EXTRACTION OF PAPAIN BY AN IONIC LIQUID-BASED AQUEOUS TWO-PHASE SYSTEM

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ABSTRACT

In this paper, N-butylpyridinium chloride ([BPy]Cl) + K_2HPO_4 aqueous two-phase systems (ATPSs) had been developed for the effective extraction of papain (PAP). Single factor experiments proved that the extraction efficiency of PAP was influenced by the temperature, pH, ionic liquid (IL) mass and the salt content. And 95.77% of PAP could be extracted into IL-rich phase under the optimum conditions. UV-vis, FT-IR and fluorescence spectrometer were used to confirm that there were no chemical interactions between PAP and [BPy]Cl, and the conformation of protein was not changed after extraction. The experimental results indicated that electrostatic interaction, salting out effect and intermolecular hydrogen bonding played important roles in the transfer process. All of the results indicated that [BPy]Cl-based ATPSs would have great potential application in bio-analysis and bio-separation.

KEYWORD: Aqueous two-phase systems, N-butylpyridinium Chloride, Extraction, Protein

1. INTRODUCTION

As a promising green solvent, ionic liquid, is a salt with melting temperatures below 100℃ – a result of their ions' delocalized charge and frequent asymmetry that prevent crystallization [1]. In recent years, ionic liquids (ILs) were attractive in many fields such as organic synthesis [2], extraction of metals [3] , biocatalysis [4] and electrochemistry [5] because of the good chemical stabilities, negligible volatility at ambient conditions and large solubilisation ability for a wide variety of compounds [6-9].

Aqueous two-phase systems (ATPSs), first used by Albertsson in the 1950s [10], are a potential liquid-liquid extraction technique which can transfer the solute from one aqueous phase to another. As new separation solvents, ILs are introduced into the ATPSs. Since the ATPSs based on ILs (ILs-ATPSs) were first reported by Rogers and coworkers in 2003 [11], it had become an important emerging technique for separation and purification of proteins, such as function guanidinium ionic liquids and potassium carbonate systems [12], 1, 1, 3, 3-tetramethylguandine acrylate and salts systems [13], glycine ionic liquids and potassium hydrogen phosphate systems [14], 1-(2-methoxyethyl)-3-methylimidazolium bromide and salts systems [15], [Hmim]BF_4 and surfactants systems [16], functionalized ionic liquids and phosphate [17]. These results indicated that the ILs-ATPSs could be served as an adequate alternative for the extraction of proteins.

In this paper, the ILs-ATPSs of [BPy]Cl/K_2HPO_4 system were established and applied to the extraction of papain (PAP). The structure formula of N-butylpyridinium chloride ([BPy]Cl) was showed in Fig .1. Factors effecting the extraction of PAP were optimized, and the mechanism of the extraction process had been investigated by using UV-vis, FT-IR and fluorescence spectrometer.

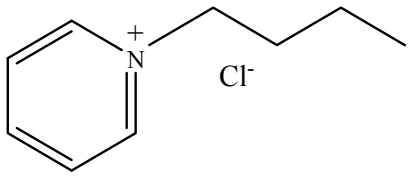

FIGURE 1 - The structure formula of N-butylpyridinium chloride ([BPy]Cl)

2. MATERIALS AND METHODS

2.1 Instrumentation

A UV-2450 UV-vis Spectrophotometer (Shimadzu, Japan) was used to determine the absorbance and the maximum absorption peak of the sample. Fourier transform infrared (FT-IR) spectrometer (Nicolet Nexus 470, Thermo Electron Co. Ltd, USA) was recorded in the range of 400-4000 cm^{-1} on a FT-IR spectrometer. Fluorescence spectrophotometer (Cary Eclipse, Varian Co. Ltd, USA) was em-

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ployed to record the emission spectra. Heto Holten Denmark (SYC-15B, Nanjing, China) was used to provide a specific temperature in the experiments.

2.2 Materials and reagents

N-butylpyridinium Chlorinated ([BPy]Cl) was purchased from Chengjie Chemical Reagent Co., Ltd. (Shanghai, China) with a quoted purity of greater than 99% and was used without further purification. K_2HPO_4, NaOH, KOH, H_3PO_4, papain (PAP), bovine serum albumin (BSA) which were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) were analytical grade reagents with a minimum mass fraction purity of 99%. The experiments were performed with double-distilled water.

