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Extraction of proteins with ionic liquid aqueous two-phase system based on guanidine ionic liquid

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ABSTRACT

Eight kinds of green ionic liquids were synthesized, and an ionic liquid aqueous two-phase system (ILATPS) based on 1,1,3,3-tetramethylguandine acrylate (TMGA) guanidine ionic liquid was first time studied for the extraction of proteins. Single factor experiments proved that the extraction efficiency of bovine serum albumin (BSA) was influenced by the mass of IL, K_2HPO_4 and BSA, also related to the separation time and temperature. The optimum conditions were determined through orthogonal experiment by the five factors described above. The results showed that under the optimum conditions, the extraction efficiency could reach up to 99.6243%. The relative standard deviations (RSD) of extraction efficiencies in precision experiment, repeatability experiment and stability experiment were 0.8156% ($n=5$), 1.6173% ($n=5$) and 1.6292% ($n=5$), respectively. UV–vis and FT-IR spectra confirmed that there were no chemical interactions between BSA and ionic liquid in the extraction process, and the conformation of the protein was not changed after extraction. The conductivity, DLS and TEM were combined to investigate the microstructure of the top phase and the possible mechanism for the extraction. The results showed that hydrophobic interaction, hydrogen bonding interaction and the salt out effect played important roles in the transferring process, and the aggregation and embrace phenomenon was the main driving force for the separation. All these results proved that guanidine ionic liquid-based ATPSs have the potential to offer new possibility in the extraction of proteins.

1. Introduction

Protein is the material basis of life. Proteins are involved in every cell and all the other important parts of the body. It is closely linked to the various forms of life activities. So, it is particularly necessary to prepare the pure proteins. However, due to the poor stability, proteins in the conditions of acids, alkali or heating are easily denatured. Therefore, the separation and purification of proteins become a bottleneck in the field of biotechnology.

The traditional protein purification methods include ammonium sulfate precipitation, salting out, electrophoresis, ion-exchange chromatography and affinity chromatography. These methods are not only time and cost consuming, but also making proteins lose activity. The aqueous two-phase system (ATPS) [1,2] emerged in recent years, is a clean alternative for traditional organic-water solvent extraction system. ATPSs are formed when two polymers, one polymer and one salt, or two salts are mixed at appropriate concentrations or at a particular temperature [3,4]. The two phases are mostly composed of water and non volatile components, thus eliminating volatile organic compounds. Aqueous two-phase system, which is a special case of liquid–liquid extraction system involving transfer of solute from one aqueous phase to another, has been used in biotechnological applications such as proteins [5,6], enzymes [7], nucleic acids [8,9], antibodies [10] and antibiotics [11,12] as non-denaturing and benign separation media recently.

Ionic liquid (IL) is a salt in the liquid state at room temperature because of its low melting point. While ordinary liquids such as water and gasoline are predominantly made of electrically neutral molecules, ionic liquids are completely composed by cations and anions, also known as low-temperature molten salt [13,14]. Ionic liquids have many distinctive properties characterized by low vapor pressure, low combustibility, excellent thermal stability, wide liquid regions, and favorable solvating properties for a range of polar and non-polar compounds [15–17]. Because of their unique properties, ionic liquids are attracting increasing attention in many fields, including organic chemistry [18–21], electrochemistry, catalysis [22], physical chemistry, and extraction/separation [23–25].

As an environmentally friendly solvent, hydrophilic ionic liquid can form an aqueous two-phase system with an appropriate salt. Ionic liquid aqueous two-phase system (ILATPS), which combines the advantages of ionic liquid and aqueous two-phase system, has many advantages that traditional aqueous two-phase system cannot match, such as low viscosity, short separation time, and the ionic liquid can be recycled. Therefore, the ILATPS is widely used in the biological

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separation and purification fields. The ionic liquid aqueous two-phase system was first found in 2002 by Dupont [26]. He had synthesized the ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate ($[C_4mim][BF_4]$) by chloride l-butyl-3-methylimidazolium ($[C_4mim]$ C1) and potassium tetrafluoroborate (KBF_4). Later in the study, he had found that hydrophilic ionic liquid $[C_4mim][BF_4]$ and KCl aqueous solution can form two phases. But the ionic liquid aqueous two-phase system was proposed in 2003 by Rogers' research team for the first time [27]. They had mixed hydrophilic ionic liquid $[C_4mim]Cl$ with K_3PO_4 to form an aqueous two-phase system, and found that the top phase enrichment of ionic liquids, the bottom phase enriched inorganic salts as well. In the previous works, some investigators

