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Extraction of Lysozyme by Hydroxyl-Functionalized Ionic Liquid Aqueous Two-Phase System

Bo Zhou, Gaozhang Gou, Shijuan Xu, Pei Zhang and Wei Liu*

Department of Chemistry, College of Science, Honghe University, Mengzi 661100, Yunnan Province, P.R. China

*Corresponding author: Tel/Fax: +86 873 3694919; E-mail: liuwei4728@126.com; zhoubo432@163.com

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An ionic liquid/aqueous two-phase system (ATPS) based on the hydroxyl-functionalized ionic liquid 3-(2,3-dihydroxypropyl)-1-methylimidazolium chloride ([2,3-dhpmim]Cl) and salt has been applied directly for extraction of lysozyme. Some effects on the extraction efficiency have been investigated systematically, such as the kind of salts, the amounts of the salt and ionic liquid and the pH of solvent. The results show that ionic liquid-ATPS can be used for extraction of lysozyme efficiently. With the pH value of solution 11.5, the concentration of K_3PO_4 34 % (mass fraction) and ionic liquid 2.5 mL, ionic liquid-ATPS had the highest extraction efficiency (87 %).

Keywords: Lysozyme, Hydroxyl-functionalized ionic liquid, Aqueous two-phase.

INTRODUCTION

As one of the most important bio-macromolecules, proteins play critical roles in the phenomena like metabolism, gene expression, signal transduction, cellular and extracellular structures and so on. In order to investigate the proteins functions, it is necessary first to obtain protein without contamination. As a matter of fact, the separation and purification of proteins are very difficult, especially from some complex mixtures[1]. Some traditional techniques to purify proteins involve several steps and are time-consuming and cost-expensive. Thus, separation and purification of proteins have become a major bottleneck in the research of proteins.

Aqueous two-phase systems (ATPSs) have been recognized as an efficient and economical technique of extraction. Typical aqueous two-phase systems (ATPS) are generated by mixing aqueous solutions of two structurally different polymers or by mixing one polymer with certain salts at high concentration[2]. Aqueous two-phase systems show some advantages, such as low energy consumption, short process time, relative reliability and a bio-compatible environment[3]. These systems are most suitable for biological samples because each phase contains 70-90 % water, which means that biomolecules will not be denatured. So this system has been widely used in proteins purification[4]. A new kind of ATPSs consisting ionic liquid (IL) and salt has been reported in 2003 by Rogers and co-workers[5].

Room-temperature ionic liquids (RTILs) have attracted growing attentions in recent years and have excellent physico-chemical properties compared with traditional organic solvents, such as negligible vapor pressure and high thermal stability[6,7]. So, the ionic liquids have taken the place of traditional volatile organic solvents in many applications, for examples chemical synthesis[8], bio-catalytic transformation[9] and analytical and separation processes[10,11]. In the present work, a kind of ATPS, consisted by hydroxyl-functionalized ionic liquid 3-(2,3-dihydroxypropyl)-1-methylimidazolium chloride ([2,3-dhpmim]Cl) with adding salts, was successfully applied to extract lysozyme. This method showed excellent separation performance with an extraction up to 87 %.

EXPERIMENTAL

Lysozyme was purchased from Sigma (St, Loius, MO, USA). N-Methylimidazolium was purchased from Linhai kaile chemical company (Zhejiang Province, China). 1,2-Dihydroxy-3-chloropropane was purchased from Darui fine chemical company (Shanghai, China). The ionic liquid ([2,3-dhpmim]Cl) was prepared according to the reference[12]. K_2CO_3, K_3PO_4 and K_2HPO_4 were bought from Guangzhou chemical company (Guangdong Province, China). Other chemicals used were of commercial grade. All reagents were used without further purification. Deionized water was used throughout.

Detection method: A TU-1810 UV-VIS spectrophotometer (Beijing Purkinje General Instrument Company, Ltd., Beijing, China) was used to collect the UV-visible data. FT-IR spectra were recorded using KBr pellet on a Nicolet 2700 spectrometer.

Preparation of phase diagram for ionic liquid/salt aqueous two-phase system: The phase diagram was prepared according to the turbidimetric titration method[5]. Briefly, a few

grams of concentrated ionic liquid ([2,3-dhpmim]Cl) solution were weighed into a test tube. Then a solution of known concentration of a salt was added drop-by-drop and mixed. The solution was clear at first. But after a certain amount of the salt was added, one further drop made the mixture turbid and separated into two phases. The mass of the mixture was noted and the composition of the two-phase system was determined at 298.15 K and ambient pressure. Keep adding a few drops of water to the two-phase system, the mixture became clear once again. In order to get sufficient data for constructing the phase diagram, the above procedure was repeated.

