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Extraction and Separation of Phycocyanin from *Spirulina* using Aqueous Two-Phase Systems of Ionic Liquid and Salt

Xifeng Zhang[1], Fenqin Zhang[1], Guanghong Luo[2,*], Shenghui Yang[2], Danxia Wang[2]

[1]The College of Agriculture and Biotechnology (CAB), Hexi University, Zhangye, P.R. China
[2]Kaiyuan Bio-tech Development Center, Hexi University, Zhangye, P.R. China
[*]Corresponding author: 464690924@qq.com

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Abstract To explore a new and simple rapid extraction and purification technique for phycocyanin, an ionic liquids(ILs)-based aqueous two-phase system(ATPS) was developed for the purification of phycocyanin from *Spirulina* extracts. Effects of various process parameters such as the concentrations of [Bmim]Cl, the concentrations of KH_2PO_4, the concentrations of crude phycocyanin, the system pH and the temperature on partitioning of phycocyanin were evaluated. The obtained data indicated that phycocyanin was preferentially partitioned into the ILs-rich phase and the ATPS composed of 23% (w/w) [Bmim]Cland 29% (w/w) KH_2PO_4 at 30°C and pH 7.0 showed good selectivity on phycocyanin. Under the optimum conditions, phycocyanin with a purity of 3.98 and yield of about 90.23 % was obtained. Therefore, ILs-based ATPS was an effective method for partitioning and recovery of phycocyanin from *Spirulina* extracts.

Keywords: *Ionic liquid, Aqueous two-phase system, phycocyanin, Purification*

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1. Introduction

Cultivation of *Spirulina* microalga is an effective process for obtaining several valuable biochemicals, such as polysaccharides [1], γ-linolenicacid [2], β-carotene [3], chlorophylla [4] and phycobiliproteins [5]. Phycobiliproteins, which are brightly colored pigments, function as a receiver of light for driving photosynthesis in the *Spirulina* microalga [6]. Microalgal phycobiliproteins are classified into three major groups: phycoerythrin, allophycocyanin, and phycocyanin [6]. The predominant pigment in the phycobiliprotein family is phycocyanin [7]. Phycocyanin is commonly used as a natural colorant in food and cosmetic industries because it is inherently blue [6]. Moreover, it can be incorporated into health foods because of its physiological properties, such as antioxidant, antiinflammatory, and hepatoprotective activities [8,9]. Because of these benefits, numerous researchers have focused on developing efficient processes for mass production of phycocyanin-producing strains [10,11] and extraction of phycocyanin from microalgae [5,12].

There are some difficulties in phycocyanin extraction because of multilayered cell walls and large amounts of contaminants. Several methods have been reported for successful separation of phycocyanin [13-17] but these methods comprised multiple steps and are time consuming, which may lead to increase in production costs and limit their widespread application.

Alternatively, Aqueous two-phase systems (ATPS) is regarded as a simple and environmentally friendly separation system. ATPS also offers many advantages, such as a low process time, low energy consumption, and an environment biocompatible to the biomolecule because each phase contains 70–90% water, which means that biomolecules will not be denatured. Hence, ATPS has been recognized as an efficient and economical method for the separation of biomolecules [18].

In recent years, as a green solvent, ionic liquid (IL) has been widely applied in various fields of chemistry. ILs are defined as salts with a melting point below 100°C and they are composed of organic cations and various anions. Compared with water and organic solvents, they exhibit a negligible volatility, non-flammability, a wide electro chemical window, high thermal and chemical stability, and strong solubility power [19,20]. The IL-ATPS has advantages with combination of IL and ATPS, such as negligible viscosity, little emulsion formation, without volatile organic solvent, quick phase separation, high extraction efficiency, and gentle biocompatible environment [21]. Therefore, this technique provided a biocompatible environment for the moderate extraction and purification of biological substances, such as proteins, enzymes and nucleic acids. Moreover, IL-ATPS allows the separation, purification and the concentration to be

integrated into one step. Thus, bioseparation engineering has desirable developing prospects in the IL-ATPS.

In the present study, IL-ATPSs formed by adding inorganic salt K_2HPO_4 into aqueous [Bmim]Cl were employed to separate phycocyanin from *Spirulina*. The extraction efficiency of phycocyanin and the influence of different process parameters (e.g., inorganic salt concentration, IL mass, crude phycocyanin concentration, pH, and temperature) were analyzed in detail. The aim of this study was to develop an extraction and purification method for phycocyanin from *Spirulina* by IL-ATPS.