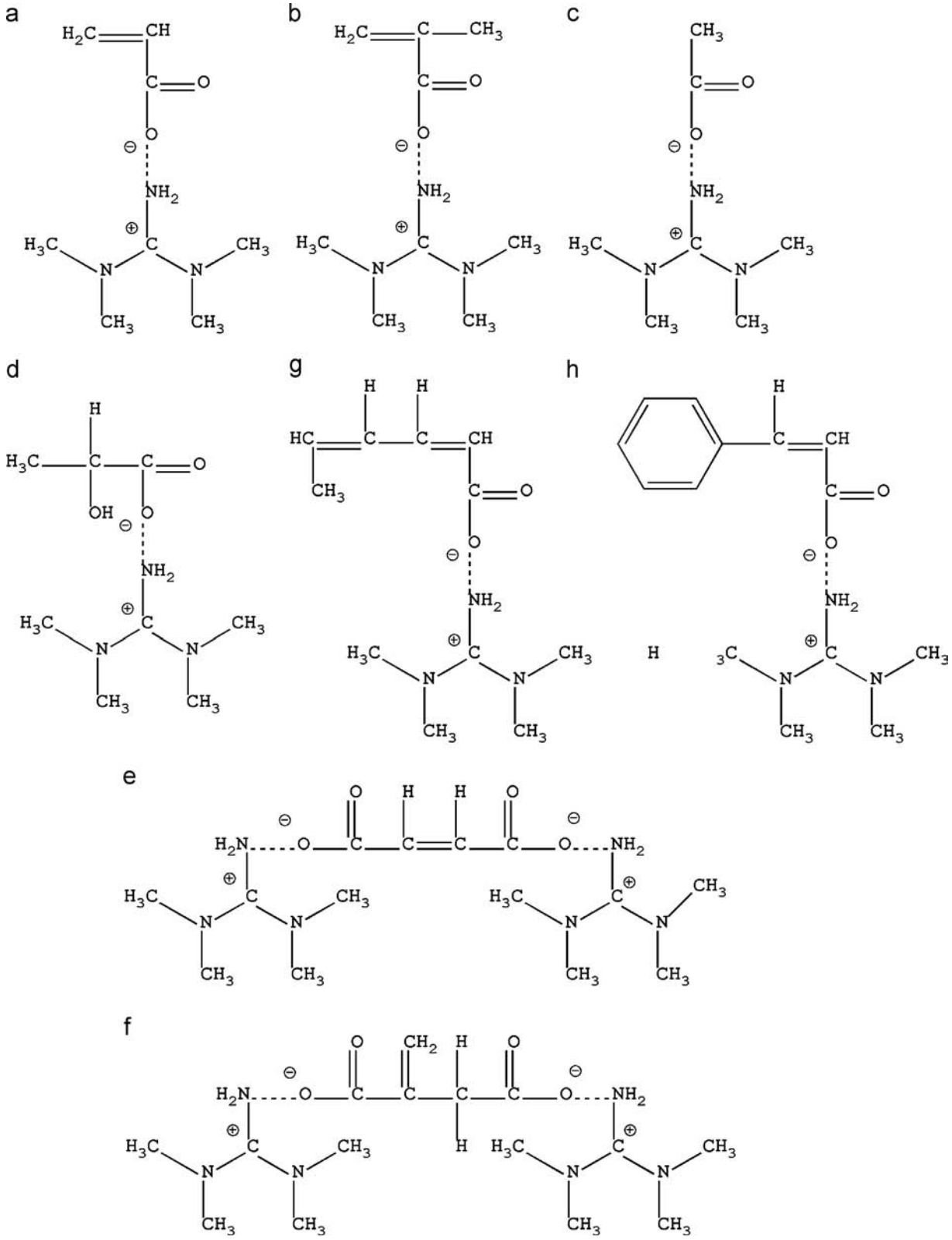

Fig. 1. The structures of the eight ionic liquids.

had studied the extraction of medicinal compounds or proteins by imidazolium ionic liquids [28–30], some others had studied the applications of guanidine ionic liquids [31] in SO_2 absorption [32], but no one had learned the separation of proteins by guanidine ionic liquids. Guanidino compounds have attracted widespread concern of the chemist and pharmacologist for the high thermal and chemical stability, high catalytic activity, and strong physiological activity. Moreover, the functional group of guanidino in the living body, plays an unique role in molecular recognition. And due to the high dispersion of cationic charge groups on the three nitrogen atoms, the guanidine ionic liquids can be discretionarily designed and synthesized.

In this paper, it is reported for the first time for the extraction of proteins by an ionic liquid aqueous two-phase system based on the guanidine ionic liquid and hydrogen phosphate. After phase separation, proteins had transferred into the IL-rich top phase. The concentrations of proteins in top phases were determined by measuring the absorbance at 278 nm for bovine serum albumin (BSA) and ovalbumin (OVA), and at 404 nm for bovine hemoglobin (BHb) using a UV2450 UV–vis spectrophotometer. In addition, FTIR, UV–vis, conductivity, DLS and TEM were used to study the mechanism of extraction process. It was suggested that aggregation and embrace phenomenon play a significant role in the separation of proteins. All these results showed that guanidine IL-based ATPSs have the potential to offer new possibility in the extraction of proteins.

2. Experimental

2.1. Chemicals

1,1,3,3-Tetramethylguanidine (> 99%, Aladdin Chemical Reagent Co., Ltd.) was used without further purification. Acrylic acid (≥99.5%) and Methacrylic acid (≥99%) were purchased from Tianjin Guangfu Fine Chemical Research Institute. Acetic acid (Sinopharm Chemical Reagent Co., Ltd.), DL-Lactic acid (West Long Chemical Co., Ltd.), Maleic acid (The Yamaura Chemical Co., Ltd.), Itaconic acid (Aladdin Chemical Reagent Co., Ltd.), Sorbic acid (The Yamaura Chemical Co., Ltd.) and trans-Cinnamic acid (Tianjin voyage Chemicals Co., Ltd.) were all of analytical grade. Bovine serum albumin, Bovine hemoglobin and Ovalbumin were all from Sinopharm Chemical Reagent Co., Ltd. Various salts, including $K_2HPO_4 \cdot 3H_2O$, K_3PO_4, K_2CO_3 and KH_2PO_4, investigated for their suitability for the formation of ionic liquid/aqueous two-phase systems, were purchased from Sinopharm Chemical Reagent Co., Ltd.

2.2. Apparatus

UV–vis spectra were measured with a UV2450 spectrophotometer (Shimadzu Corporation). FTIR spectra were recorded on a Spectrum One FTIR Spectrometer (Perkin Elmer, U.S.). ^{1}H NMR and ^{13}C NMR spectra were measured with a Varian Inova-400 spectrometer (Varian, U.S.). A LCQ-Advantage mass spectrometer (Thermo Finnigan, U.S.) was used for the measurement of the mass of ILs. The DLS measurements were carried out using a Zetasizer Nano-ZS90 (Malvern Instruments, U.K.). After being dried, the samples were imaged under a JEOL JEM-3010 transmission electron microscopy.

