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Thermodynamic studies of partitioning behavior of cytochrome c in ionic liquid-based aqueous two-phase system

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ABSTRACT

The ionic liquid/aqueous two-phase extraction systems (ATPSs) based on imidazolium ionic liquids were used to extract cytochrome c. Effects of the alkyl chain length of the ionic liquid cations, concentration of potassium citrate, temperature and pH on the extraction efficiency have been investigated. The thermodynamic parameters (ΔG_T°, ΔH_T° and ΔS_T°) associated with Cyt-c partitioning in aqueous two phase systems were determined. Thermodynamic studies indicated that the partitioning of Cyt-c was driven by both hydrophobic and electrostatic interactions in the extraction process. Under the optimum conditions, experiment results showed that 94% of the cytochrome c could be extracted into the ionic liquid-rich phase in a one-step extraction. The structural characterization of Cyt-c in the IL ATPS was investigated by UV–vis and circular dichroism (CD) spectra. The results demonstrated that no direct bonding interaction observed between ionic liquid and cytochrome c, while the native properties of the cytochrome c were not altered. Compared with traditional liquid–liquid extractions based on toxic organic solvents, ionic liquid/aqueous two phase extraction offers clear advantages due to no use of volatile organic solvent and low consumption of imidazolium ionic liquids.

1. Introduction

Aqueous two-phase systems (ATPS) is a very promising technology for novel separation and pretreatment [1–3]. It is a liquid–liquid extraction technique that has been used to establish bioprocesses for the primary recovery and partial purification of a variety of biological products, including proteins, genetic material, nanoparticles, low molecular weight products, cells and cell organelles [4,5]. In recent years, ionic liquids (ILs) based ATPS have attracted much attention in the field of separation and purification [6–26] due to their "green" characteristics such as negligible volatility and non-flammability under ambient conditions, large liquid range, high thermal and chemical stability, strong solubility power and the feasibility of structural and functional tenability [8,9]. First IL-based ATPS have been applied to the extraction of testosterone and epitestosterone in human urine or the extraction of major opium alkaloids in Pericarpium papaveris using $[C_4mim][Cl]/K_2HPO_4$ systems [10,11]. An IL-based ATPS using Ammoeng^{TM} 110 has been applied to investigating the main driving forces of protein partitioning within these systems [12]. The mechanism of ionic liquid ATPS formation and the extraction behavior of tocopherol homologues were discussed using $[Bmim]BF_4/Na_2CO_3$ ATPS [14]. The extraction of proteins by IL-based ATPS was first achieved by Du et al. who extracted proteins from human body fluids by employing a $[C_4mim][Cl]/K_2HPO_4$ system [15]. Furthermore, a wealth of products, such as polyphenolic compounds [16], tocopherol homologues [17], anionic dyes [18], codeine and papaverine [19], and biomolecules [20–22] have been successfully separated by the IL-based ATPSs. To the best of our knowledge, less information has been reported for the activity of protein in IL-based ATPS. In earlier studies, phosphate and sulfate salts were commonly used to generate ATPS. These salts, however, led to high phosphate or sulfate concentration in the effluent streams, causing environment problems [27–30]. One way to reduce the amount of salt discharged into the wastewater is to substitute these inorganic salts by citrate, which is biodegradable and nontoxic [26,28–31]. Furthermore, a series of salts were investigated for the information of ATPS with $[C_4mim][Br]$. The ability of the salts studied for phase separation follows the order: $K_3PO_4 > K_2HPO_4 > K_3Cite$ (potassium citrate) $> K_2CO_3$ [26]. Potassium citrate was finally selected because of its biodegradability, nontoxicity, as well as a better ability to promote phase separation. This is the first time on Cyt-c extraction using 1-hexyl-3-methyl-imidazolium bromide ($[C_6mim][Br]$)/potassium citrate aqueous two-phase system.

Cytochrome c (Cyt-c) is a representative hemeprotein that mediates electron transfer in the mitochondrial respiratory chain. In 1993, extraction of Cyt-c in sodium dodecylbenzenesulfonate microemulsions was reported by Claude Jolivalt for the first time [32]. Tatsuya Oshima separated the Cyt-c successfully using the extraction system with $^tOct^{[6]}CH_2COOH$ [33]. Cyt-c was also

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extracted from an aqueous solution into IL phases with DC18C6 [34–36] because the lysine residues of Cyt-c can form a supramolecular complex with crown ethers [37,38]. Ionic liquid was for the first time employed for selective isolation of heme-protein species by Wang [39]. In addition, the hemoglobin was extracted by employing the ionic liquid microemulsion system, which suggested that the electrostatic interaction was among the main driving forces for the transfer of hemoglobin from aqueous phase into the microemulsion [40].

In the present study, we employed Cyt-c from horse heart as a model protein. The objectives of this work were as follows: (a) to extract the Cyt-c into ILs-rich phase in the absence of dicyclohexano-18-crown-6; (b) to investigate the influence of the different parameters, such as IL type, concentration of potassium citrate, pH and temperature on the partitioning of Cyt-c; (c) to obtain the main driving force for the extraction process. Under the optimum conditions, the Cyt-c retained 91% of its activity and an enrichment factor was up to 8.8. Furthermore, UV–visible and CD spectroscopic investigations indicated that there was no direct chemical bond between the Cyt-c and IL.

