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Design of Novel Biocompatible and Green Aqueous two-Phase Systems containing Cholinium L-alaninate ionic liquid and polyethylene glycol di-methyl ether 250 or polypropylene glycol 400 for separation of bovine serum albumin (BSA)

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ABSTRACT

Recently, cholinium aminoate ionic liquids (Ch[*AA*] ILs) are gaining more attention due to their low toxicity and high biodegradability and this makes them a good candidate when we consider the formation of their aqueous two-phase systems (ATPSs) with suitable polymers that can be used for protein separation. In this work, the phase diagram of novel ATPSs composed of cholinium L-alaninate (Ch[L-Ala]) and polyethylene glycol dimethyl ether 250 at different temperatures or polypropylene glycol 400 at 298.15 K were experimentally determined. The effect of temperature and type of polymer on the binodal curve and tie-lines were investigated systematically. The phase separation abilities in these ATPSs were evaluated using Setschenow-type equation on the basis of a salting-out coefficient obtained from fitting the tie-lines to this equation and the trends showed that two-phase area was expanded by increasing temperature. These results were also confirmed by the trend observed with the effective exclude volume values. Two empirical equations including Merchuk were used for representing binodal data. Reliability of tie-lines was verified by Othmer-Tobias and Bancraft equations. Different versions of NRTL models were used to correlate the obtained tie-line data. In addition, the partition behavior of a model protein, i.e. bovine serum albumin (BSA), was studied by measuring of partition coefficient and extraction efficiency in mentioned systems. The results show that BSA could be enriched effectively into the IL- rich phase with a single step extraction efficiency of about 80% under the optimized conditions.

1. Introduction

Proteins play important roles in many biological processes. These biomaterials have found biotechnological, therapeutic and diagnostic applications [1]. The conventional protein separation methods such as ammonium sulfate precipitation, salting out, electrophoresis [2], ion exchange [3], and affinity chromatography [4] are complex, high cost and difficult to scale up. Recently research workers focused their attention on using liquid-liquid extraction method for separation of proteins [5]. The traditional liquid-liquid extraction method, however, cannot be used for protein separation, because the proteins are easily denatured or lose their biological activities in organic solvents. To resolve this problem ATPS containing a water-soluble polymer and another polymer or certain inorganic/organic salt have been proposed [6] and used successfully for protein separation [7–9]. However, limited ranges of the polarities of coexisting phases in these ATPSs make some limitations in their use. Improvement in this respect has been made by introducing the new IL-based ATPSs [10] which have many advantages such as high efficiency and technological simplicity and considered to be a clean alternative to the traditional liquid-liquid extraction systems [11,12]. ILs are considered as "green solvents"; but well known ILs having imidazolium and pyridinium cations and combined with fluorinated or other anions are poorly biodegradable and highly toxic [13–16]. Therefore, researchers have decided to prepare new benign and non-toxic ILs from environmentally friendly precursors [17–21], such as cholinium chloride ([*Ch*]Cl) which is called "choline". It was found that ILs containing cholinium cation possess remarkable biodegradability as well as low toxicity and therefore are suitable for separation of proteins especially of bovine serum albumin (BSA) [15,20,22,23]. Then the researchers realized that anion component of cholinium-based ILs has also a great influence on the overall toxicity, and decided to use amino acids (AA) as the anion component of IL. By this procedure researchers have succeeded in synthesizing various ILs derived from AA which have low toxicity and may be used for protein separation [24]. The first report corresponds to the synthesis of ILs composed of imidazolium cations and amino acid anions by Fukumoto and coworkers [25]. Recently some Ch[*AA*]-ILs were prepared and their

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physicochemical properties were investigated [26-30]. Polyethylene glycol (PEG) which is a hydrophilic polymer has been used in aqueous two-phase partitioning studies [31]. Polyethylene glycol di-methyl ether (PEGDME) is a polymer that has a similar structure to the PEG, thus it can be used to form ATPSs with cosmotropic (i.e. water-structuring) salts. In addition, PEGDME is an aprotic, polar, and low toxic polymer which is chemically inert and thermally stable. This polymer is one of the most powerful solvents available today for a wide range of applications in pharmaceutical and chemical processes. It is used for the synthesis of active pharmaceutical ingredients (APIs) or extraction of naturally occurring actives from plants. PPG 400 is a biocompatible and biodegradable polymer, with a lower critical solution temperature (LCST) in binary mixtures with water (LCST ≈ 46 °C), and could be recovered conveniently by heating at a temperature above the LCST [15]. In order to design a suitable and high-performance process for separation and purification of proteins, it is important to have liquid-liquid equilibrium data including binodal and tie-lines for these new ATPSs. Recently in this respect phase behavior of ATPSs containing choline amino acid ILs and K_3PO_4 and their applications for protein partitioning have been studied [32]. There is also another report regarding the study of liquid-liquid equilibria of ATPSs containing Ch[*AA*]-ILs and polypropylene glycol with molar mass 400 $g \cdot mol^{-1}$ (PPG_{400}) and their performance in partitioning of some proteins [33]. Here, we present new ATPSs containing Ch[L-Ala] and polyethylene glycol di-methyl ether with molar mass 250 $g \cdot mol^{-1}$ ($PEGDME_{250}$) or PPG_{400}. For these systems, there is no information regarding phase behavior or protein partitioning.

