A protein extraction and separation has been reported using the ionic liquid (IL)-based aqueous two-phase system (ATPS). Although ILs have many benefits, there was a problem of unfavorable physical properties such as high viscosity, or high-cost production of ILs. Recently protic ionic liquids (PILs) are emerging as an alternative to conventional ionic liquids. PILs are easily produced through a reaction between Brønsted acid and base at a relatively low cost. In this study, PILs (pyrrolidinium formate [Pyrr][HCOO] and propionate [Pyrr][C₂H₅COO] and inorganic salts (K₃PO₄, K₂HPO₄) as phase-forming constituents of ATPS, were used for the protein extraction.

From the binodal experiments, the phase separation abilities are [Pyrr][HCOO] > [Pyrr][C₂H₅COO] and K₃PO₄ > K₂HPO₄. Because proteins precipitated at the interface when using K₃PO₄, ATPS composed of [Pyrr][HCOO] and K₂HPO₄ was selected for the protein extraction. The proteins were successfully extracted to PIL-rich phase. The distribution ratio of each protein was in the order hemoglobin < cytochrome C < α-chymotrypsin < albumin. The partition behavior of proteins in PIL-based ATPS was complex and influenced by combined effect of hydrophobic, electrostatic interactions and salting out. FT-IR spectroscopic characterization indicated that secondary structures of hemoglobin and cytochrome C were retained in PIL-rich phase after extraction.

1. Introduction

In separation and recovery of proteins which were fragile molecules, mild operation conditions are required to retain their structural integrity and chemical functionality. Purification of proteins is generally achieved by liquid-liquid extraction, precipitation, chromatography, and electrophoresis, but they have limitations such as high costs, laborious, etc [1]. Such downstream-processing accounts for over 80% of the production cost. Aqueous two-phase system (ATPS) have focused as an alternative to the traditional processes due to the mild conditions and ease of scaling it up [2]. ATPS has been applied to a few industrial scale protein extraction from crude feedstock [3]. Conventional ATPSs consist of two-phase system of aqueous rich polymer/polymer (e.g. dextran/polyethylene glycol (PEG)) and polymer/salt (e.g. PEG/inorganic salt). In addition to polymer-based ATPS, hydrophilic organic solvents [4,5] and ionic liquids (ILs) [6] have appeared as a phase-forming constituent. ILs can be divided into two distinct families, aprotic ionic liquids (AILs) and protic ionic liquids (PILs). AIL-based ATPSs have applied to the protein separation [7]. Protein extraction with AIL-based ATPSs is promising because of ease of phase separation, high
extraction efficiency, and high stability of proteins distributed in AIL-rich phase [1]. Although AILs have many benefits, they also have unfavorable physical properties such as high viscosity, or high-cost production. Recently, PILs are emerging as an alternative to AILs. PILs are easily produced through a neutralization reaction between Bronsted acid and Brønsted base at a relatively low cost. Camêlo et al. [8] reported successful genipin extraction with ATPSs formed by acetonitrile and PIL, monoethanolammonium dihydrogen citrate. Lucena et al. [9] reported the use of PIL (hydroxyethylammonium propionate) as adjuvant in the extraction of radish peroxidase with ATPS formed by PEG and ammonium sulfate. The extraction efficiency of this enzyme was improved by adding PIL. Few studies have focused on PIL as a phase-forming constituent in ATPS.

In this study, we investigated the protein extraction with novel ATPS formed by PIL and inorganic salt. To our best knowledge, this is a first example of ATPS formed by PIL and inorganic salt. We selected pyrrolidinium-based PILs and potassium phosphates as phase-forming constituents. These are because physical properties of pyrrolidinium-based PILs were investigated [10] and potassium phosphates are frequently used in protein extraction with AIL-based ATPS [7].