Extraction of lysozyme: A proper amount of ionic liquid, K_3PO_4 and the solution of lysozyme were added into a graduated glass tube. The volume of the glass tube was calibrated. The mixture was diluted to the mark with deionized water and gently stirred by magnetic stirrer for 0.5 h which was sufficient to separate the mixture into two liquid phases. The temperature of the systems was controlled at T = 298.15 K in a thermostated water bath. The phase separation quickly occurred after cessation of the stirring process. The volume of top and bottom phases was recorded. Then the sample was collected from the ionic liquid-rich upper phase and diluted for analysis. A mass balance check was made between the initial mass of lysozyme and the amounts in the upper and lower phases on the basis of equilibrium compositions. The relative error in the mass balance was within ± 2 %. To avoid interference from phase components, samples were diluted and analyzed against blanks containing the same phase compositions but without lysozyme. Phosphate buffer was used to adjust the pH by adding the aqueous solution of KOH or H_3PO_4.

UV-visible and FT-IR spectra: The spectra of lysozyme in [2,3-dhpmim]Cl/K_3PO_4 aqueous two-phase system were determined to study the lysozyme conformation before and after extraction. During each experiment, the top ionic liquid-rich phase containing lysozyme was collected to measure the UV-visible spectra. The blanks containing the same phase components but without lysozyme was used as reference solution. FT-IR spectra were recorded with a resolution of 2 cm^{-1}. A mimic system containing lysozyme, [2,3-dhpmim]Cl and water was used to obtain the FT-IR spectra. The spectrum of lysozyme in [2,3-dhpmim]Cl was measured by mixing 3 mg of lysozyme, 3 mg of moisture [2,3-dhpmim]Cl with 200 mg KBr as a pellet pressed with a KBr Die Kit. The spectrum of pure lysozyme in deionized water was also collected using KBr pellet method.

RESULTS AND DISCUSSION

Three kinds of salts, such as K_2HPO_4, K_2CO_3 and K_3PO_4, had been studied their ability for the formation of ionic liquid aqueous two-phase system. From the Fig. 1, it was obvious that the ability of salts for ionic liquid aqueous two-phase system follows the order: $K_3PO_4 > K_2HPO_4 > K_2CO_3$. At the same time, it indicated that the less salt (K_3PO_4) was needed to form the ionic liquid-ATPS. So, in this study, K_3PO_4 was finally selected for further investigations.

Effect of the amount of K_3PO_4 on the extraction efficiency: The amount of K_3PO_4 on the extraction efficiency of lysozyme was studied. 2.0 mL of K_3PO_4 solution with different

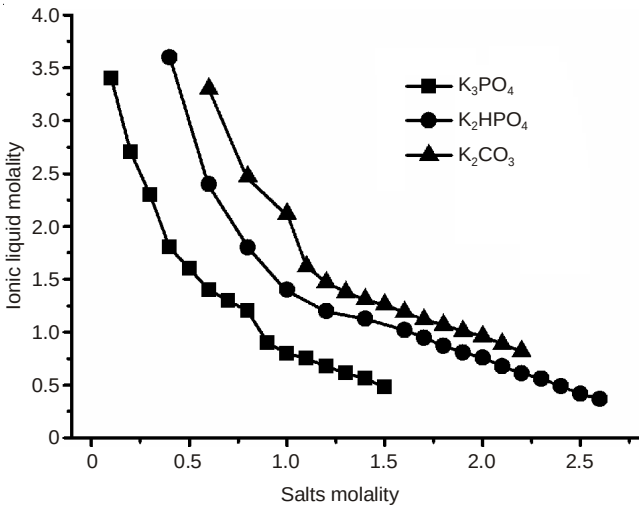

Fig. 1. Phase diagram of ionic liquid/Salts aqueous two-phase system

concentrations (10-40 %) was added to system while the amounts of ionic liquid and lysozyme were fixed. The results were shown in the Fig. 2. From the Fig. 2, the extraction efficiency of lysozyme increased with the increase of K_3PO_4 concentration from 10 to 34 %. When the concentration exceeded 34 %, the extraction efficiency did not increase obviously. So, the optimal concentration of K_3PO_4 solution was 34 %.