2.3 The extraction of papain by ILs-ATPSs

A graduated glass tube was prepared by weighing and adding a suitable amount of [BPy]Cl, K_2HPO_4 and PAP solution. The mixture was gently stirred for 10 min to attain equilibrium at 25℃. The mixture was centrifuged at 2000 rpm for 5 min to ensure a complete phase separation. The concentration of PAP was determined by the Bradford protein assay (Bovine Serum Albumin (BSA) was used as a standard sample to build a calibration curve of PAP) [18] with Coomassie Brilliant Blue G250 at 595 nm. After the volume of top phase and bottom phase was recorded, the mixtures (0.1 mL) were collected from the top and bottom phases and diluted to 1.0 mL, respectively, then 5.0 mL of G-250 solution was added, and the tube was shaken and allowed to stand for 5 min before analysis. The contents of papain were measured at 595 nm by a UV-vis spectrophotometer. The calibration curve of PAP was $A = 0.6187C - 0.0086$ with $R^2 = 0.9992$, where A was the UV absorbance, and C was the concentration of PAP in the range of 0-1.0 mg/mL.

Partition coefficients (*K*), phase volume ratio (*R*), extraction efficiency (*E*) were calculated by Eq. (1), (2), (3), respectively:

$$R = \frac{V_t}{V_b} \quad (1)$$

$$K = \frac{C_t}{C_b} \quad (2)$$

$$E = \frac{CtVt}{CtVt + CbVb} \quad (3)$$

where C_t and C_b were the equilibrium concentrations of the partitioned protein in the top and bottom phases, respectively; V_t and V_b were the volume of the top phase and the bottom phase, respectively.

2.4 Structural characterization of PAP in the ILs-ATPSs

The spectras of PAP in [BPy]Cl/K_2HPO_4 systems were recorded to study the conformation of the proteins. The top IL-rich phase containing PAP was collected to measure the UV–vis spectra when the system attained equilibrium. The blanks containing the same phase composition but without PAP was used as reference solution. FT-IR spectrometer was used to research the architectural features of PAP before and after extraction. The fluorescence spectrum of PAP was determined to study the protein conformation before and after extraction by mixing different concentrations of [BPy]Cl. The excitation wavelength was 280 nm [19].

3 RESULTS AND DISCUSSION

3.1 The establishment of ILs-ATPSs

A given amount of [BPy]Cl and K_2HPO_4 was mixed and stirred thoroughly. The phase separation quickly occurred after cessation of the shaking process. The phenomenon was shown in Fig. 2, the appearance of a clear interface in the system between the two phases, without precipitate or turbidness appearing. Top phase was concentrated by [BPy]Cl, and bottom phase was concentrated by K_2HPO_4. And the volume of the top and the bottom phase no longer changed with time.

3.2 Effect of [BPy]Cl amount

The effect of the ILs amount in the systems was investigated on the extraction of PAP. As indicated in Fig. 3(a), the extraction efficiency was insensitive to the concentrations of ILs. 92.2 - 94.2% of the extraction efficiency was obtained under the condition of 0.4 g - 2.0 g [BPy]Cl. It was reported that the proteins were usually insoluble in the hydrophilic ILs [20], so there was no direct bonding interaction involved between proteins and ILs. Our experimental data indicated that the proteins were dissolved in IL-rich phase. Because of the presence of water, the hydrogen bond association between the cation and anion of the ILs was partially disrupted [21]. The proteins transferred to

