RESEARCH ARTICLE

High-value utilization of egg yolk: One-step extraction, flotation, purification, and characterization of phosvitin in egg yolk through aqueous two-phase flotation

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Aqueous two-phase flotation (ATPF) separation technologies are extensively applied to separate biologically active molecules. In comparison to traditional separation methodologies, ATPF has several advantages including simple operation, high separation efficiency, low cost, low pollution, gentle mass transfer, and easy industrialization. To realize high-value utilization of egg yolk, a simple, cheap, and effective method for the extraction of egg yolk phosvitin (Pv) was established based on the ATPF system consisting of polyethylene glycol (PEG2000), K_2HPO_4, and capture agent PEG2000-NH_2-Fe^{2+}. The influences of the concentrations of PEG2000, K_2HPO_4, and PEG-NH_2-Fe^{2+} as well as pH on Pv extraction were evaluated. Response surface methodology experiments were performed to optimize the effective parameters. Purified Pv was characterized using dodecyl sodium sulphate-polyacrylamide gel electrophoresis, fluorescence (FL) spectroscopy, ultraviolet spectroscopy, and Fourier transform infrared (FTIR) spectroscopy. The hydrophobicity, emulsifying property, and antioxidant activity of Pv obtained by ATPF were also explored. The optimal condition was obtained for ATPF with 28 mL 25% K_2HPO_4 (w/w), 5 mL 40% PEG2000 (w/w), and 6.1% PEG-NH_2-Fe^{2+}, while loading 2 mL pretreated Pv solution (Pv (w):water (v) = 10:1). Under optimal conditions, Pv extraction efficiency of 92.15 ± 0.35% with purity of 90.72 ± 0.43% was achieved. The results showed that Pv purity obtained by ATPF was satisfactory, and there were no significant differences in the structure and function of Pv separated by ATPF and standard Pv. Therefore, ATPF was found to be a highly efficient method for the separation and purification of Pv from egg yolk.

Keywords: polyethylene glycols; aqueous two-phase flotation system; phosvitin; egg yolk; extraction.

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Introduction

Aqueous two-phase flotation (ATPF) is a novel methodology for separation and enrichment with several advantages such as high concentration coefficient, no need for toxic organic reagents in the extraction process, *etc*. [1]. Therefore, it can be applied in the separation of biomolecules such as proteins, enzymes, and phenols. Phosvitin (Pv) is one of the main phosphorylated proteins present in egg yolk. As the most hydrophilic protein in nature, it contains about 60% of total phosphorus of egg yolk [2]. Pv contains 217 amino acids (45 kDa) with isoelectric point (pI) approximately 4.0 [3]. It has a balanced composition of amino acids and can provide various nutrients for the human body. In addition, it has a variety of functional features including surface hydrophobicity, emulsification, antioxidant activity, and bactericidal functions.

Currently, the methods of Pv isolation from egg yolk mainly include chromatographic column separation and purification, organic solvent separation, thermal precipitation, polyethylene glycol precipitation, *etc*. Researchers have conducted a great number of studies on Pv extraction. Conventional solvent extraction of Pv requires large amounts of organic solvents and is not environmentally friendly. Supercritical extraction needs expensive equipment and is not efficient. Column chromatography is difficult for large-scale production. Therefore, it is essential to develop easy and cost-effective methods for the separation of Pv from egg yolk. Jiang *et al*. successfully separated α-Lactalbumin from whey using polyethylene glycol (PEG) 1000/citrated ATPF with concentration coefficient of 5.33 ± 0.05% and flotation efficiency of 87.54 ± 0.76% and obtained α-Lactalbumin with the purity of 96.78 ± 0.79% [4]. It was revealed that ATPF could be employed to extract whey and vegetable proteins, solving the problem of organic solvent residue. However, the high viscosity of this system could result in slow separation rate. Pakhale *et al*. used a PEG/phosphate ATPF for the separation and purification of bromelain. Under optimal conditions, concentration coefficient of 4.26 and extraction rate of 91.47% were obtained. This research revealed that a system consisting of PEG and inorganic salts provided high extraction rates for spinach protease. However, this research lacked theoretical modeling of the extraction system [5]. Carlos *et al* used PEG400/ammonium sulfate ATPF system to separate and purify polyphenols from camu-camu pulp residues. Under optimal conditions, this system provided efficiency of 81.02 % and a concentration factor twice that of conventional aqueous two-phase extraction [6]. PEG8000 and potassium phosphate ATPF were also applied to separate and purify cyclodextrin glycosyltransferase from *Bacillus cereus*. Under the optimal condition, concentration coefficient of 21.8 and extraction rate of 97.1% were obtained [7].

According to the results of previous studies, the ATPF method was confirmed to be a simple and efficient technique, which provided a novel method for the separation and purification of biomolecules from natural products. This research employed an ATPF system consisting of PEG2000 and phosphate for the extraction and separation of Pv to establish a cheap, simple, efficient, and environmentally friendly separation process to separate Pv from egg yolk, so as to realize high Pv utilization rate and minimize environmental impacts.