2.3. Synthesis and characterization of ILs

According to the literature, eight kinds of ionic liquids (as shown in Fig. 1) consisting of 1,1,3,3-tetramethylguanidinium cation and different anions (as shown in Table S1), were directly synthesized by neutralization of 1,1,3,3-tetramethylguanidine (TMG) and eight different kinds of acids. The synthetic route of the ionic liquids was shown in Fig. S1. As an example, 1,1,3,3-tetramethylguandine acrylate (TMGA) was synthesized via direct neutralization of 1,1,3,3-tetramethylguanidine (TMG) and acrylic acid (AA) without any solvents. TMG (50.60 mL, 0.4 mol) was firstly added to a dried 100 mL Single-necked flask. The flask was equipped with a magnetic stirrer and the temperature was controlled at 25 °C. AA (33.93 mL, 0.4 mol) was then added dropwise with a dropping funnel in 2 h. The reaction was continued for 4 h. Finally, the product was vacuum distilled at 70 °C to remove unreacted reactants. 1,1,3,3-tetramethylguandine acrylate, viscous liquid with slight yellow was produced with a yield of 96.32%. The preparation methods of other ILs were similar with TMGA's. The structures of all the synthetic ILs were confirmed by FT-IR, ^{1}H NMR and ^{13}C NMR spectra, which were shown in Fig. S2 and Table S2, respectively.

2.4. Preparation of binodal curves for ATPSs

The phase diagrams were prepared by the turbid titration method. A few moles of ionic liquid were weighed into a 10 mL tube. A known concentration of K_2HPO_4 solution was then added dropwise to the centrifuge tube until the mixture became turbid or cloudy. The volume of the salt solution added was recorded and the composition of this mixture was calculated. Addition of a few drops of water made the mixture clear again, and the above procedure was repeated to obtain sufficient data to construct a liquid–liquid equilibrium binodal curve.

2.5. Extraction and determination of proteins in both phases

An appropriate amount (3.0 mmol) of the ionic liquids were taken into a 10 mL centrifuge tube. Then, 3 mL K_2HPO_4 aqueous solutions with a concentration of 0.50 g/mL were added and 25 mg proteins were mixed in. Afterwards, the mixture was shaken vigorously in a shaker for 30 min to ensure the transfer of proteins into IL-rich top phase. The schematic diagram of extraction process was shown in Fig. 2. After extraction, the concentrations of proteins in top phases were determined by measuring the absorbance at 278 nm for bovine serum albumin (BSA) and ovalbumin (OVA), and at 404 nm for bovine hemoglobin (BHb) using a UV2450 UV–vis spectrophotometer.

Partition coefficient (K) of the proteins between the phases was calculated by

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

Phase volume ratio (R) is defined as volume ratio of the top phase to the bottom phase:

$$R = \frac{V_t}{V_b}$$

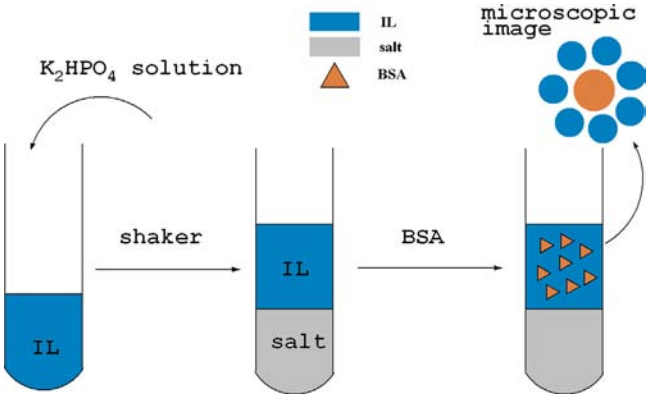

Fig. 2. The extraction process of ILATPS.

The extraction efficiency (E) was calculated by the following equation:

$$E = \frac{C_t V_t}{C_t V_t + C_b V_b} = \frac{KR}{1 + KR}$$

where C_t and C_b are the concentrations of proteins in the IL-rich top phase and the phosphate-rich bottom phase, respectively. V_t and V_b stand for the volume of the top phase and bottom phase, separately.

3. Results and discussion

3.1. Phase diagram

Phase diagram data are required for the design of aqueous two-phase extraction process. The binodal curves determined at 25 °C for the TMGA/salt and IL/K_2HPO_4 systems were shown in Fig. 3a and b, respectively. These binodal curves provide information about the concentrations of ILs and salts required to form aqueous two-phase systems. Fig. 3a shows the phase diagram for the aqueous two-systems of 1,1,3,3-tetramethylguandine acrylate (TMGA) with a series of salts. It is clear that the ability of these salts for phase separation follows the order: $K_3PO_4 > K_2HPO_4 > KH_2PO_4 > K_2CO_3$, indicating that less grams of K_3PO_4 were needed to form an aqueous two-phase system. However, K_2HPO_4 was chosen in this study because of its high solubility in water (1600 g/L), strong phaes-forming ability, as well as the capability to provide a suitable pH range for phase separation. It can be seen from Fig.3b that the ability of the ILs for phase separation follows the order: Acetate > Sorbate > Itaconate > Acrylate > Methacrylate > Lactate > Cinnamate > Maleate. Maybe, the phase-forming ability is associated with their viscosity. It is inferred that the lower viscosity results in the higher solubility in water, and the higher solubility in water leads to the stronger phase-forming ability.

In a system consisting of K_2HPO_4 (0.50 g/mL) and aqueous solution (3.0 mL), effect of the amount of ionic liquid on the formation of ATPS at room temperature was studied. The results were shown in Fig. 4a. A further increase in the moles of TMGA guaranteed an increase in the volume of IL-rich top phase, and a decrease in the volume of bottom phase. The volume ratio of top phase to bottom phase ($R = V_t/V_b$) was calculated. Finally it was found that the coefficient R linearly increased by increasing amount of ionic liquid with a slope of 0.18321.