2. Experimental

2.1 Chemicals

Organic acids (formic and propionic acid) and pyrrolidine were used as PIL constituents in this study. PIL was synthesized according to the procedure shown in the previous paper [10]. Pyrrolidine (0.5 mol) was placed in a three-neck round-bottom flask immersed in an ice bath. To the pyrrolidine acid (0.5 mol) was added drop-wise over 1 h under vigorous stirring. After the addition of the acid, the reaction mixture was stirred for 4 h. The residual reactants and water were removed under reduced pressure. Molecular structures and abbreviations of PIL used in this study are shown in Table 1. Proteins used in this study were cytochrome C (Cyt) from bovine heart (Sigma-Aldrich, C2037), hemoglobin (Hem) from bovine blood lyophilized powder (Sigma-Aldrich, H2500), albumin from bovine serum (BSA) (Wako, 014-25781) and \( \alpha \)-chymotrypsin (Chy) from bovine pancreas, Type II, lyophilized powder (Sigma-Aldrich, C4129). Average molecular weights and pI values of the proteins investigated are summarized in Table 2. All the remaining reagents were of analytical grade and used without further purification.

2.2 Preparation of phase diagram

The binodal curves were determined at 25°C by cloud point method. A \( \text{K}_2\text{HPO}_4 \) or \( \text{K}_3\text{PO}_4 \) aqueous solution of known concentration was placed in a 100 mL conical flask and to the flask PIL was added drop-wise until the clear solution turned turbid or two phase systems were formed. Then, de-ionized water was added drop-wise to the flask to obtain a clear one phase system and more PIL was added again to afford two phase systems. The composition of this mixture was noted and the experiments were repeated to obtain the binodal curve.

2.3 Aqueous two-phase extraction experiment

Solid inorganic salts, water and PIL were mixed with the composition listed in Table 3. Total weights were 5 g for extraction of Cyt and Hem and 10 g for extraction BSA and Chy. After inorganic salt was dissolved in water and the salt solution was kept to 25°C, protein was dissolved in the solution. And then to the solution PIL was added to form two phases. The solution was mixed thoroughly and left standing for
more than 24 h in a thermostated bath at 25°C. The volumes of the top and bottom phases were recorded. The pHs of salt-rich phase were measured and their values were between 9.8 and 9.9. The concentrations of Cyt and Hem were determined with an UV-vis spectrophotometer (Shimadzu UV2550) at 400 nm. The concentrations of BSA and Chy were determined by HPLC (Shimadzu LC-20ADsp) with an UV detector at 280 nm (Shimadzu SPD20A). Analysis was performed using an InertSustain C18 (GL Sciences) column and an eluent solution (water) as a mobile phase (1.0 mL min⁻¹). Extractability, $E$, and distribution ratio, $D_A$ were defined as follows.

$$E = \frac{C_T V_T}{C_{0T} V_{0T}}$$  \hspace{1cm} (1)

$$D_A = \frac{C_T}{C_{0T}}$$  \hspace{1cm} (2)

where $C$ and $V$ are the concentration of protein and the solution volume. Subscripts T and B denote the top (PIL rich) and bottom (salt rich) phases, respectively, and 0 denotes the initial state.

| Table 1. Molecular structures and abbreviations of PIL. |
|--------------------------------------------------------|
| Protic ionic liquid | Structure | Abbreviation |
|---------------------|-----------|--------------|
| Pyrrolidinium formate | ![Structure](image) | [Pyrr][HCOO] |
| Pyrrolidinium propionate | ![Structure](image) | [Pyrr][C₂H₂COO] |

| Table 2. Average molecular weight and pl values of proteins. |
|-------------------------------------------------------------|
| Proteins | M (Da) | pI |
|-----------|--------|----|
| BSA       | 66000  | 4.9 |
| α-Chymotrypsin | 25000  | 8.1 |
| Cytochrome C | 12000  | 10  |
| Hemoglobin | 64500  | 7   |

2.4 FT-IR spectra of proteins

To examine the change in the secondary structure of proteins, the FT-IR measurements (Shimadzu IRAffinity-1, MIRacle 10) of proteins (Cyt and Hem) were performed by an ATR method. Aqueous salt solution containing proteins before extraction and PIL-rich solution after extraction were measured. FT-IR of BSA and Chy could not be measured because of background interference.
Table 3. Composition of aqueous two-phase system.