2. Materials and methods

2.1. Materials

Bovine serum albumin and Coomassie Blue G250 were obtained from Sinopharm Chemical Reagent Co., Ltd. Cytochrome c (C7752) was purchased from Sigma. 1-Ethyl-3-methyl-imidazolium bromide ([C_2mim]Br), 1-butyl-3-methyl-imidazolium bromide ([C_4mim]Br) and 1-hexyl-3-methyl-imidazolium bromide ([C_6mim]Br) made by Lanzhou Greenchem ILS, LICP. CAS. China. Ascorbic acid was purchased from Tianjin KaiTong Chemical Company. PEG was obtained from Shanghai Lingfeng. Potassium citrate and citric acid were purchased from Tianjin Guangcheng Chemical Company. All the reagents were of analytical grade. Pure water was used throughout.

2.2. Aqueous two-phase diagrams

Binodal curves were determined by the turbidimetric titration method [30,41]. The liquid–liquid equilibrium data (5 °C, 25 °C, 35 °C) for the aqueous 1-butyl-3-methyl-imidazolium bromide ([C_4mim][Br])/potassium citrate system were reported by Zafarani-Moattar and Hamzehzadeh [26]. In present work, the temperature was fixed at 30 °C (±0.5 °C) using a bath of circulating water. Stock solutions of ILs 50% (w/w) and potassium citrate 55% (w/w) were employed. IL ([C_2mim]Br, [C_4mim]Br, [C_6mim]Br) was added dropwise to potassium citrate solutions or vice versa, until the solution turned turbid. The starting and added solution masses were measured on an analytical balance with a precision of ±0.001 g. The composition of the mixture was calculated and taken as a binodal point. A small amount of water was added to biphasic system until the turbidity disappeared. The above steps were repeated until enough points were obtained to form the binodal curves. The phase diagrams are shown in Fig. 1.

2.3. Extraction procedure

To a 10 mL graduated tube, a suitable amount of potassium citrate buffer (50%, w/w), Cyt-c solution and ionic liquid were added. To polyethylene glycol (PEG)/potassium citrate system, ATPSs were prepared by weighing out appropriate amounts of 50% (w/w) PEG stock solution, 40% (w/w) potassium citrate buffer solution, Cyt-c solution and water in order to achieve the desired final system composition. All systems were mixed using a vortex mixer and centrifuged for 10 min at 1500 rpm. Then the mixture was allowed to set for 30 min at 30 °C. The volume of each phase was measured when the two phases were clear. The phase ratio ($R = V_t/V_b$) (where V_t and V_b are the volumes of the top and bottom phases, respectively) and enrichment factor (F) (defined as $F = V_{water}/V_t$) were calculated.

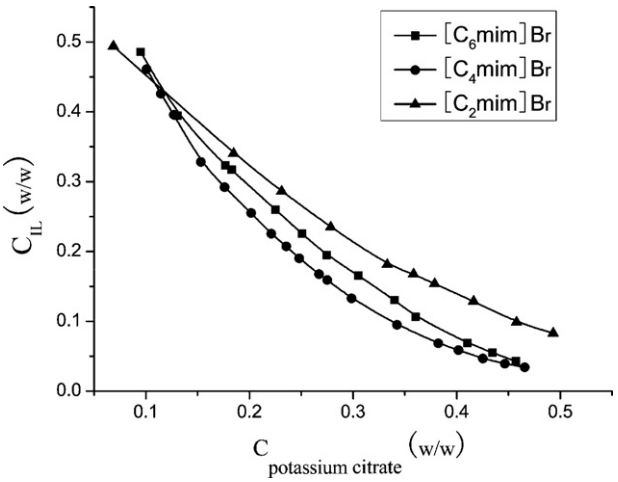

Fig. 1. Phase diagrams for the ILs ([C_2mim]Br, [C_4mim]Br and [C_6mim]Br)/potassium citrate systems recorded at 30 °C. The phase diagrams were determined by turbidometric titration.

The distribution of Cyt-c is described by partition coefficient (K) (given as $K = c_t/c_b$, where c_t and c_b are the concentrations of the Cyt-c in the top and bottom phase, respectively). The extraction recovery (Y_e/%) was defined as the quantity ratio of the Cyt-c extracted in the top phase to the total Cyt-c in the system.

2.4. Determination of the thermodynamic functions associated with Cyt-c partition

The Gibbs energy change associated with the Cyt-c partition in the IL-based ATPS at a given temperature can be calculated by the known equation (1). The enthalpic change and entropic change were obtained from the slope and intercept of the linear equation [42].

$$\Delta G_T^\circ = -RT \ln K \quad (1)$$

$$\ln K = \frac{-\Delta H_T^\circ}{RT} + \frac{\Delta S_T^\circ}{R} \quad (2)$$

2.5. The concentration and activity of cytochrome c determination

The Cyt-c concentration was determined by the Bradford method [43] using Coomassie Blue G250 with bovine serum albumin (BSA) as standard. The optical density was measured at 595 nm.