In order to investigate its effect on the formation of the two-phase system and the capability for phase separation, various concentrations of K_2HPO_4 (3.0 mL) were added at room temperature to the system

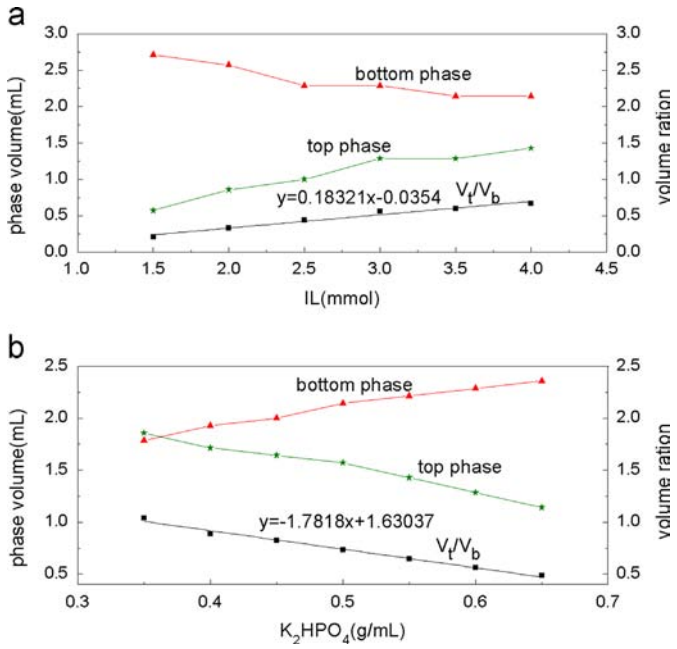

Fig. 4. (a) Effect of the amount of TMGA on the formation of ATPS at 25 °C. 3 mL K_2HPO_4 solution (0.50 g/mL) was added into the ATPS and (b) effect of the amount of K_2HPO_4 on the formation of ATPS at 25 °C. 3.0 mmol TMGA was added into the ATPS.

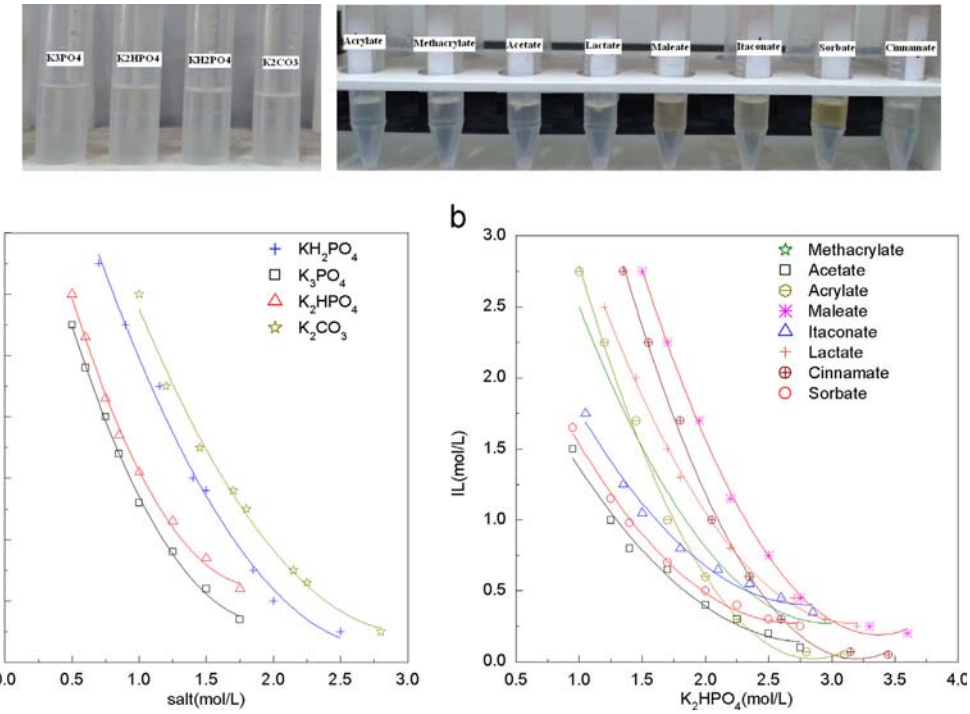

Fig. 3. Phase diagram of IL/salt aqueous two-phase systems: (a) TMGA/salt and (b) IL/K_2HPO_4.

with a certain amount of TMGA (3.0 mmol). The results were shown in Fig. 4b. The experiments indicated that further increases in the concentration of K_2HPO_4 ensured a slight increase in the volume of bottom phase, and a decrease in the volume of top phase. Moreover, the volume ratio of top phase to bottom phase ($R=V_t/V_b$) was calculated, and it was found that the coefficient R decreased linearly by increasing concentration of K_2HPO_4 with a slope of −1.7818.

3.2. Single factor experiments

3.2.1. Effect of the kinds of ionic liquids

Eight kinds of ionic liquids with the common cation of 1,1,3, 3-tetramethylguanidine prepared have been investigated for the extraction of three proteins (BSA, BHb, OVA). The extraction efficiencies were showed in Table S3. It is obvious that different ionic liquids have different abilities to extract various proteins, and BSA is completely transferred into TMGA-rich top phase after extraction. Therefore, when we take BSA as the object of this research, TMGA is the best extraction solvent. As an example, the effect on different kinds of ionic liquids for the extraction of BSA was shown in Fig. 5a.