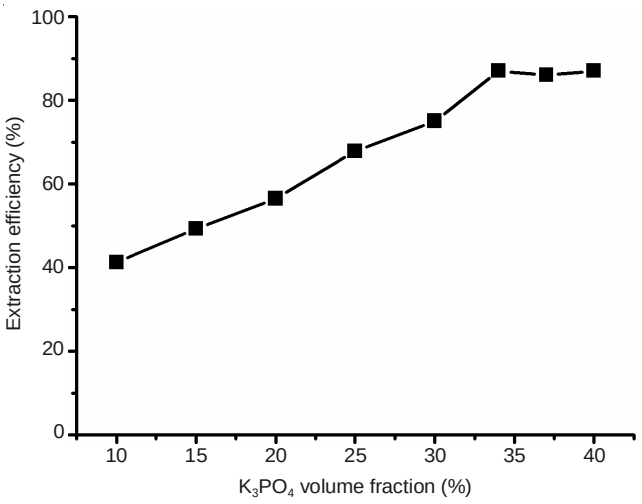

Fig. 2. Effect of K_3PO_4 concentration on the extraction efficiency

Effect of pH: The pH of the solvent has an important effect on the protein. Extractions of lysozyme in different pH solvents have been investigated. The isoelectric point of lysozyme is about 11. So, pH range of 8-12 was chosen to investigate the pH dependence of lysozyme extraction. The results were shown in Fig. 3. As could be seen from the Fig. 3, the extraction efficiency of lysozyme was decreased slightly with the increase of pH values. It might be caused by the charged state of lysozyme affected by the pH of solvent. When the pH value of solvent was below the isoelectric point of lysozyme, the ionic liquid-ATPS had high extraction efficiency relatively. Therefore, it could be concluded that the electrostatic interaction between lysozyme and cation in the ionic liquid had an important effect on the extraction efficiency. So, the optimal pH was 11.5.

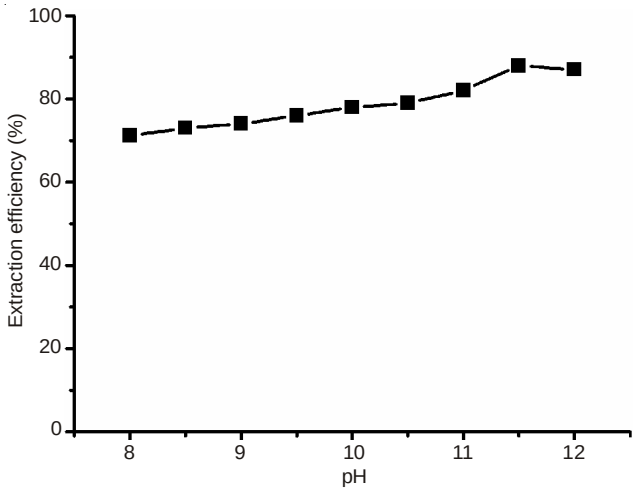

Fig. 3. Effect of pH values on the extraction efficiency

Effect of the mass of ionic liquid: The amount of ionic liquid has an important effect on the extraction efficiency too. Different amounts of ionic liquid were added to systems while the amounts of Na_3PO_4 and lysozyme were fixed, respectively. The influence of the amount of ionic liquid on the extraction efficiency was demonstrated in the Fig. 4. The extraction efficiency increased obviously at the beginning. When the amount of ionic liquid was higher than 2.5 mL, the extraction efficiency increased very slightly. So, the optimal amount of ionic liquid was 2.5 mL.

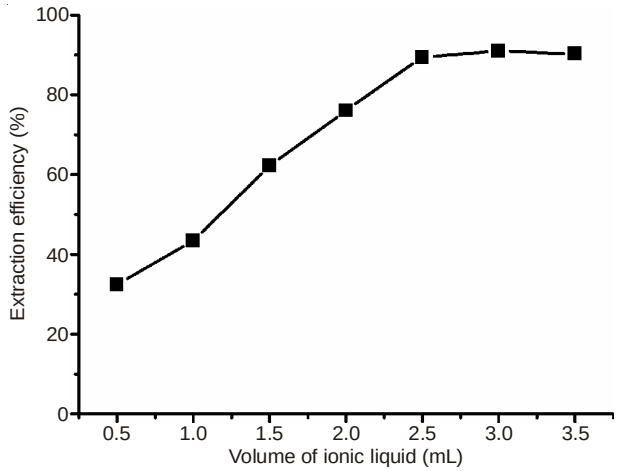

Fig. 4. Effect of ionic liquid amount on the extraction efficiency

Study of the lysozyme conformation before and after extraction: The study's aim is to get purified lysozyme without contamination in the process of extraction. In order to examine the lysozyme conformation before and after extraction, UV-visible and FT-IR spectra were used.