The activity of Cyt-c was estimated by reduction of heme groups after the addition of ascorbic acid as follows: 2.0×10^{-4} mol/dm^3 of ascorbic acid and 4.0×10^{-6} mol/dm^3 of Cyt-c were mixed in a quartz cell to measure the UV spectrum. The initial reduction rate coefficient, k, was determined by the following equation [33,44]:

$$\frac{\ln(A_{max} - A)}{A_{max} - A_{min}} = -kt \quad (3)$$

where A is the absorbance at 550 nm, A_{max} and A_{min} are the maximum and minimum absorbances at 550 nm during the assay, respectively.

All experiments and measurements were conducted in triplicate.

Fig. 2. Extraction of Cyt-c employed PEG and IL /potassium citrate systems at 30 °C. 1: PEG1000 (18%, w/w)/potassium citrate (20%, w/w); 2: PEG2000 (18%, w/w)/potassium citrate (20%, w/w); 3: PEG4000 (18%, w/w)/potassium citrate (20%, w/w); 4: PEG6000 (18%, w/w)/potassium citrate(20%, w/w); 5: PEG10000 (18%, w/w)/potassium citrate(20%, w/w); 6: [C_2mim]Br (6.25%, w/w)/potassium citrate (41.7%, w/w), no phase separation; 7: [C_4mim]Br(6.25%, w/w)/potassium citrate (41.7%, w/w); 8: [C_6mim]Br (6.25%, w/w)/potassium citrate (41.7%, w/w). [Cyt-c] = 20 μg/g.

3. Results and discussion

3.1. The preliminary extraction of the Cyt-c

To find out the optimal aqueous two phase system, PEG-based and IL-based ATPSs were tested in preliminary studies. As shown in Table 1 and Fig. 2, the extraction efficiencies of Cyt-c in IL-based ATPSs are substantially higher than that in PEG-based ATPSs. The results indicate that the Cyt-c inclines to transfer into the upper phase with a certain level of enrichment using IL-based ATPSs. However, for all the PEG-based systems studied, the Cyt-c tends to remain in bottom phase. The IL-based system and 20 μg/g Cyt-c were employed in the subsequent studies. The effect of Cyt-c concentration on its extraction efficiency was nil (data is not shown).

3.2. Studies of ionic liquid aqueous two-phase systems and effect of the amount of potassium citrate on phase separation

A phase diagram data is required for the design of aqueous two-phase extraction process and for the development of models that predict the partitioning of proteins [8]. Fig. 1 illustrates the phase diagrams of [C_2mim]Br, [C_4mim]Br and [C_6mim]Br/potassium citrate systems at 30 °C. It is obvious that the ability of ILs for two-phase formation followed the order: [C_2mim]Br < [C_4mim]Br < [C_6mim]Br, which is in accordance with the order hydrophobicity of the ILs.

The effect of the amount of potassium citrate on phase behavior of ILs-based ATPS was also investigated. The results are shown in Fig. 3(A) and (B). The phase volume ratio (R) exhibited an increase with the increase of potassium citrate mass at the beginning. On the contrary, the effect of potassium citrate on the enrichment factor (F) was just reverse. It could be explained by the fact that the top phase volume increase greatly at first as the amount of potassium citrate increase [10]. Under the experiment condition, the enrichment factors (F) of [C_2mim]Br, [C_4mim]Br and [C_6mim]Br/potassium citrate systems were 11–125, 10–96 and 8–14, correspondingly. Note that there is no emulsion formation in the extraction process.

3.3. Effect of the alkyl chain length of the ILs

For the sake of illustrating the possible effect of alkyl chain length on the extraction procedure, four different alkyl chain length ILs ([$C_n$mim]Br, n = 2, 4, 6) were used for the experiments. The results were shown in Fig. 4. At any given mass of potassium citrate studied, the extraction efficiency of Cyt-c is found to follow the trend: [C_6mim]Br > [C_4mim]Br > [C_2mim]Br. In general, there are several competing interactions among the IL, the inorganic salt and water, such as hydrogen-bonding, interactions, π···π interactions,

Table 1
Extraction of Cyt-c used PEG and IL/potassium citrate systems at 30 °C. [Cyt-c] = 20 μg/g.

Component 1	Component 2	Y_b (%)	Y_e (%)
PEG1000 (18%, w/w)	Potassium citrate (20%, w/w)	92	8
PEG2000 (18%, w/w)	Potassium citrate (20%, w/w)	100	0
PEG4000 (18%, w/w)	Potassium citrate (20%, w/w)	100	0
PEG6000 (18%, w/w)	Potassium citrate (20%, w/w)	100	0
PEG10000 (18%, w/w)	Potassium citrate (20%, w/w)	100	0
[C_2mim]Br (6.25%, w/w)	Potassium citrate (41.7%, w/w)	No phase separation	
[C_4mim]Br (6.25%, w/w)	Potassium citrate (41.7%, w/w)	51	49
[C_6mim]Br (6.25%, w/w)	Potassium citrate (41.7%, w/w)	30	70

Y_b: the quantity ratio of the Cyt-c remained in the bottom phase to the total Cyt-c in the system and Y_e: the quantity ratio of the Cyt-c extracted in the top phase to the total Cyt-c in the system.