3.2.2. Effect of the mass of ionic liquid

As an example, the effect on BSA distribution of the mass of TMGA was studied, and the results are illustrated in Fig. 5b. TMGA/K_2HPO_4 (0.5 g/mL, 3.0 mL) aqueous two-phase system was used. 20 mg BSA was added. As indicated in Fig. 5b, 88–100% of BSA was transferred into IL-rich top phase, and the extraction efficiency was increased with the addition of ionic liquid. When TMGA is weighed 3.5 mmol, the efficiency is the highest with 99.4547%. It is the reason that the ionic liquid can form aggregates, and with the advanced concentration of IL, the number of IL particles is growing that more BSA aggregates can be enveloped by IL aggregates.

3.2.3. Effect of the amount of K_2HPO_4

As an example, Fig. 5c shows the effect on BSA distribution of the amount of K_2HPO_4. TMGA (3.5 mmol)/K_2HPO_4 (3.0 mL) aqueous two-phase system was used and 20 mg BSA was added. As indicated in this figure, extraction efficiency changes with the various concentrations of K_2HPO_4 over the range of 0.35–0.65 g/mL. It is clear that the extraction efficiency was increased with the increasing concentration of K_2HPO_4 solution between 0.4–0.55 g/mL, and when the concentration of K_2HPO_4 was higher than 0.55 g/mL, the extraction efficiency had fallen rapidly. The protein structure is dependent on the hydrophobic/hydrophilic balance. K_2HPO_4 is a strong salting-out salt and large amount of K_2HPO_4 in protein solution can increase the hydrophobic interaction, leading to the reduction of the solubility of protein in water. And the reason for the decrease of extraction efficiency when K_2HPO_4 concentration was higher than 0.55 g/mL might be that the protein molecular structure is also controlled by the surface water of protein. When the bottle phase is highly hydrophilic, hydrogen bonding interaction between the surface water of protein and amino acid residue drives protein to transfer into IL-rich top phase. Therefore, it is likely that the hydrophobic interaction, salting-out effect and hydrogen bonding interaction acts as an important driving force to make protein transfer into IL-rich top phase.

3.2.4. Effect of the mass of protein

In order to discuss the effect on extraction efficiency of the mass of protein, TMGA(3.5 mmol)/K_2HPO_4 (0.5 g/mL and 3.0 mL) aqueous two-phase system was adopted and the results illustrated in Fig. 5d. As can be seen from Fig. 5d, the extraction efficiency of BSA was nearly 90% when the mass of BSA was between 10–25 mg, while out of this range, the extraction efficiency was decreased to 70% or even lower. So, too few or too many mass of protein was adverse to protein distribution. It is obvious that the number of

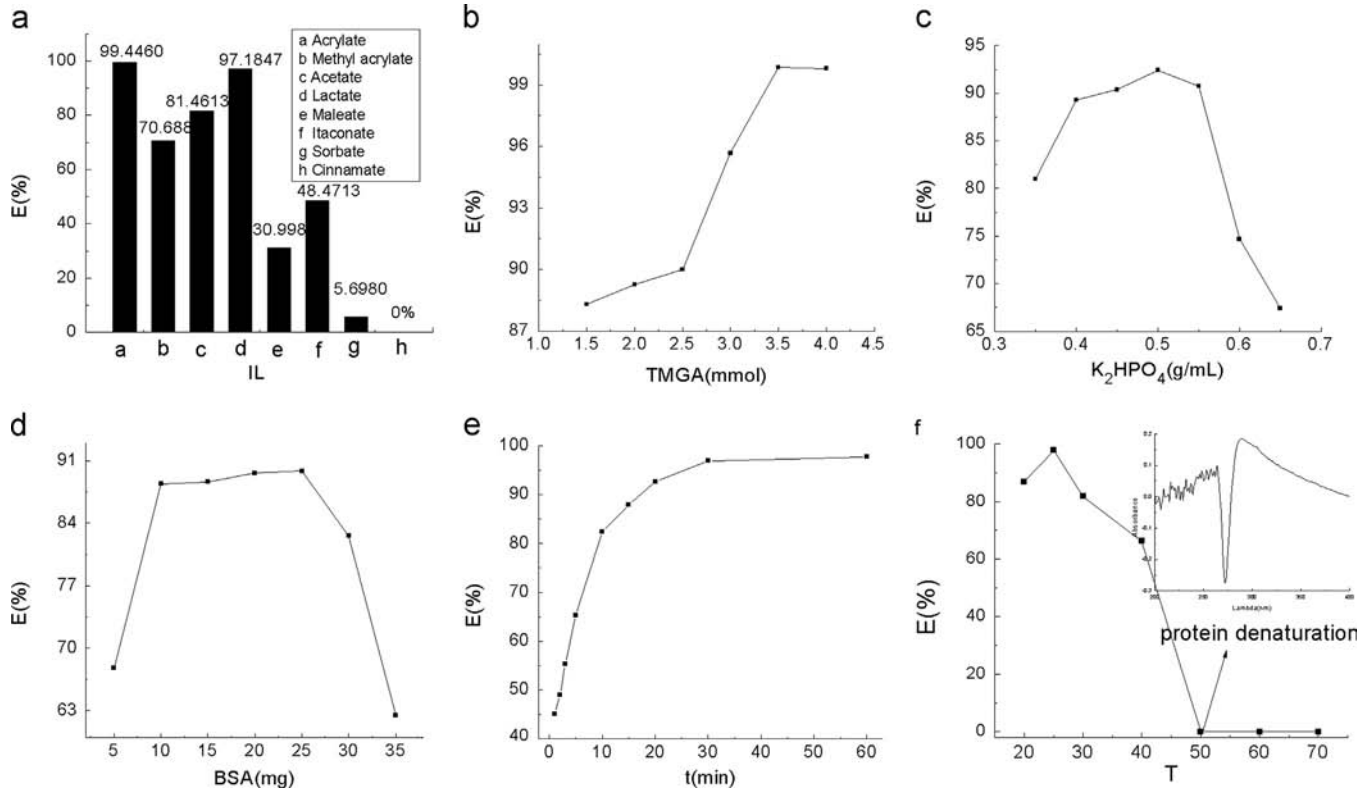

Fig. 5. Effect of kinds of IL (a), mass of TMGA (b), concentration of K_2HPO_4 (c), mass of BSA (d), separation time (e), and temperature (f) on the extraction efficiency in TMGA/K_2HPO_4 aqueous two-phase system.