2. Materials and Methods

2.1. Materials and Reagents

Spirulina was acquired from Kaiyuan Bio-tech Development Center (Zhangye, China). The salts and other analytical grade chemicals were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) Water used for preparation of aqueous solutions was from a Millipore Direct-Q Water system(resistivity, 18.2 MΩ·cm).

2.2. Preparation of Crude Phycocyanin

Spirulina platensis dried powder were suspended at 0.06 g/mL in 20 mmol/L Tris-HCl buffer (contain 10 mmol/L EDTA, pH6.5). Phycocyanin was extracted by repeated freezing (-20°C) and thawing at room temperature until the blue color becomes in acetate buffer. Cell debris was removed by centrifugation at 5000 rpm for 10 min and the extract thus obtained was termed as crude extract.

2.3. Preparation of ILATPS

ATPSs were prepared in 10 mL centrifuge tubes by adding the appropriate amount of [Bmim]Cl, K_2HPO_4 and crude extract. Distilled water was used to bring the final weight of the system to 10 g. Another mixture with the same phase components but without crude phycocyanin was prepared as a blank to avoid interference. The mixture was stirred well to make the K_2HPO_4 dissolve completely. The phase-separation was speeded up by centrifugation at a rolling speed of 4000 rpm for 5 min, two clear phases formed. The partitioning experiments were done at room temperature (25°C), the top phase was mainly composed of IL and phycocyanin, and the bottom phase was the salt-rich solution containing impurities. The volume of each phase was noted down.

2.4. Effect of Concentration of [Bmim]Cl on Partitioning of Phycocyanin

To study the effect of concentration of [Bmim]Cl, [Bmim]Cl of different mass were mixed with 29%(w/w)KH_2PO_4 to form ATPS Partitioning was performed as described previously.

The phycocyanin concentration during the extraction process was determined according to the procedure used by Chen et al. [22]. The equilibrium concentrations of phycocyanin in the top and bottom phases were determined spectrophotometrically at 620 and 652 nm using an SP-721 spectrophotometer (Shanghai Spectrum Instrument Co.LTD, China)., respectively. The phycocyanin concentration was estimated using Eq. (1)

$$C(mg/mL) = \frac{OD620 - 0.474OD650}{5.34} \quad (1)$$

where C is the phycocyanin concentration (mg/mL), and OD620 and OD652 are the optical density of the sample at 620 and 652 nm, respectively.

The phase ratio (R) is defined as

$$R = \frac{Vi}{Vs} \quad (2)$$

where V_i and V_s stand for the volume of the IL-rich phase and salt-rich phase, respectively.

The partition coefficient (K) is defined as

$$K = \frac{Ci}{Cs} \quad (3)$$

where C_i and C_s are the concentrations of phycocyanin in the IL-rich phase and salt-rich phase, respectively.

The extraction efficiency (E) of phycocyanin in the IL-rich phase is determined from the

$$E = \frac{RK}{1+RK} \quad (4)$$

The purity of phycocyanin was defined as the ratio of absorbance at 620 nm to 280 nm, wherein OD_{620} is the maximum absorbance of phycocyanin and OD_{280} is the absorbance of total proteins [23]

2.5. Effect of Concentration of K_2HPO_4 on Partitioning of Phycocyanin

To study the effect of K_2HPO_4 on partitioning of the phycocyanin in ATPS, K_2HPO_4 at different concentrations (23, 25, 27, 29 and 31% w/w) were mixed with 23%(w/w) [Bmim]Cl in ATPS. Based on E and Purity, the ATPS rendering the most effective partitioning was chosen for further study.

2.6. Effect of Crude Phycocyanin Concentration on Partitioning of Phycocyanin

To study the effect of crude phycocyanin on partitioning of the phycocyanin in ATPS, crude phycocyanin at different concentrations (1 mg/mL, 3 mg/mL, 5 mg/mL, 7 mg/mL and 9 mg/mL) were mixed with 23%(w/w)[Bmim]Cl and 29%(w/w)KH_2PO_4 in ATPS. Based on E and Purity, the ATPS rendering the most effective partitioning was chosen for further study.