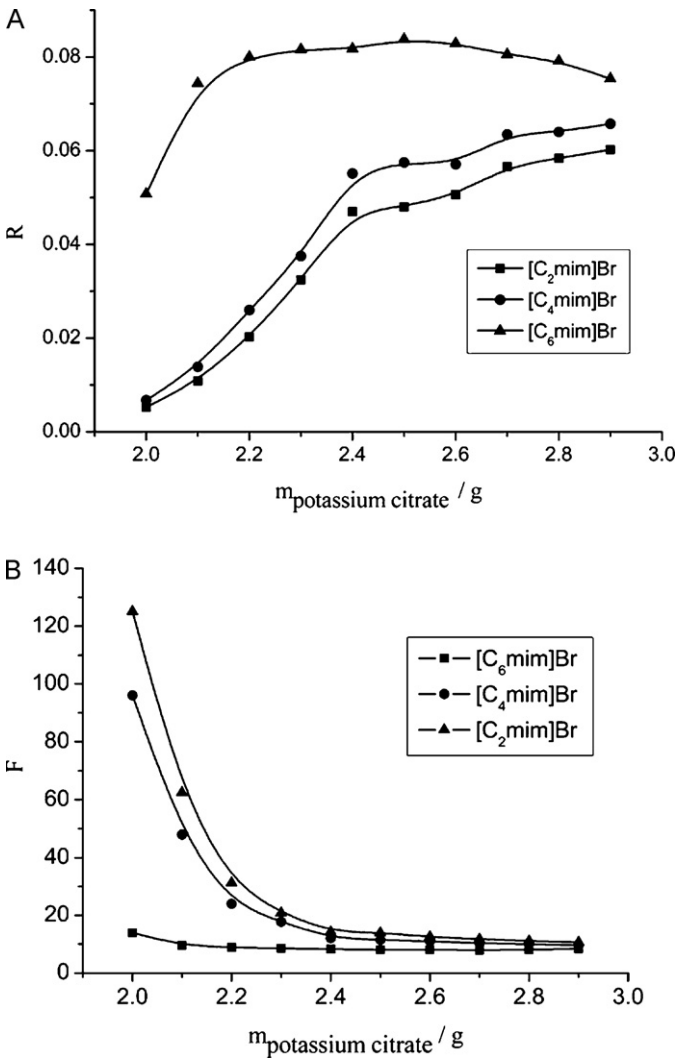

Fig. 3. Effect of the mass of potassium citrate on: (A) the phase volumes ratio (R) and (B) enrichment factor (F) at 30 °C. Water (2.5 g)/[C_2mim]Br (0.5 g), water (2.4 g)/[C_4mim]Br (0.4 g) and water (2.3 g)/[C_6mim]Br (0.3 g) were added in ATPSs.

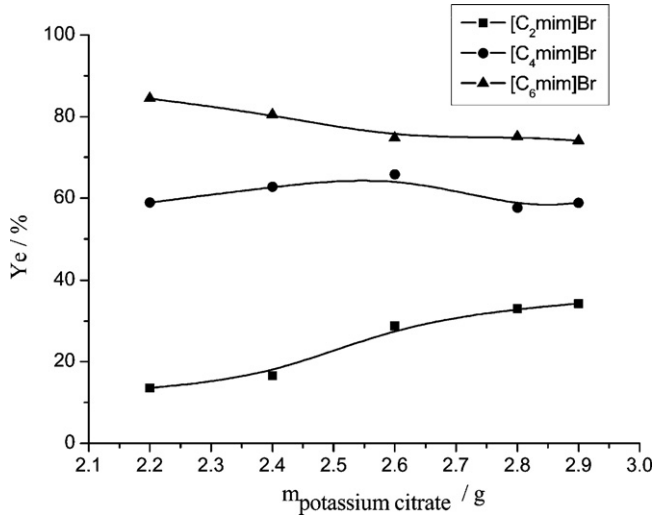

Fig. 4. Effect of alkyl chain of ILs on the extraction recovery of Cyt-c. [C_2mim]Br (0.5 g), [C_4mim]Br (0.4 g), [C_6mim]Br (0.3 g), and potassium citrate buffer [4.5 g (50%, w/w)] were added for the ATPS formation. [Cyt-c] = 20 μg/g, pH = 7.0, T = 30 °C.

Table 2
The transfer thermodynamic properties for Cyt-c in [C_6mim]Br/potassium citrate ATPS at pH 7.0. Potassium citrate buffer [4.5 g (50%, w/w)] and [C_6mim]Br (0.3 g) were added for the ATPS formation. [Cyt-c] = 20 μg/g.