protein aggregates is growing with the increasing mass of BSA. So it is believed that in the beginning, the extraction efficiency increases quickly before 10 mg. But the extraction efficiency shows little change between 10 mg and 25 mg, which is the best scope for BSA extraction. However, an extraction system has a limited ability of extraction, so the system is near saturation when BSA addition is 25 mg. When BSA addition is more than 25 mg, the extraction quantity may have a little increase, but the extraction efficiency decreases rapidly.

3.2.5. Effect of the separation time

Separation time is an obvious influencing factor of the extraction efficiency. The extraction of BSA was carried out over a time range of 1–60 min, and the time dependence of the extraction efficiency is illustrated in Fig. 5e. TMGA (3.5 mmol)/K_2HPO_4 (0.5 g/mL and 3.0 mL) aqueous two-phase system was adopted and 20 mg BSA was employed. It is shown that within 10 min, the amount of BSA transferred into IL-based top phase grows linearly with the increase of separation time. In the range of 10–30 min, the extraction efficiency obviously increases from 82.3702% to 96.8964%. And when separation time is increased to 1 h, the extraction efficiency is no longer growing, keeping an extraction efficiency of almost 97%. Therefore, 30 min was chosen to be an appropriate separation time.

3.2.6. Effect of the temperature

As an example, the extraction efficiency of BSA was studied over a temperature range of 20–70 °C, and the results were presented in Fig. 5f. The TMGA (3.5 mmol)/K_2HPO_4 (0.5 g/mL and 3.0 mL) aqueous two-phase system was used and 20 mg BSA was employed. It is obvious that at temperature below 50 °C, BSA transferred into IL-based top phase remained virtually unchanged, keeping high extraction efficiency. When temperature kept at 50 °C or higher, BSA was denaturated because of heat-treated. But according to further research, denaturation of BSA at 50 °C was reversible while denaturation of BSA at 60 °C and 70 °C was irreversible. Therefore, the temperature was controlled not to exceed 50 °C to keep protein unchanged in the separation process. The possible reason for this phenomenon is that the hydrophobic interaction enhances with the increased temperature, but when temperature is high to destroy the hydrogen bonding interaction between the surface water of protein and amino acid residue, the formation of hydrogen bond is less influenced by hydrophobic interaction so that hydrophobic effect weakened. It is worth mentioning that the suitable temperature is 25 °C, making the separation process more convenient.

3.3. Orthogonal experiment

The optimize conditions were determined through orthogonal experiment by mole number of IL (factor A), concentration of K_2HPO_4 (factor B), mass of protein (factor C), temperature (factor D) and separation time (factor E). The results of orthogonal experiment on five factors and four levels were shown in Table S4. K_1, K_2, K_3 and K_4 mean the average extraction efficiencies of each factor in each of the level. R is the difference value between the highest and the lowest values of K. The bigger the difference of R, the more influence the factor has on the extraction efficiency. Table S4 shows that the major and minor orders of the five influencing factors are ACBDE from primary to secondary, and the optimize conditions are $A_2B_3C_4D_2E_3$, when the extraction efficiency reaches up to 99.6243%.

3.4. Methodological study

Method in this study was validated by the precision experiment, repeatability experiment and stability experiment. Apparatus precision was investigated by the measurement of the top phase solution for five times in parallel under the same conditions. The relative standard deviation (RSD) of extraction efficiency is 0.8156% ($n=5$), explaining the precision of the UV–vis spectra is excellent. And take five copies of the same sample measured respectively under the same conditions. The value of RSD is 1.6173% ($n=5$), indicating that the detection method has good repeatability. Stability experiment was performed by taking a sample detected continuously in five days under the same conditions. The results show the relative standard deviation (RSD) of extraction efficiency is 1.6292% ($n=5$), proving the sample is recoverable within five days.

3.5. Extraction mechanism

3.5.1. UV–vis and FT-IR spectra

In order to examine the protein conformation before and after extraction, UV–vis and FT-IR spectra were investigated for BSA. Fig. S3 shows the UV–vis spectra of BSA in pure water and in IL-rich top phase after extraction. It is clear that the maximum absorption peak of BSA in IL-rich top phase still existed at 278 nm, and the figures of BSA before and after extraction were similar, suggesting that there are no chemical bonds between BSA molecules and ILs.

FT-IR spectra provide useful information for identifying the presence of certain functional groups or chemical bonds in a molecule or an interaction system, attributable to the unique energy absorption bands for specific bonding environments or interactions [33]. Proteins are irregular polymers made up essentially of 20 amino acids with four levels of spatial structure. The primary structure in a polypeptide chain is its amino acid sequence, while linear segments of the polypeptide chain (i.e., a —helices, b—sheets, and b—turns) constitute the secondary structure, the conformation of which is stabilized by the main-chain hydrogen bonds [34]. Amide is the basic unit of the peptide bond: amide I is assigned to both C═O stretching vibration and ring stretching vibrations, while amide II is assigned to C–N stretching vibrations. The absorption bands most widely used as structure probes in protein FT-IR spectroscopy have been the amide I vibrations, which fall between 1690 and 1600 cm^{-1} [35]. Fig. S4 shows the FT-IR spectra of pure ionic liquid, pure BSA and BSA in IL-rich top phase after extraction. It is clear that before extraction, the absorption band of TMGA (1577 cm^{-1}) and the two absorption bands of BSA (the amide I band at 1640 cm^{-1} and the amide II band at 1542 cm^{-1}) were identifiable in the spectra. When after extraction, the two absorption bands have changed to be 1620 cm^{-1} and 1562 cm^{-1} in the IL-BSA complexity. There was no disappearance or little shift of the peak, suggesting the conformation of the protein was not changed after extraction.