In this work, the binodal and tie-line data are reported for {$PEGDME_{250}$ + Ch[L-Ala] + water} system at $T =$ (298.15, 308.15 and 318.15) K. Similar information was obtained for {PPG_{400} + Ch[L-Ala] + water} system at 298.15 K. In addition, the critical concentration of IL needed to induce ATPS has been calculated at each temperature using linear least square regression method. To survey the driving force formation of these two-phase systems the free energies, enthalpies and entropies of cloud points were calculated at the working temperatures. Setschenow-type equation [34] was applied to investigate phase forming ability in these systems via obtaining salting-out coefficient from fitting tie-line data to this equation. Binodal data were treated with binodal model [35] and the effective exclude volumes were calculated with results indicating the same trend as predicted by the Setschenow-type equation. In order to study the effect of polymer type on the phase diagram, the measured liquid-liquid equilibrium of ATPSs containing ($PEGDME_{250}$+ Ch[L-Ala]) and (PPG_{400} + Ch[L-Ala]) were compared at $T = 298.15$ K. For first time, the performance of $PEGDME_{250}$ as a phase forming component with Ch[L-Ala] was investigated for separation of a model protein, i.e., BSA. The effects of temperature and type of polymer were searched on the BSA separation via calculation of partitioning coefficient and efficiency of BSA. The reliability of experimental tie-line compositions was examined by the Othmer-Tobias and Bancraft equations [36,37]. Two empirical nonlinear expressions [38,39] were used for reproducing the experimental binodal data. The local composition models e-NRTL [40] and m-NRTL [41] were used to fit the experimental tie-line data.

2. Experimental section

2.1. Materials

Choline chloride (99% purity) was obtained from Daejung of Korea. L-Alanine (99% purity), BSA (98% purity), $PEGDME_{250}$ (99.5% purity), PPG_{400} (99.5% purity), potassium hydroxide (99% purity) and methanol (99% purity) were purchased from Merck. In all experiments, the polymers and IL were used without further purification and double distilled deionized water with conductivity 0.055 $\mu S \cdot cm^{-1}$ was used.

2.2. Synthesis of Ch[L-Ala]-IL

Ch[L-Ala] was synthesized via a neutralization reaction. First, cholinium hydroxide was prepared by refluxing of stoichiometric mixing ([*Ch*]Cl) and potassium hydroxide in methanol as a solvent in a round flask for 12 h at 333.15 K under nitrogen atmosphere [42]. When the reaction was completed, a rotary evaporator was employed to remove the methanol in the product at a temperature 343.15 K. Upon complete substation of chloride, no precipitation of AgCl is be found by addition of a few drops of $AgNO_3$ solution. The obtained product was dissolved in distilled water to prepare aqueous cholinium hydroxide ([*Ch*]OH) solution. The concentration of prepared aqueous [*Ch*]OH solution was determined by titration of the solution with 0.100 $mol \cdot L^{-1}$ HCl standard solution. Next, the as-prepared aqueous solution of [*Ch*]OH was neutralized with stoichiometric amino acid and reaction mixture was stirred magnetically for 12 h at room temperature under nitrogen atmosphere. The most of water in the system was then removed by rotary evaporation, and the resulting product was dissolved in a mixed solvent of methanol/acetonitrile (1:9) and filtered to remove any unreacted amino acid. Finally, the solvent was removed again, and all the Ch[L-Ala] was dried at 343.15 K for at least 48 h under vacuum before use. The water mass fraction of IL was determined by Karl-Fischer titration, and the value was about 0.05%wt. The purity and yield of the synthesized IL were >0.98 and 80%, respectively.

2.2.1. Characterization of the Ch[L-Ala]

The purity of synthesized IL was analyzed by FT-IR and ^{1}H NMR to confirm the absence of any major impurities, and this IL is found to be >0.98 in mass fraction. FT-IR spectra and ^{1}H NMR data of cholinium L-alaniante are in good agreement with those reported in the literature [32,33,43]. The FT-IR spectrum and ^{1}H NMR data for Ch[L-Ala] are given as follows:

Ch[L-Ala]:$\bar{\nu} =$ 3336, 2923, 2863, 1583, 1484, 1395, 1090, 957, 838 cm^{-1}.

^{1}H NMR (400 MHz, D_2O) = 1.06 (3H, d, CH_3CH), 3.03 (9H, s,–N(CH_3)), 3.19 (1H, q, $CHNH_2$), 3.35 (2H, m, CH_2OH), 3.89 (2H, m, CH_2CH_2N).

2.3. Determination of binodal curve

The binodal curves for a ternary system including of Ch[L-Ala], water and $PEGDME_{250}$ or PPG_{400} were determined at mentioned temperature, by clouding point titration method as described in previous works [44,45]. In briefly, an aqueous solution of Ch[L-Ala] with a known concentration was prepared and titrated with an aqueous polymer solution until the mixture becomes cloudy which indicates the two-phasic region. Then water was added dropwise to the mixture to make a clear solution (monophasic region). An analytical balance (Shimatzu, 321-34,553, Shimatzu Co., Japan) with a precision of $\pm 1.10^{-7}$ kg was used to determine mass fractions of the components.

2.4. Determination of tie-lines

For determination of tie-lines, the appropriate amounts of polymer, Ch[L-Ala] and deionized water in the biphasic region were mixed in 2-ml microcentrifuge tubes and vigorously stirred. Then, the samples were placed in the thermostat to equilibrate for a minimum of 24 h so that they could be separated into two clear phases. Temperature was controlled by a thermostat (JULABO model MB, Germany) with an accuracy of ±0.02 K. Samples from both phases were carefully withdrawn using a small syringe. The concentration of IL in the top and bottom phases were determined by a UV–vis spectrophotometer (Model: SPECORD 40-Series Analytik Jena AG- Germany) at 216 nm. Refractive indexes of both phases in each sample were measured by a refractometer (ATAGO DR-A1, Japan) with a precision of ±0.0001 at $T = 298.15$ K to quantify $PEGDME_{250}$ or PPG_{400} concentration as described previously