T (K)	K	ΔG_T° (kJ/mol)	$T\,\Delta S_T^\circ$ (kJ/mol)	ΔH_T° (kJ/mol)
278.15	13.14	−5.95	30.43	24.61
288.15	17.98	−6.99	31.53	
298.15	22.97	−7.77	32.62	
308.15	35.92	−9.17	33.71	
318.15	51.42	−10.42	34.81	

hydrophobic interactions, and electrostatic interactions [20–24]. In the present work, the results indicated that the longer alkyl chain length of ILs resulted in better extraction efficiency. This observation is likely related to the hydrophobicity of the IL, which will turn stronger with the increase of the alkyl chain length [39,45]. Therefore, the hydrophobic interactions play a critical role in Cyt-c extraction process. The hydrophobic interactions between Cyt-c and the IL-rich top phase in ILATPS probably are the main driving force for the Cyt-c extraction.

3.4. Effect of temperature on the extraction efficiency

The effect of temperature on the extraction efficiencies of Cyt-c were investigated using [C_6mim]Br/potassium citrate system. The results are shown in Table 2 and Fig. 5. It can be observed that the extraction of Cyt-c is a spontaneous process ($\Delta G_T^\circ < 0$; $\Delta S_T^\circ > 0$). Meanwhile, the value of T ΔS_T° is always greater than that of ΔH_T°. It indicates that partition of the protein is controlled by entropy changes, which is the characteristic of hydrophobic interaction [46]. The hydrophobic interaction is a type of entropic forces. In the case of hydrophobic interactions, the entropy change is positive [47]. The ΔS_T° calculated for the Cyt-c extraction is indeed positive. The entropy and enthalpy change for the extraction of protein is characterized by positive values, and therefore endothermic and entropically driven [12]. Therefore, the thermodynamic studies also demonstrate that the hydrophobic interactions are the main droving force for the extraction of Cyt-c in [C_6mim]Br/potassium citrate aqueous two phase system. The similar mechanism was reported for BSA partitioning in IL/phosphate system [8,12]. As shown in Fig. 5, a linear correlation was found between ln K and 1/T within the temperature range of 5–45 °C. Over the investigated temperature range, the enthalpy change (ΔH_T°) is constant and the partition

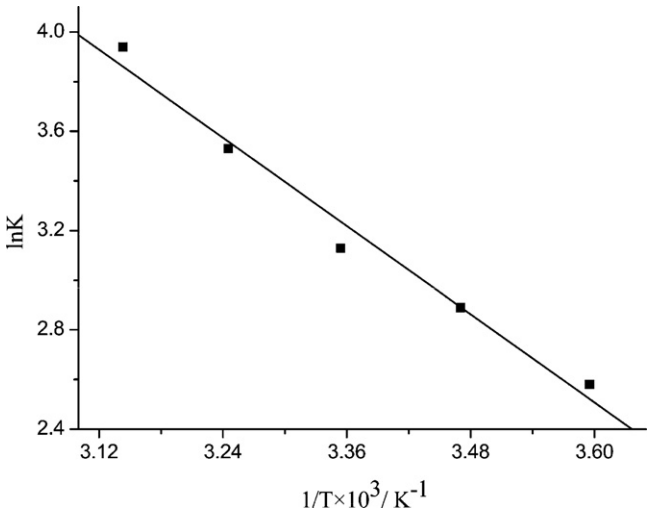

Fig. 5. The plot of ln K versus 1/T produces a straight line. [C_6mim]Br (0.3 g) and potassium citrate buffer [4.5 g (50%, w/w)] were added for the ATPS formation. [Cyt-c] = 20 μg/g, pH = 7.0.

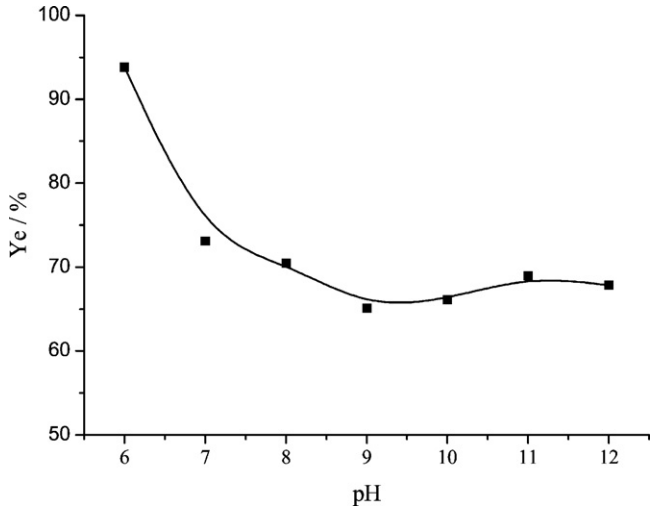

Fig. 6. Effect of pH on the extraction recovery of Cyt-c. [C_6mim]Br (0.3 g) and potassium citrate buffer [4.5 g (50%, w/w)] were added for the ATPS formation. [Cyt-c] = 20 μg/g, T = 35 °C.

coefficient (K) of the Cyt-c increases as the temperature rises. However, Zafarani-Moattar [26] reported that the phase-forming ability decreased as the temperature increased. The following studies were carried out at 35 °C.