2.7. Effect of pH on Partitioning of Phycocyanin

In order to investigate the influence of pH, an IL-based ATPS consisting of 23%(w/w)[Bmim]Cl and 29%(w/w)KH_2PO_4 was prepared. The pH of the systems was adjusted by HCl or NaOH. Based on E and Purity, the ATPS rendering the most effective partitioning was chosen for further study.

2.8. Effect of Temperature on Partitioning of Phycocyanin

The model system to evaluate the effect of temperature on phycocyanin partitioning was prepared as described above. Phase separation was induced by storing the samples in a water bath for 1-2 h at a temperature of 25, 30, 35, 40 and 45°C, respectively. The performance of partitioning were measured to study the effect of temperature.

2.9. Sodium Dodecyl Sulphate-Gel Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by following Deutscher's method [24], using a 30% polyacrylamide slab gel. Electrophoresis was run at 50 V, 12.5 mA, for 3–4 h. The gel was stained with a solution that was 0.05%(w/w) Coomassie Brilliant Blue R250, 50%(v/v) methanol and 12%(v/v)acetic acid. The gel was destained using a buffer that was identical to the staining solution except that it contained no Coomassie Brilliant Blue.

3. Results and Discussion

3.1. Effect of Concentration of [Bmim]Cl on Partitioning of Phycocyanin

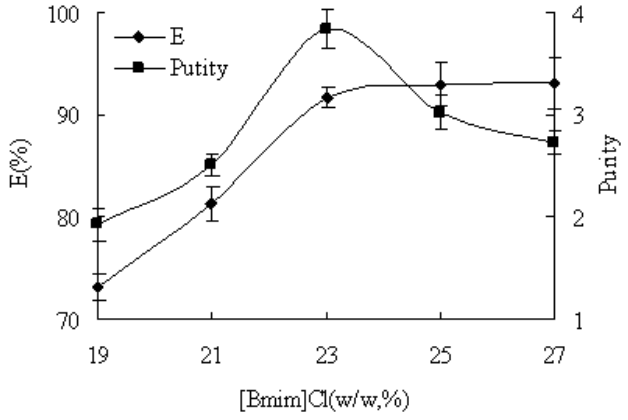

Figure 1. The influence of different concentration of [Bmim]Cl in ATPS on the extraction efficiency and purity of phycocyanin

The effect of [Bmim]Cl amount in the system on the extraction efficiency and purity of phycocyanin is shown in Figure 1a. The extraction efficiency of phycocyanin increased with increasing [Bmim]Cl amount when the concentration of KH_2PO_4 was controlled at 29% (w/w).The purity of phycocyanin in the top phase increased as the concentration of [Bmim]Cl increased till the concentration reached 23%. At the concentration above 23%, The extraction efficiency still increased but purity of phycocyanin decreased. This observation can be attributed to the advanced amount of [Bmim]Cl. In general, imidazolium cation of the ILs has an aromatic π system. It appears that the π–π interaction between the imidazolium cation and the aromatic residues of the proteins was a possible driving force for the extraction of proteins [25]. The probably reason was that more phycocyanin was partitioned to the top phase as the concentration increased to a higher value while residual proteins partitioned to the top phase also increased. As a result purity of phycocyanin in top phase decreased.

3.2. Effect of KH_2PO_4 on Partitioning of Phycocyanin

The effect of KH_2PO_4 concentration on the extraction efficiency and purity of phycocyanin is shown in Figure 2. The extraction efficiency and purity of phycocyanin showed an increased trend with increasing KH_2PO_4 concentration from 23% to 29%. The maximum extraction efficiency and purity of phycocyanin were obtained when the concentration of KH_2PO4 was controlled at 29%. This result may be attributed to the salt-out effect. The hydrophobicity of the bottom phase increased with increasing inorganic salt concentration, in which the solubility of phycocyanin decreased because of competition between salt ions and phycocyanin for water molecules through intermolecular hydrogen bonds [26]. This competition yields less phycocyanin dissolved into the salt-rich phase, thereby lowering the extraction efficiency and purity.