3.5.2. The microscopic structure of IL-rich top phase

Examination of the microscopic structure of the IL-rich top phase is a further understanding of the separation process. Fig. S5 shows the concentration dependence of the conductivity for aqueous TMGA solutions at 25 °C. The conductivities exhibit typical behavior with two linear fragments, and the concentration at which the two linear fragments intersect is assigned to the critical aggregation concentration (CAC). The CAC value of the TMGA was shown to be nearly 0.10 g/mL. As the IL concentration is greater than the CAC, the IL aggregates would be formed. Therefore, it is proved that aggregates of ionic liquid were formed in the top phase of the ATPS (0.2 g/mL IL+2.5 mg/mL BSA) investigated in the present work. The size distribution of the IL aggregates was determined by DLS at different concentrations.

As shown in Fig. S6, the aqueous TMGA solution is not microscopically homogeneous, but polydisperse. The average size is 250.4, 374.9, 560.7, 831.8 and 1196.0 nm, when the concentration is 0.10,

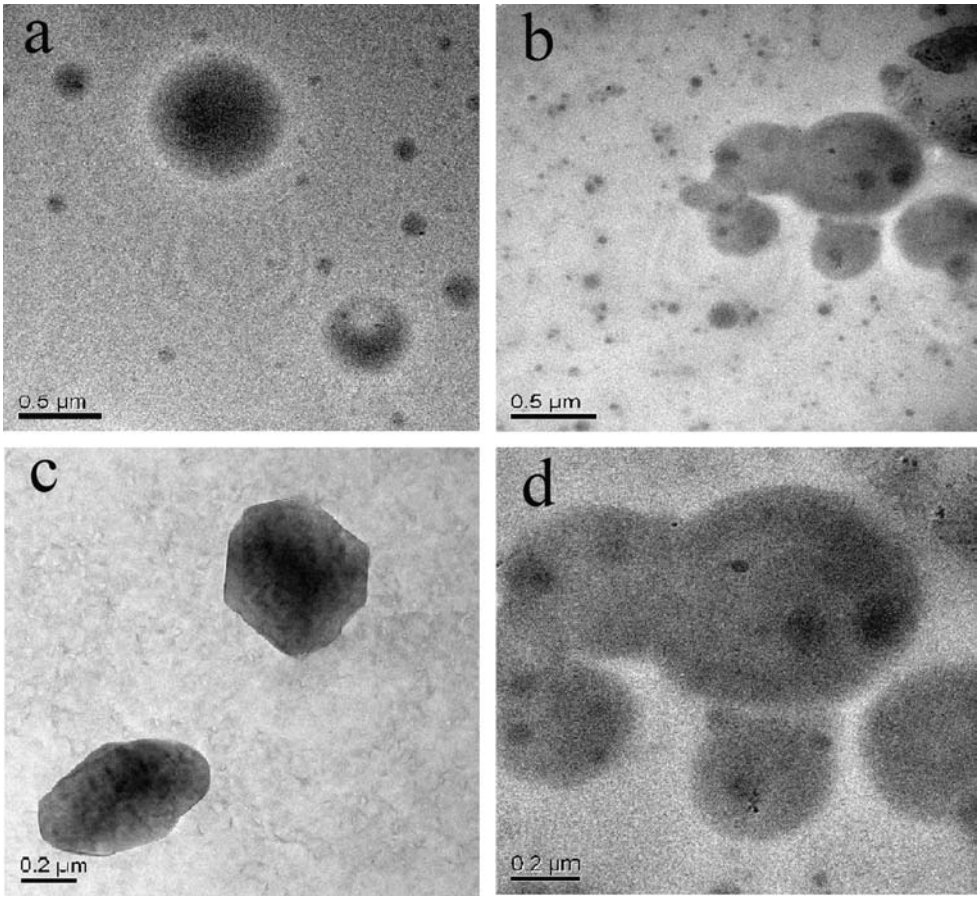

Fig. 6. TEM images of the aggregates for IL (0.2 g/mL), BSA (2.5 mg/mL) and IL (0.2 g/mL)+BSA (2.5 mg/mL). (a) IL; (c) BSA; (b and d) IL+BSA.

0.20, 0.30, 0.40, 0.50 g/mL, respectively. The size of IL aggregates increases with the increasing concentration of IL. Fig. S7 shows the DLS results of the aqueous ionic liquid solution, aqueous protein solution and aqueous IL+protein solution. It is shown that the size of mixture aggregate is smaller than IL aggregate and protein aggregate.

Then, TEM images of IL (0.2 g/mL), protein (2.5 mg/mL) and mixture (0.2 g/mL IL+2.5 mg/mL protein) were measured as a result of Fig. 6. Fig. 6a shows the conformation of IL aggregate (0.5 μm), Fig. 6c shows the appearance of protein aggregate (0.2 μm), and Fig. 6b and d shows the distribution of mixture aggregate in 500 nm and 200 nm, respectively. From Fig. 6b and d, it can be seen that the IL aggregates encircle the BSA aggregate (as in the schematic diagram of the extraction process Fig. 2). Therefore, it is appropriate to state that the formation of IL aggregates and IL-protein aggregates is the main driving force in the uptake of protein by ILATPS.