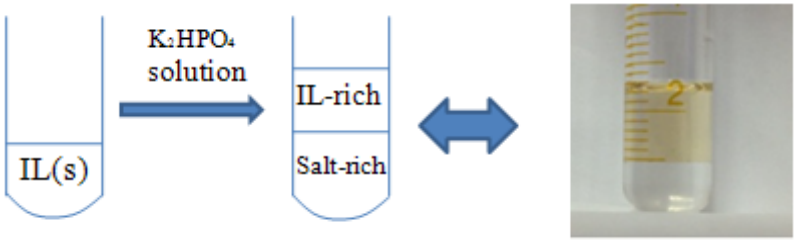

FIGURE 2 - The formation of [BPy]Cl/K_2HPO_4 systems

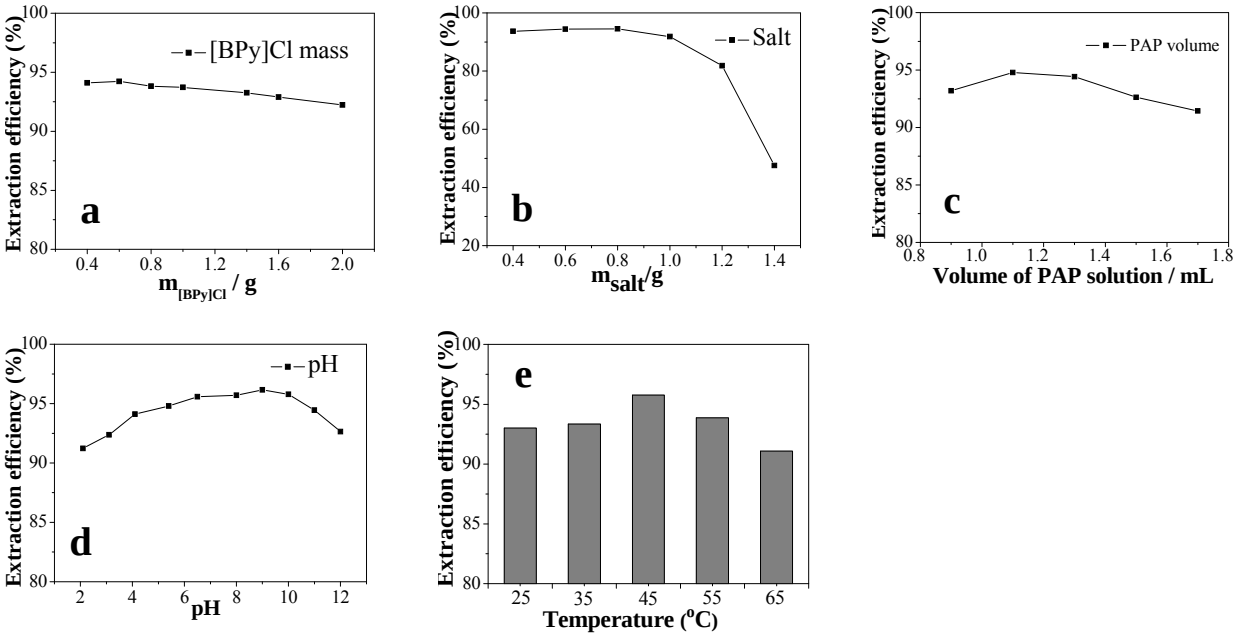

FIGURE 3 - Effect of PAP extraction conditions. a: [BPy]Cl amount (0.4, 0.6, 0.8, 1, 1.4, 1.6, 2 g); b: salt amount (0.4, 0.6, 0.8, 1.0, 1.2, 1.4 g); c:volume of PAP solution (0.9, 1.1, 1.3, 1.5, 1.7 mL); d: pH (2, 3, 4, 5, 6, 8, 9, 10, 11, 12), and e: temperature (25, 35, 45, 55, 65℃).

the IL-rich phase required the fracture of electrostatic interactions between proteins and oppositely charged ILs. So, the amount of ILs did not affect the extraction efficiency of proteins significantly.

3.3 Effect of K_2HPO_4 amount

The amount of K_2HPO_4 in [BPy]Cl/K_2HPO_4 system was a significant factor affecting the extraction of PAP. The amount of K_2HPO_4 ranged from 0.4 -1.4 g was studied, and the result was shown in Fig. 3(b). When K_2HPO_4 was 0.6 g, the extraction efficiency of PAP could reach 94.4%. At high salt concentration, the hydrophobic interactions were increased. So the protein could be enriched in the top phase. With the mass of K_2HPO_4 continued to increase, the content of PAP in the top phase decreased. This phenomenon might be caused by the moving of ILs away from the salt-rich phase to the IL-rich phase [22]. And the salt-rich phase was highly hydrophilic and the IL-rich phase would be correspondingly lower. PAP was more easily to form hydrogen bonding with the water in the salt-rich phase. Therefore, the mass of K_2HPO_4 was selected as 0.6 g for the subsequent experiments.

3.4 Effect of the volume of PAP solution

The influence of the volume of PAP solution on the extraction was investigated by varying the volume of PAP solution (4 g/L) from 0.9 mL to 1.7 mL. The result was shown in Fig. 3(c), the extraction efficiency of PAP increased as the volume of PAP solution increased to 1.1 mL. Then the extraction efficiency slightly decreased when adding more PAP solutions. The maximum extraction efficiency could reach 94.7%. Therefore, the volume of PAP solution was selected as 1.1 mL for the following experiments.