3.5. Effect of pH on the extraction efficiency

In order to examine the effect of pH, the systems were prepared in different buffers with the pH ranging from 6 to 12 (Fig. 6). [C_6mim]Br/potassium citrate aqueous systems cannot form two phase at pH below 6.0. The protein carries a net negative charge when the pH of the system is higher than the isoelectric point (pI) of the protein. While the pH of the system is below its pI, the result will be reversed. As Cyt-c became more positive (pI = 10.3) by decreasing pH (10.0–6.0) of the system, an increase of the extraction recovery was observed (Fig. 6). It can be explained that the positively charged amino acids on a proteins' surface interact with the inorganic salts' anions which allow the interaction with the positively charged IL-cation [12]. The recovery achieved the minimum value when the pH of the system was close to the pI of the Cyt-c. As the pH value is above the pI of the Cyt-c, the recovery

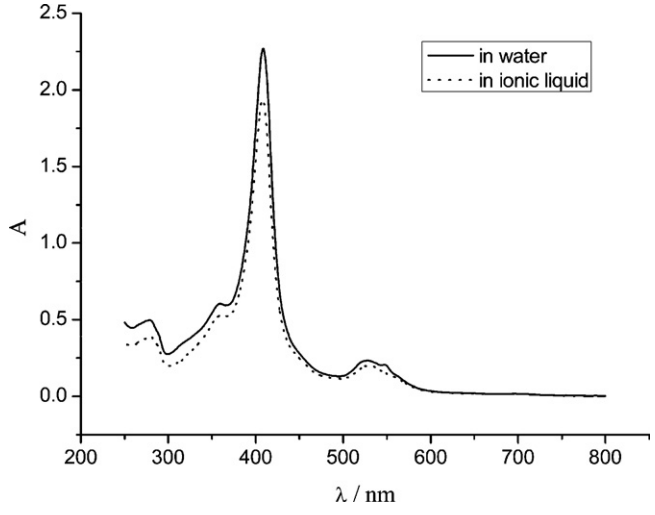

Fig. 7. UV–vis spectra of Cyt-c in pure water and in [C_6mim]Br-rich top phase. Solid line: [Cyt-c] = 25 μM; broken line: [Cyt-c] = 20 μM.

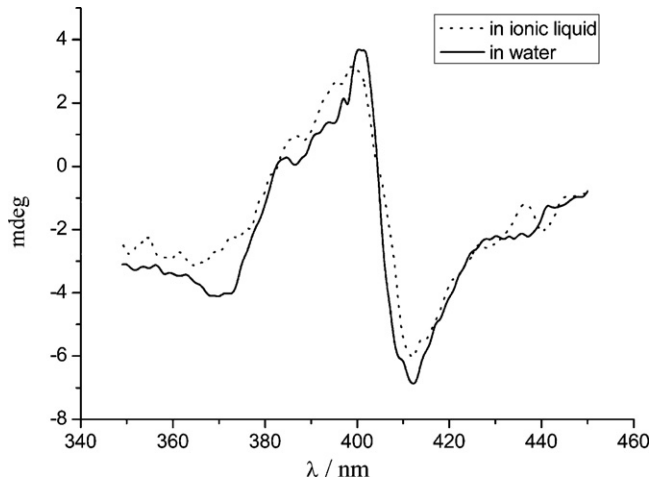

Fig. 8. CD spectra of Cyt-c in pure water and in [C_6mim]Br-rich top phase. Solid line: [Cyt-c] = 25 μM; broken line: [Cyt-c] = 20 μM.

is slightly enhanced. The outcome can be interpreted by another mechanism. The negatively charged residues of proteins interact with the positively charged imidazolium cation. To sum up, the experiment indicates that the pH of the IL-based ATPS plays an important role in the Cyt-c partitioning. It is confirmed that electrostatic interactions are also the main driving force governing Cyt-c partitioning in [C_6mim]Br/potassium citrate system.

3.6. Structural characterization and activity of Cyt-c in the extraction system

As shown in Fig. 7, the UV–vis absorption peak of Cyt-c is at 409 nm. It is obvious that the absorptions for Cyt-c are almost identical in despite of the composition of the matrices. The result clearly indicates that no direct bonding interaction generated when the Cyt-c was extracted into the top [C_6mim]Br-rich phase.

Next, the secondary structure and activity of Cyt-c in the pure water and top IL-rich phase after extraction were investigated respectively. Fig. 8 shows the CD spectra of Cyt-c before and after extraction. A negative (412 nm) peak and positive (400 nm) peak generated due to a split cotton effect are observed in the two medium. It is indicated that the secondary structure of the Cyt-c in IL-rich phase is unaltered. Unfortunately, the tertiary structure