| Salt (wt%) | PIL (wt%) | Water (wt%) |
|-----------|-----------|-------------|
| 25.0      | 30.0      | 45.0        |
| 27.5      | 30.0      | 42.5        |
| 30.0      | 30.0      | 40.0        |
| 32.5      | 30.0      | 37.5        |
| 35.0      | 30.0      | 35.0        |

3. Results and Discussion

3.1 Phase diagram of aqueous two-phase system consisting of PIL and inorganic salts

Figure 1 shows on the bimodal curves of the ATPS consisting of PIL and inorganic salts at 25°C plotted in mass fraction. The closer the bimodal curves to the origin of coordinates, the less amount of PIL required for the formation of ATPS under the same concentration of salt. From the figure, the phase separation abilities are [Pyrr][HCOO] > [Pyrr][C2H5COO] and K3PO4 > K2HPO4. In our previous paper [4], it is reported that K3PO4 had higher salting-out effect than K2HPO4. Anions in ionic liquids with lower hydrogen bond basicity values coordinate less with water molecules and are more easily salted out by the inorganic salts [11]. Hydrogen bond basicity value of [P4442][HCOO] was reported to be lower than that of [P4442][C2H5COO] [12], suggesting that [Pyrr][HCOO] is more susceptible to salting-out agent than [Pyrr][C2H5COO] (P4442 denotes tributylethylphosphonium cation).

3.2 Extraction of proteins with ATPS

In the case of using K3PO4, proteins precipitated at the interface because of its high salting-out ability. In the following experiment, [Pyrr][HCOO] and K3HPO4 were used to form ATPS.

Table 4 shows the extractability and distribution ratio of protein by the ATPS using PIL and K2HPO4. In Table 4, the top and bottom phases were PIL-rich and salt-rich, respectively. The all proteins were successfully extracted to PIL-rich phase. The increase in salt concentration caused the increase in top phase volume and a decrease in bottom phase volume because of the salting-out effect. The extractability and distribution ratio of proteins generally also increased with increasing the salt concentration. It is known that the logarithm of the distribution ratio \((D)\) of an ionic species is linearly related to the ionic strength \((I)\) in
polymer-based ATPS [13]

\[ \ln D = C - BI \]  

(3)

where \( C \) and \( B \) are constants which depends on the physical properties of protein and salt. Plots based on Eq. (3) for all proteins were shown in Figure 2. It was found that Eq. (3) is valid for the protein extraction with PIL-based ATPS.

Table 4. Extraction of proteins by the aqueous two-phase systems.

| Proteins | Salt concentration [wt%] | Bottom phase [cm³] | Top phase [cm³] | \( E \) [%] | \( D_A \) [-] |
|----------|--------------------------|--------------------|----------------|-----------|-------------|
| Cyt      | 25.0                     | 2.3                | 1.4            | 67.9      | 1.29        |
|          | 27.5                     | 2.2                | 1.5            | 69.9      | 1.58        |
|          | 30.0                     | 2.2                | 1.7            | 69.3      | 1.74        |
|          | 32.5                     | 2.1                | 1.8            | 67.3      | 1.76        |
|          | 35.0                     | 2.1                | 1.9            | 70.5      | 2.16        |
| Hem      | 25.0                     | 2.3                | 1.3            | 37.9      | 0.34        |
|          | 27.5                     | 2.2                | 1.5            | 45.9      | 0.58        |
|          | 30.0                     | 2.2                | 1.6            | 57.3      | 0.98        |
|          | 32.5                     | 2.2                | 1.8            | 59.4      | 1.20        |
|          | 35.0                     | 2.1                | 2.0            | 61.8      | 1.54        |
| BSA      | 25.0                     | 5.4                | 2.4            | 73.9      | 1.25        |
|          | 27.5                     | 4.6                | 3.1            | 79.0      | 2.53        |
|          | 30.0                     | 4.3                | 3.2            | 81.6      | 3.30        |
|          | 32.5                     | 4.3                | 3.2            | 82.7      | 3.56        |
|          | 35.0                     | 3.9                | 3.4            | 82.2      | 4.03        |
| Chy      | 25.0                     | 5.2                | 2.1            | 68.1      | 0.86        |
|          | 27.5                     | 4.9                | 2.9            | 68.4      | 1.28        |
|          | 30.0                     | 4.5                | 3.4            | 70.2      | 1.78        |
|          | 32.5                     | 4.2                | 3.8            | 73.4      | 2.50        |
|          | 35.0                     | 3.8                | 4.2            | 74.2      | 3.18        |