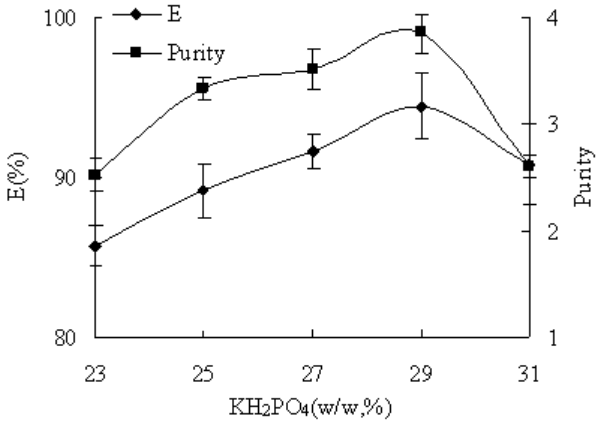

Figure 2. The influence of different concentration of KH_2PO_4 in ATPS on the extraction efficiency and purity of phycocyanin

3.3. Effect of Crude Phycocyanin Concentration on Partitioning of Phycocyanin

The effect of crude phycocyanin concentration on the extraction efficiency and purity is presented in Figure 3. The extraction efficiency and purity of phycocyanin showed an increased trend with increasing crude phycocyanin concentration from 1 to 3mg/mL. However, the extraction efficiency and purity of phycocyanin were decreased slightly with further increase of crude phycocyanin concentration from 3 to 5mg/mL. This phenomenon could be attributed to the fact that increasing the crude phycocyanin concentration causes the aggregation of phycocyanin molecules and the enhancement of intra-molecular hydrogen bonds, which weaken the interactions between phycocyanin and water molecules. Moreover, excessive addition of crude phycocyanin caused accumulation at the interface, which negatively affected mass transfer. The extraction system has a limited ability of extraction with a certain amount of ionic liquid. These results suggest that the crude phycocyanin concentration should be 3 mg/mL.

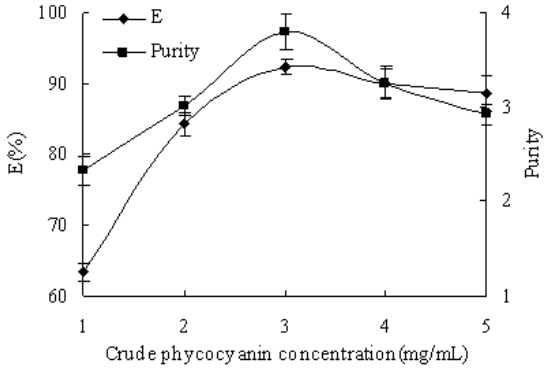

Figure 3. The influence of different concentration of crude phycocyanin in [Bmim]Cl-KH_2PO_4 ATPS on the extraction efficiency and purity of phycocyanin

3.4. Effect of pH on Partitioning of Phycocyanin

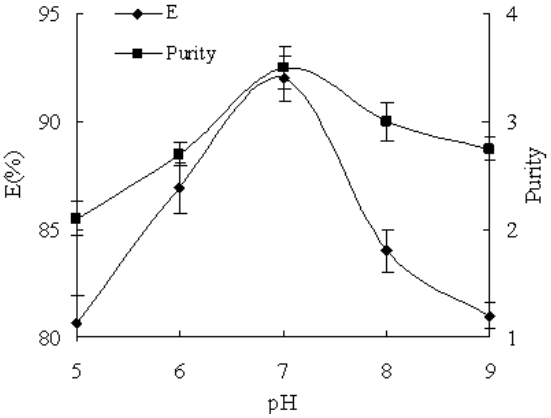

Figure 4. Effect of pH in [Bmim]Cl-KH_2PO_4 ATPS on the extraction efficiency and purity of phycocyanin

From Figure 4, the highest extraction efficiency and purity shown by ILATPS was at pH7. This showed that at neutral pH phycocyanin partition better behaviour compared to acidic or alkaline conditions. pH changed the partition behaviour of phycocyanin as evidenced by lower purity on systems with pH 5, pH 6.0, pH 8.0 and pH 9.0 compared to pH 7.0. The pI of phycocyanin was 4.3. Above the isoelectric point (pI), phycocyanin with negative charge prefer the top phase in ATPS, while proteins with positive charge normally partition selectively to the bottom phase. The manipulation of pH in ILATPS correlated with electrochemical interactions between protein and solvent in the system. These electrochemical interactions played an important role in partition behaviour of phycocyanin [27]. Another reason was hydrophobic interaction. The driving force originated from the hydrophobic interactions between the exposed amino acid residues on the surface of the protein and the imidazole ring of the IL cations. Interaction of the two substances contained an aromatic ring with π electrons, and π–π conjugation was produced when the imidazole and amino groups were close to each other. Thus, electrostatic interactions and π–π conjugation between the charged groups in proteins and the ionic group of ILs have significant functions in determining the dependence of the extraction efficiency and purity of proteins on the pH of aqueous solutions [28]. Therefore, the pH should be controlled at 7 in this study.