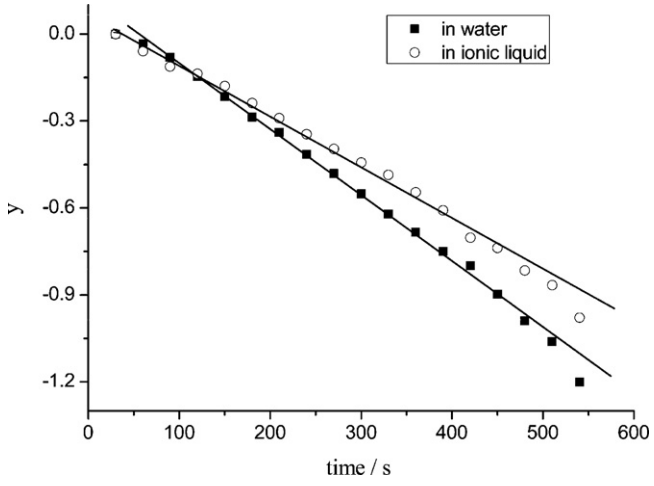

Fig. 9. Activity of the Cyt-c was measured by reaction of Cyt-c with ascorbic acid. [Ascorbic acid] = 2.0×10^{-4} mol/dm^3 and [Cyt-c] = 4.0×10^{-6} mol/dm^3. The absorbance was determined at 550 nm. $y = \ln\{(A_{max} - A)/(A_{max} - A_{min})\}$.

could not be measured accurately due to the impermeability of IL for short-wavelength light.

The activities of Cyt-c as an electron-transfer protein before and after extraction process were evaluated by the initial reduction rate coefficient (k). As shown in Fig. 9, $\ln\{(A_{max} - A)/(A_{max} - A_{min})\}$ against reaction time (t) gave satisfactory linear relationships. The initial reduction rate coefficient (k) of the reactions before and after extraction were 0.0022 and 0.0020, respectively, which obtained by linear least-square analysis (Fig. 9). Under the extraction conditions, the Cyt-c retained 91% of its activity, compared to the initial protein solution before the extraction step.

4. Conclusion

Extraction of Cyt-c using [C_6mim]Br/potassium citrate has been successfully accomplished. Thermodynamic studies indicated that the partitioning of Cyt-c was driven by both hydrophobic interactions and electrostatic interactions in the extraction process. The enthalpy was calculated to be 24.61 kJ/mol, which suggested that the extraction process of the Cyt-c was endothermic. The optimal system consisted of 0.3 g [C_6mim]Br and 4.5 g potassium citrate buffer (50%, w/w, pH 6.0) at 35 °C. Under these conditions, a high recovery (94%) and activity (91%) were obtained in the IL-rich top phase. UV–vis and CD spectra of Cyt-c in IL-rich phase proved that the structure of Cyt-c has not been altered after extracted into IL-rich phase. The results demonstrated that this aqueous two-phase system has the potential to be applied to extraction and concentration of Cyt-c.

