPa.s and 1.403 for 50% v/v respectively.

Conclusions

The purpose of this study was to evaluate the extraction of a large complex protein; Rubisco in IL based ATPS and the stability of proteins (BSA, IgG1 and Rubisco) in aqueous ionic liquid solutions. Aqueous two phase extraction of Rubisco was investigated in PEG /Potassium citrate system and Iolilyte 221 PG/Sodium-Potassium Phosphate system. The results demonstrate that ionic liquid based ATPS showed higher partitioning of Rubisco than PEG based system. However the SEC-HPLC studies shows lack of structural stability of Rubisco. This is further supported by the studies done in aqueous solution of Iolilyte 221 PG. Similar studies on BSA and IgG1 in Iolilyte 221 PG showed formation of aggregates at higher concentration of ionic liquid. BSA in aqueous Cyphos 108 solution formed aggregates and IgG1 and Rubisco were precipitated with increasing concentration.

The empirical findings in the study suggest that higher concentration of the ionic liquids tested, results in protein aggregation. The study also reveals that the size and complexity of the protein influences protein aggregation and subsequent stability in the ionic liquid. From the study done using Cyphos 108 it is evident that the anion also strongly influences the stability of protein. A careful selection of IL with respect to anion can help in designing an IL suitable for extracting protein biomolecules. The current investigation shows that the Rubisco is structurally and functionally stable at ~10%v/v concentration of ionic liquid. Nevertheless it is important to understand that each IL is different and one size fits all does not apply. To summarize, these results help in understanding the behaviour of protein in high concentration of IL.

Notes and references

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