4. Conclusions

This is the first report for the extraction of proteins with an ionic liquid aqueous two-phase system (ILATPS) based on guanidine ionic liquid. The greatest benefit of the proposed method is that the adapted extraction solvent is green and environmentally friendly. In comparison with the imidazole ionic liquids, guanidine ionic liquids display more superiority. Guanidino compounds have attracted widespread concern of the chemist and pharmacologist for the high thermal and chemical stability, high catalytic activity, and strong physiological activity. Moreover, the functional group of guanidino in the living body, plays an unique role in molecular recognition. And due to the high dispersion of cationic charge groups on the three nitrogen atoms, the guanidine ionic liquids can be discretionarily designed and synthesized. In addition, besides the single factor experiments, the orthogonal experiment was performed to acquire the optimum conditions of extraction of proteins with this ILATPS. These data fully illustrated the choices of factors in this system. It is worth to mention that the aggregation and embrace phenomenon is the virtual governing force controlling the BSA extraction. As the other driving forces involved in the partitioning of protein between the IL-rich and phosphate phase, the aggregation and embrace phenomenon could be applied to a variety of different samples and exhibited potential value.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.06.011.

References

[1] A.M. Azevedo, P.A.J. Rosa, I.F. Ferreira, Sep. Purif. Technol. 65 (2009) 33.
[2] J.S. Becker, O.R.T. Thomas, M. Franzreb, Sep. Purif. Technol. 65 (2009) 49.
[3] L. Li, F. Liu, X.X. Kong, Anal. Chim. Acta 452 (2002) 327.

[4] H. Walter, G. Johansson (Eds.), Methods in Enzymology, vol. 228, Academic Press, London, 1994.
[5] N.L.P. Dallora, J.G.D. Klemz, P.A.P. Filho, Biochem. Eng. J. 34 (2007) 94.
[6] D.H. Chen, X.W. Chen, Y. Shu, J.H. Wang, Talanta 75 (2008) 1270.
[7] C.K. Su, B.H. Chiang, Process Biochem. 41 (2006) 261.
[8] G.A. Gomes, A.M. Azevedo, M.R. Aires-Barros, D.M.F. Prazeres, Sep. Purif. Technol. 65 (2009) 27.
[9] J.H. Wang, D.H. Cheng, X.W. Chen, Z. Du, Z.L. Fang, Anal. Chem. 79 (2007) 620.
[10] A.M. Azevedo, A.G. Gomes, P.A.J. Rosa, I.F. Ferreira, A.M.M.M.O. Pisco, M.R. Aires-Barros, Sep. Purif. Technol. 65 (2009) 18.
[11] B. Mokhtarani, R. Karimzadeh, M. Amini, S.D. Manesh, Biochem. Eng. J. 38 (2008) 248.
[12] M.M. Bora, S. Borthakur, P.C. Rao, N.N. Dutta, Sep. Purif. Technol. 45 (2005) 156.
[13] T. Welton, Chem. Rev. 99 (1999) 2084.
[14] R.D. Rogers, K.R. Seddon, Science 302 (2003) 793.
[15] M.J. Earle, J.M.S.S. Esperanca, M.A. Gilea, J.N. Canongia Lopes, L.P.N. Rebelo, J.W. Magee, K.R. Seddon, J.A. Widegren, Nature 439 (2006)834 439 (2006).
[16] C. Carrillo-Carrion, B.M. Simonet, M. Valcarcel, Analyst 137 (2012) 1159.
[17] F.Y. Du, X.H. Xiao, X.J. Luo, G.K. Li, Talanta 78 (2009) 1184.
[18] Y.S. Vygodskii, E.I. Lozinskaya, A.S. Shaplov, K.A. Lyssenko, M.Y. Antipin, Y.G. Urman, Polymer 45 (2004) 5042.
[19] C.E. Song, E.J. Roh, Chem. Commun. 10 (2000) 842.
[20] F. Favre, H. Olivier-Bourbigou, D. Commereuc, L. Saussine, Chem. Commun. 15 (2001) 1363.
[21] M.J. Earle, P.B. McCormac, K.R. Seddon, Chem. Commun. 20 (1998) 2249.
[22] S. Shah, M.N. Gupta, Bioorg. Med. Chem. Lett. 17 (2007) 928.
[23] J.G. Huddleston, H.D. Willauer, R.P. Swatloski, A.E. Visser, R.D. Rogers, Chem. Commun. 16 (1998) 769.
[24] G.T. Wei, Z.S. Yang, C.J. Chen, Anal. Chim. Acta 488 (2003) 188.
[25] M. Matsumoto, K. Mochiduki, K. Fukunishi, K. Kondo, Sep. Purif. Technol. 40 (2004) 103.
[26] J. Dupont, C.S. Consorti, P.A.Z. Suarez, Org. Synth. 15 (2002) 243.
[27] R.D. Rogers, K.E. Gutowski, G.A. Broker, J. Am. Chem. Soc. 125 (2003) 6633.
[28] Y. Yuan, Y. Wang, R. Xu, M. Huang, H. Zeng, Analyst 136 (2011) 2305.
[29] H. Zeng, Y. Wang, J. Kong, C. Nie, Y. Yuan, Talanta 83 (2010) 590.
[30] Yuzhi Xiao Lin, Xiaojie Wang, Songyun Liu, Huang, Qun Zeng, Analyst 137 (2012) 4085.
[31] Haixiang Gao, Buxing Han, Junchun Li, Tao Jiang, Zhimin Liu, Weize Wu, Yanhong Chang, Jianmin Zhang, Synth. Commun. 34 (2004) 3089.
[32] Dong An, Linbo Wu, Bo-Geng Li, Shiping Zhu, Macromolecules 40 (2007) 3393.
[33] L. Jin, R.B. Bai, Langmuir 18 (2002) 9770.
[34] R.J. Simpson, Purifying Proteins for Proteomics—A Laboratory Manual, Cold Spring Harbor Laboratory PressCold Spring Harbor, NY649.
[35] J.W. Brauner, C.R. Flach, R. Mendelsohn, J. Am. Chem. Soc. 127 (2005) 109.