Acknowledgments

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References

[1] J.S. Becker, O.R.T. Thomas, M. Franzreb, Sep. Purif. Technol. 65 (2009) 46–53.
[2] A.G. Gabriela, A.M. Azevedo, M.R. Aires-Barros, D.M.F. Prazeres, Sep. Purif. Technol. 65 (2009) 22–30.
[3] J. Benavides, M. Rito-Palomares, J. Chem. Technol. Biotechnol. 83 (2008) 133–142.
[4] G. Johansson, H. Walter, Int. Rev. Cytol. 192 (2000) 33–60.
[5] A.M. Azevedo, P.A.J. Rosa, I.F. Ferreira, A.M.M.O. Pisco, J. de Vries, R. Korporaal, T.J. Visser, M.R. Aires-Barros, Sep. Purif. Technol. 65 (2009) 31–39.
[6] M.J. Ruiz-Angel, V. Pino, S. Carda-Broch, A. Berthod, J. Chromatogr. A 1151 (2007) 65–73.
[7] A. Berthod, M. Ruiz-Angel, S. Carda-Broch, J. Chromatogr. A 1184 (2008) 6–18.
[8] Y.C. Pei, J.J. Wang, K. Wu, X.P. Xuan, X.J. Lu, Sep. Purif. Technol. 64 (2009) 288–295.
[9] X. Han, D.W. Armstrong, Acc. Chem. Res. 40 (2007) 1079–1086.
[10] C.Y. He, S.H. Li, H.W. Liu, K.A. Li, F. Liu, J. Chromatogr. A 1082 (2005) 143–149.
[11] M.G. Freire, C.M.S.S. Neves, I.M. Marrucho, J.N. Canongia Lopes, L.P.N. Rebelo, J.A.P. Coutinho, Green Chem. 12 (2010) 1715–1718.
[12] S. Dreyer, P. Salim, U. Kragl, Biochem. Eng. J. 46 (2009) 176–185.
[13] Y.H. Chen, Y.G. Wang, Q.Y. Cheng, X.L. Liu, S.J. Zhang, J. Chem. Thermodyn. 41 (2009) 1056–1059.
[14] Q.W. Yang, H.B. Xing, Y.F. Cao, B.G. Su, Y.W. Yang, Q.L. Ren, Ind. Eng. Chem. Res. 48 (2009) 6417–6422.
[15] Z. Du, Y.L. Yu, J.H. Wang, Chem. Eur. J. 13 (2007) 2130–2137.
[16] F.Y. Du, X.H. Xiao, X.J. Luo, G.K. Li, Talanta 78 (2009) 1177–1184.
[17] C.X. Li, J. Han, Y. Wang, Y.S. Yan, X.H. Xu, J.M. Pan, Anal. Chim. Acta 653 (2009) 178–183.
[18] Y.C. Pei, J.J. Wang, X.P. Xuan, J. Fan, M.H. Fan, Environ. Sci. Technol. 41 (2007) 5090–5095.
[19] S.H. Li, C.Y. He, H.W. Liu, K.A. Li, F. Liu, J. Chromatogr. B 826 (2005) 58–62.
[20] J.F.B. Pereira, A.S. Lima, M.G. Freire, J.A.P. Coutinho, Green Chem. 12 (2010) 1661–1669.
[21] C.L.S. Louros, A.F.M. Cláudio, C.M.S.S. Neves, M.G. Freire, I.M. Marrucho, J. Pauly, J.A.P. Coutinho, Int. J. Mol. Sci. 11 (2010) 1777–1791.
[22] M. Domínguez-Pérez, L.I.N. Tomé, M.G. Freire, I.M. Marrucho, O. Cabeza, J.A.P. Coutinho, Sep. Purif. Technol. 72 (2010) 85–91.
[23] C.M.S.S. Neves, S.P.M. Ventura, M.G. Freire, I.M. Marrucho, J.A.P. Coutinho, J. Phys. Chem. B 113 (2009) 5194–5199.
[24] S.P.M. Ventura, C.M.S.S. Neves, M.G. Freire, I.M. Marrucho, J. Oliveira, J.A.P. Coutinho, J. Phys. Chem. B 113 (2009) 9304–9310.
[25] K.E. Gutowski, G.A. Broker, H.D. Willauer, J.G. Huddleston, R.P. Swatloski, J.D. Holbrey, R.D. Rogers, J. Am. Chem. Soc. 125 (2003) 6632–6633.
[26] M.T. Zafarani-Moattar, S. Hamzehzadeh, J. Chem. Eng. Data 54 (2009) 833–841.
[27] H. Hustedt, Biotechnol. Lett 8 (1986) 791.
[28] T.S. Porto, G.M. Medeiros e Silva, C.S. Porto, M.T.H. Cavalcanti, B.B. Neto c, J.L. Lima-Filho, A. Converti, A.L.F. Porto, A. Pessoa Jr., Chem. Eng. Process 47 (2008) 716.
[29] G. Tubío, B. Nerli, G. Picó, J. Chromatogr. B 852 (2007) 244.
[30] M.T. Zafarani-Moattar, A.A. Hamidi, J. Chem. Eng. Data 48 (2003) 262–265.
[31] J. Vernau, M.R. Kula, Biotechnol. Appl. Biochem. 12 (1990) 397–404.
[32] C. Jolivalt, M. Minier, H. Renon, Biotechnol. Prog. 9 (1993) 456–461.
[33] T. Oshima, H. Higuchi, K. Ohto, K. Inoue, M. Goto, Langmuir 21 (2005) 7280–7284.
[34] B. Odell, G.J. Earlam, Chem. Soc. Chem. Commun. (1985) 359–361.
[35] K. Shimojo, K. Nakashima, N. Kamiya, M. Goto, Biomacromolecules 7 (2006) 2–5.
[36] K. Shimojo, N. Kamiya, F. Tani, H. Naganawa, Y. Naruta, M. Goto, Anal. Chem. 78 (2006) 7735–7742.
[37] D. Paul, A. Suzumura, H. Sugimoto, J. Teraoka, S. Shinoda, H. Tsukube, J. Am. Chem. Soc. 125 (2003) 11478–11479.
[38] A. Suzumura, D. Paul, H. Sugimoto, S. Shinoda, R.R. Julian, J.L. Beauchamp, J. Teraoka, H. Tsukube, Inorg. Chem. 44 (2005) 904–910.
[39] D.H. Cheng, X.W. Chen, Y. Shu, J.H. Wang, Talanta 75 (2008) 1270–1278.
[40] S. Yang, D.H. Cheng, X.W. Chen, J.H. Wang, Sep. Purif. Technol. 64 (2008) 154–159.
[41] M.T. Zafarani-Moattar, R. Sadeghi, Fluid Phase Equilib. 181 (2001) 95–112.
[42] D. Spelzini, B. Farruggia, G. Pico, J. Chromatogr. B 821 (2005) 60–66.
[43] M.M. Bradford, Anal. Biochem. 72 (1972) 248–254.
[44] T. Ono, T. Miyazaki, M. Goto, F. Nakashio, Solv. Extr. Res. Dev. Jpn. 3 (1996) 1–9.
[45] M.G. Freire, C.M.S.S. Neves, P.J. Carvalho, R.L. Gardas, A.M. Fernandes, I.M. Marrucho, L.M.N.B.F. Santos, J.A.P. Coutinho, J. Phys. Chem. B 111 (2007) 13082–13089.
[46] H. Bianco-Peled, S. Gryc, Langmuir 20 (2004) 169–174.
[47] http://www.chaperone.sote.hu/Forces.html (2011-6-3).