3.5. Effect of the Temperature on Partitioning of Phycocyanin

To further confrim the extraction temperature studied on the influence of extraction eficiency and purity of phycocyanin in IL-based aqueous two-phase systems, the phycocyanin content on its distribution behavior was also studied. In light of Figure 5, as the temperature increased from 25 to 30°C, the extraction efficiency of the phycocyanin increased from 90.15% to 93.03%. When the temperature was kept at 30°C or higher, the extraction yield and purity decreased correspondingly. The possible reason for this phenomenon is that the increased extraction temperature could reduce the viscosity of the ionic liquid, enhance the solvent solubility and diffusion capacity. However, when the temperature continues to rise, the extraction rate was reduced. On the one hand, it means that the temperature was high to destroy the hydrogen bonding interaction between the surface water of protein and amino acid residue. On the other hand, as the temperature rises, the extraction rate is reduced resulting in a tendency for the liquid to be homogeneous. So the extraction was carried out at 30°C because of the relatively high extraction yield and purity.

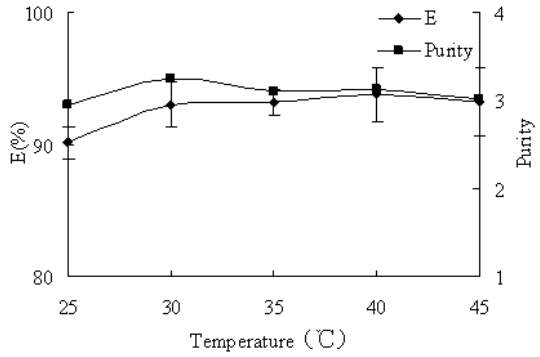

Figure 5. Effect of temperature in [Bmim]Cl-KH_2PO_4 ATPS on the extraction efficiency and purity of phycocyanin

3.6. SDS-PAGE

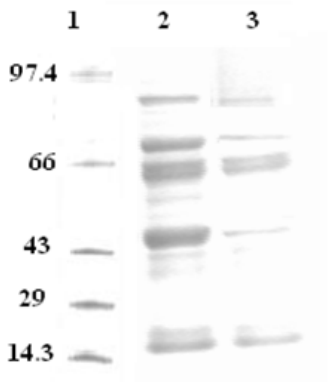

Figure 6. SDS-PAGE of phycocyanin purified by aqueous two-phase extraction: Lane 1- Marker; Lane 2- Crude extract; Lane 3- Top phase

The purity of phycocyanin obtained from ILATPS was confirmed using SDS-PAGE as shown in Figure 6. Lane 1 indicated the molecular marker, while lane 2 was the crude extract of phycocyanin and lane 3 was the phycocyanin after ILATPS extraction. From the SDS-PAGE, an increase in the purity of phycocyanin is observed after the ILATPS extraction.

3.7. Recovery of IL

Considering that ILs can impact on the water ecosystems and ILs remain very expensive in comparison with the conventional extractants, it is significantly important to recycle ILs in using IL-based ATPS, especially when dealing with their application on a large scale and in related wastewater streams [28].

In this study, a large number of [Bmim]Cl remain in the IL-rich phase after ILATPS extraction. In the light of previous study [27], the IL-rich phase containing [Bmim]Cl was initially concentrated to remove water under reduced pressure, and then extracted into CH_2Cl_2 solution. [Bmim]Cl can be recycled after removing CH_2Cl_2.

4. Conclusions

Purification of phycocyanin from the crude extract using [Bmim]Cl/KH_2PO_4 ATPS is reported for the first time. During this study, a systematic approach was used to find the optimized conditions to purify phycocyanin. The process parameters involved in the purification of phycocyanin were discussed and their optimization was described in detail. A method for purification of phycocyanin in an ATPS was proposed. With a 23%[Bmim]Cl and 29%KH_2PO_4 ATPS, phycocyanin with a purity of 3.98 and yield of about 90.23% was obtained. This method enhances both the purity and the yield of the phycocyanin beyond that obtained by the conventional salting-out step. Experimental results obtained here demonstrated the feasibility of an ATPS for the purification of phycocyanin.

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