Fig. 5 showed the UV-visible spectra of lysozyme in pure water and in [2,3-dhpmim]Cl-rich phase, respectively. The results demonstrated that the spectra of lysozyme before and after extraction had the same absorption in different solvents. Their maximum absorption wavelengths were at the same position (280 nm).

FT-IR spectroscopy is considered as one of the classical experimental methods in offering information about structure of proteins. FT-IR spectroscopy in rich-ionic liquid phase was

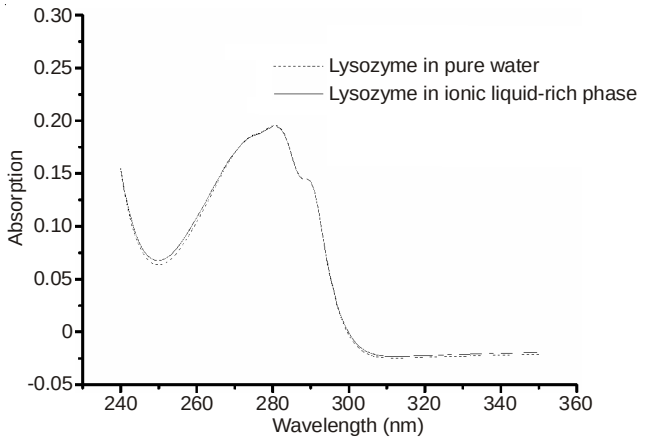

Fig. 5. UV-visible spectra of lysozyme in pure water and in ionic liquid-rich phase

gained and compared with the spectroscopy of pure lysozyme to find out the effect of ionic liquid on the secondary structure of lysozyme. Fig. 6 showed the FT-IR spectra of lysozyme in pure water and rich-ionic liquid phase, respectively. From the Fig. 6, it could be seen that two spectra were very similar. There had little shift or little disappearance of the peak for the major IR bands, which suggested that the conformation of the lysozyme was not obviously changed in the ionic liquid-rich phase.

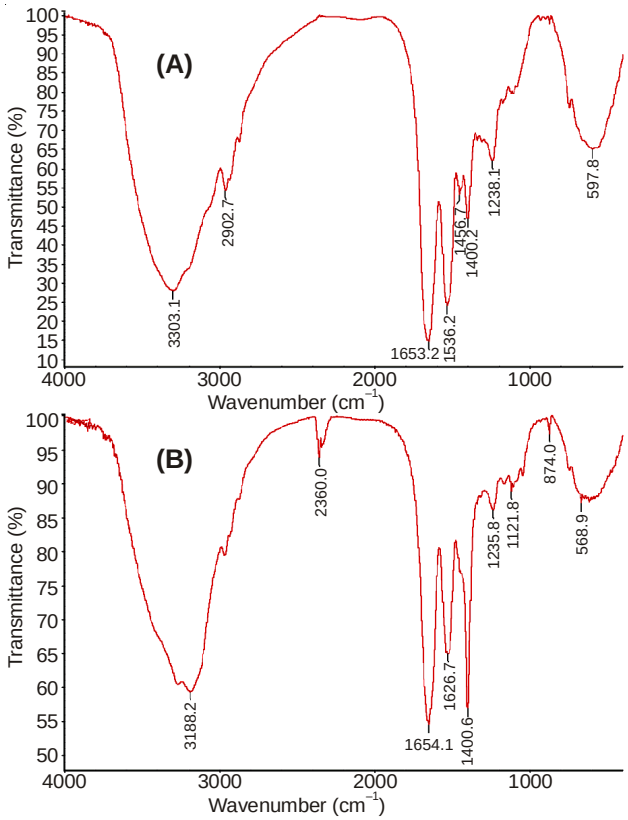

Fig. 6. FT-IR spectra of lysozyme (A), in pure water (B), in ionic liquid-rich phase

Conclusion

In this work, a simple and fast extraction technique for lysozyme has been studied. The results have proved that the

ionic liquid aqueous two-phase system is an effective extraction system for lysozyme. On the optimal conditions, the system has the highest extraction efficiency (87 %). The electrostatic interaction and salting-out affect may be the main causes to drive the lysozyme into the ionic liquid-rich phase.

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