3.5 Effect of pH

In order to examine the effect of pH, the systems were prepared in different buffers with the pH ranging from 2.0 to 12.0. The results were shown in Fig. 3(d), the extraction efficiency of PAP increased with increasing solution pH value, and the maximal extraction efficiency of 96.2% was obtained at pH 9. On one hand, proteins were amphoteric substances and existed as anions, cations, or neutral molecules depending on the different types of amino acids on their surface. They existed as cations when pH was lower than the isoelectric points (pI = 8.75) and existed as anions when pH was higher than the pI. At the pI, both functional groups were charged but the molecule as a whole carried no net charge [23]. On the other hand, $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-} coexisted in K_2HPO_4 solution. With the increasing of pH, the amount of $H_2PO_4^-$ and HPO_4^{2-}decreased, while PO^{3-} increased in the investigated pH range [14]. Therefore, it was deduced that the electrostatic interactions among the charged proteins, pyridine cations and phosphate anions played important roles for the partitioning of PAP.

3.6 Effect of temperature

The influence of temperature on the extraction of PAP was investigated in the range of 25-65℃, and the results were presented in Fig. 3(e). It was observed that when the temperatures was below 45℃, the PAP transferred into the IL-rich phase, the extraction efficiency increased gradually and the maximum extraction efficiency could reach 95.77%. But when the temperature was higher than 45℃,

the extraction efficiency decreased. A possible reason for this phenomenon was that the hydrophobic interaction was enhanced with the increasing of temperature. But as the temperature continued to rise, it was too high to maintain the hydrogen bonding force between the water molecules at the protein surface and the amino acid residue [24].

3.7 Spectroscopy studies before and after the extraction

The extraction of PAP using ATPSs provided a novel approach for the separation and purification of proteins. In order to facilitate subsequent biological investigations, UV-Vis, FT-IR and fluorescence spectroscopy were investigated to examine the conformations of PAP before and after extraction.

3.7.1 UV-vis spectroscopy

The UV-vis absorbance of proteins are mainly attributable to the lateral chains of aromatic series of amino acids, so in the ultraviolet region the chemical environment around the amino acids can be used to deduce the structure of the proteins. Fig. 4 showed the UV-vis spectra of PAP in pure water and in [BPy]Cl-rich phase after extraction. The maximum absorption wavelength of PAP was unchanged in pure water and in [BPy]Cl-rich phase. This indicated that there were no chemical interactions between PAP and the [BPy]Cl in the extraction process.

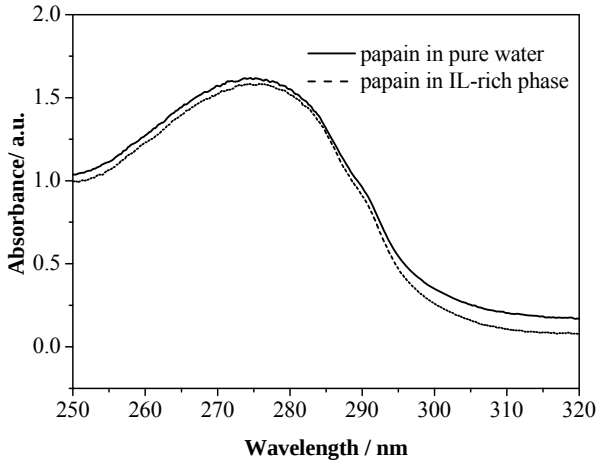

FIGURE 4 - UV-vis spectra of PAP in pure water and in [BPy]Cl-rich phase

3.7.2 FT-IR spectroscopy

FT-IR spectroscopy is one of the classical experimental methods being useful in providing information on structure features of proteins. The primary structure in a polypeptide chain is its amino acid sequence, while linear segments of the polypeptide chain (i.e., a-helices, β-sheets, and β-turns) constitute the secondary structure [25]. Of all the amide bands, amides I and II are the most informative bands to study the secondary structure of proteins. Amide I band represents primarily the C-O stretching vibration of the amide groups and occurs in the region 1690–1600 cm^{-1}, while amide II band represents the C-N stretching vibrations and occurs in the region 1575–1480 cm^{-1} [26]. FT-IR spectra of PAP, [BPy]Cl, and PAP in [BPy]Cl were illustrated in Fig. 5, the two marker bands (i.e., the amide I band at 1633 cm^{-1} and the amide II band at 1509 cm^{-1}) were easily identifiable in the FT-IR spectra of PAP. The major IR bands of the IL were also remained in this region after extraction. This result agreed well with the changes observed in the UV-vis spectra, which further confirmed the conclusion that the chemical construction of PAP was not destroyed after extraction.