The highest distribution ratio of each protein in Table 4 are in the order Hem < Cyt < Chy < BSA. In extraction of proteins with AIL-based ATPS [1,14], hydrophobic, electrostatic interactions, and salting out effects were considered the driving forces for the efficient partitioning of proteins. The distribution ratio of proteins increased with increasing pH [15], suggesting that more net negative charge of the proteins can be extracted, that is, proteins having lower pI values preferentially transferred to the IL-rich phase. Moreover,
the proteins with large molecular weight are better extracted in the IL-rich phase than small proteins [1].
Based on Table 2 and the above discussion, the order of extractability, Cyt < Chy < BSA, can be explained.
However, although hemoglobin had a moderate pI value and high molecular weight, its distribution ratio was
lowest among the proteins investigated. The empirical equation [15] is extensively used to describe the
solubility of proteins (S) in salt solution at higher concentration range (m: molality).
\[ \ln S = \beta - mK_s \] (4)
where \( \beta \) is a constant which depends on pH and temperature and \( K_s \) is the salting-out constant which depends
on the physical properties of protein and salt. The salting-out constant of hemoglobin was reported to be
smaller than other proteins [16]. Therefore, the salting-out effect may not affect extraction of hemoglobin
much. As the results, the partition behavior of proteins in PIL-based ATPS was complex, not influenced by a
single effect, and it is found to be a result of combined effect of hydrophobic, electrostatic interactions, and
salting out.

3.3 Structure characterization of proteins

The amide I vibration of FT-IR spectra in the protein solution, absorbing near 1650 cm\(^{-1}\), is assigned
to mainly C=O stretching vibration. It depends on the secondary structure of the proteins and therefore is
most commonly used for secondary structure analysis [17]. FT-IR spectra of aqueous salt solution containing
Cyt and Hem before extraction and of PIL-rich solution after extraction were shown in Fig. 3. For both
proteins, band positions of amide I vibration were 1652 cm\(^{-1}\). \( \alpha \)-Helix and \( \beta \)-sheet structures absorb
predominantly at 1654 and 1633 cm\(^{-1}\), respectively [17]. Therefore, secondary structure of both proteins are
found to be mainly \( \alpha \)-helix. After extractions of both proteins, the amide I vibrations are shifted only slightly
to the low frequency side. This suggested secondary structures of proteins were retained in PIL-rich phase.
Chaotropic cations and kosmotropic anions of IL have been reported to be the best combination for the
thermal stability of cytochrome C in hydrated IL [18]. Because larger cation is generally kosmotropic,
pyrrolidinium cation among IL cations is expected to be chaotropic. On the other hand, formate ions are
neither a structure maker nor a structure breaker [19]. Therefore, cytochrome C in PIL-rich phase showed
no significant structural change.

4. Conclusion

In this study, an ATPS composed of pyrrolidinium-based PIL and inorganic salts was applied to extract
proteins. From the binodal experiments, the phase separation abilities are [Pyr][HCOO] > [Pyr][C\(_2\)H\(_3\)COO]
and K\(_3\)PO\(_4\) > K\(_2\)HPO\(_4\). Because proteins precipitated at the interface when using K\(_3\)PO\(_4\), ATPS composed of
[Pyr][HCOO] and K\(_2\)HPO\(_4\) was selected for the protein extraction. The distribution ratio of each protein was
in the order Hem < Cyt < Chy < BSA. The partition behavior of proteins in PIL-based ATPS was complex
not influenced by a single effect, but influenced by combined effect of hydrophobic, electrostatic interactions
and salting out. Structural characterization of proteins using FT-IR spectroscopy demonstrated that secondary
structures of proteins and after the extraction were retained in PIL-rich phase.

Thus an ATPS composed of pyrrolidinium-based PIL and inorganic salts was found to be promising
for purification of proteins under the mild condition.
Figure 3. FT-IR spectra of cytochrome C (A) and hemoglobin (B) before and after extraction.

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