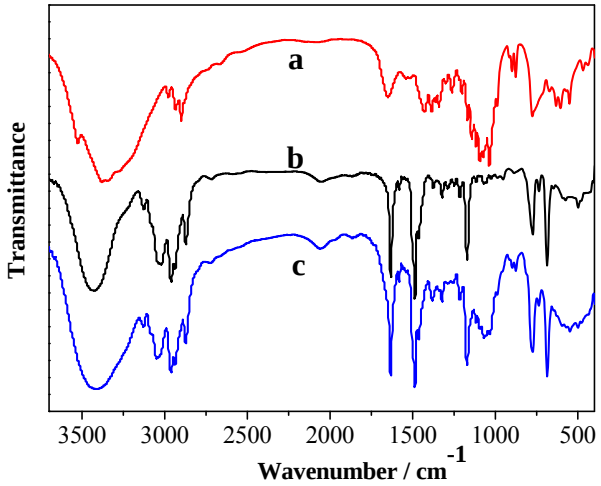

FIGURE 5 - FT-IR spectra of (a) pure PAP; (b) pure [BPy]Cl; (c) PAP in [BPy]Cl.

3.7.3 Fluorescence spectroscopy

Fluorescence spectroscopy was used to monitor changes on the tertiary structure [27] induced by the interaction with [BPy]Cl. Fig. 6 showed the fluorescence emission spectra of PAP in the presence of [BPy]Cl with different concentrations at an excitation of 280 nm. The virtually identical fluorescence spectra suggested that there was no fluorescence quenching. This conclusion was further confirmed by our UV-vis measurements and FT-IR spectra. Hence, we tentatively concluded that the [BPy]Cl-based ATPSs provided a gentle environment for PAP.

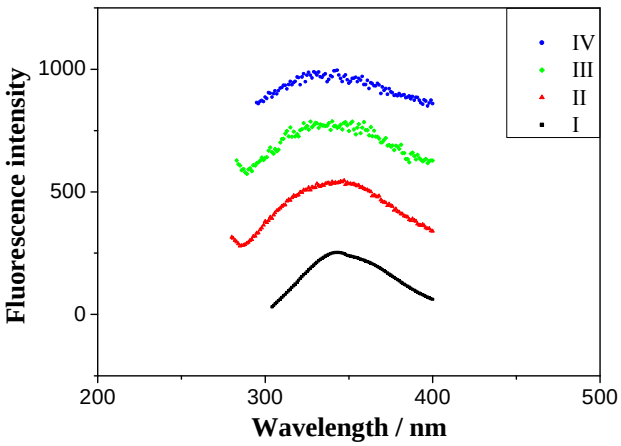

FIGURE 6 - Fluorescence emission spectra of PAP, I: $C_{(PAP)}$ = 1 mg/mL, $C_{(IL)}$ = 0; II: $C_{(PAP)}$= 1 mg/mL, $C_{(IL)}$ = 1 mg/mL; III: $C_{(PAP)}$ = 1 mg/mL, $C_{(IL)}$ = 3 mg/mL; IV: $C_{(PAP)}$ = 1 mg/mL, $C_{(IL)}$ = 9 mg/mL

4. CONCLUSIONS

This paper systematically investigated the extraction of PAP in [BPy]Cl/K_2HPO_4 systems. And 95.77% of PAP can be extracted into the IL-rich phase under the optimal conditions: 0.6 g [BPy]Cl, 0.6 g K_2HPO_4, and 1.1 mL PAP (4 g/L) at 45℃, and pH 8. Undoubtedly, electrostatic interaction, salting out effect and intermolecular hydrogen bonding played important roles for the shifting of proteins. UV-vis, FT-IR and fluorescence spectrometer were used to examine the conformations of PAP before and after extraction. No chemical interactions between PAP and [BPy]Cl were observed during the extraction process. The high extraction efficiency and the friendly conditions indicated that [BPy]Cl/K_2HPO_4 systems for the selective separation of proteins have potential application in bio-analysis and bio-separation.

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The authors have declared no conflict of interest.

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