Materials and methods

Preparation and purification of ATPF

In the flotation column, ATPF was prepared by mixing 2 ml of pre-treated Pv (Sigma Corporation (Burlington, MA, USA) solution (10 mg/mL) and 28 mL K_2HPO_4 stock solution at different concentrations (w/w) of 18, 22, 24, 28, and 30% at room temperature. After mixing evenly, nitrogen was purged into the flotation column at a flow of 60 mL/min. When the flow of bubbles in the flotation column stabilized, 5 mL of PEG2000 (Aladdin, Shanghai, China) and PEG-NH_2-Fe^{2+} solutions provided by the department of Chemistry, Northeast Agriculture University (Harbin, Heilongjiang, China) at a certain mass fraction were added to the flotation pillar. The floating device was fabricated by our research group. The purging was stopped after nitrogen gas brought Pv to the upper phase. When the upper and lower phase interfaces of the system were stable, they were taken out for subsequent measurements, and each experiment was repeated 3 times.

Single factor experiment and

Effects of different parameters including PEG 2000 (A) at 25, 30, 35, 40, and 45% (w/w), K_2HPO_4 (B) at 18, 22, 24, 28, and 30% (w/w), PEG-NH_2-Fe^{2+} (C) at 0, 2.5, 5, 7.5, and 10%, and pH (D) at 6.0, 7.0, 8.0, 9.0, and 10.0 on the separation were evaluated. To optimize the conditions and investigate the interactions among parallel factors, response surface methodology based on single factor testing was applied. Experimental factors mainly included mass fractions of PEG,

K_2HPO_4, and pH. Experimental results were taken as extraction rate (Y) and purity (P). Each experiment was repeated three times.

Determination of protein

Bradford method was applied to determine the total protein content of the upper phase obtained by ATPF [8]. The standard used was bovine serum albumin. Meanwhile, a blank control was prepared to determine the effects of the interfering substances in the upper phase. Three parallel tests were performed for each sample group. The yield and purity of Pv were employed to evaluate extraction efficiency. The preliminary extraction rate of egg yolk high phosphor protein was calculated as follows.

$$Y = \frac{m}{M \times 12} \times 100\%$$

where Y was yield (%). *M* was raw egg yolk mass (g). *m* was final sample total mass after freeze-drying (mg). The 12 was a coefficient representing 1 g egg yolk containing 12 mg Pv on average. 1 mL sample solution (1 mg/mL) was added to digestion solution, which consisted of 1 mL concentrated sulfuric acid and 50 mg catalyst (copper sulfate and potassium sulphate at 1:4 molar ratio). After heating at 100°C until white smoke appears, the reaction mixture was cooling down before adding 30% H_2O_2. The solution was heated at 250°C again until it became colorless. The final volume of the mixture was fixed to 50 mL using ultrapure water. 1 ml ablation solution was mixed with 3 ml constant phosphate reagent using ultrapure water and 6 mol/L sulfuric acid, 2.5% ammonium molybdic acid solution, and 10% ascorbic acid solution at 2:1:1 ratio. The absorbance of the mixture was measured at 660 nm after incubating at 45°C water bath for 20 min. Phosphorus content was determined using the same method as Pv determination above, and the purity was calculated as below.

$$P = \frac{p1}{p2} \times 100\%$$

where P was purity (%). *p1* was phosphorus content (%) in the sample protein. *p2* was phosphorus content (%) in Pv standard.

Characterization of Pv

SDS-PAGE kit (Beijing Soleberg Technology Co., Beijing, China) was employed for the preparation of resolving gel (12%) and stacking gel (5%), and the molecular weight range of the precipitated protein marker was 14.4 to 116.0 kDa. Voltage values for concentrated and separated gels were 80 and 120 V, respectively. The gel was left in the stationary solution for 30 min after electrophoresis and was then stained twice with staining solution before multiple decolorizations until gel background became light.

Spectral analysis

2 mg sample and 200 mg KBr were evenly ground and pressed to a tablet. In the measurements, the resolution was set at 4/cm, scanning number was set at 32, and scanning wavelength range was 400 – 4,000/cm. Peak Fit v4.12 software (Grafiti LLC, Palo Alto, California, USA) was used to analyze protein conformation. The structures of extracted Pv through ATPF system (Pv-F) and Pv standards were evaluated on a PerkinElmer LS55 fluorescence spectrometer (PerkinElmer, Shelton, CT, USA). The protein sample was solved in 10 mmol/L phosphoric buffer (pH 7.2) and diluted accordingly. Fluorescence spectra were measured at 280 nm with emission spectrum scanning range from 315 to 415 nm. The width of emitting and exciting slits was 5 nm. Sample buffer system was used as a blank control to subtract fluorescence spectrum baseline [9]. A UV-2550 ultraviolet spectrophotometer (Nihon Isandotsu Corporation, Kyoto, Japan) was applied to measure the absorbance of Pv-F and Pv standard solutions in the range of 250 - 360 nm.

Determination of surface hydrophobicity

Pv-F and Pv standard solutions with different concentrations in the range of 0.1 to 0.5 mg/mL were prepared in each test. 12.5 µL of 8 mmol/L 8-aniline-1-naphthalonic acid (ANS) solution prepared from 10 mmol/L stock solution (pH 7.0) was combined with 3 mL of the above sample solutions, and fluorescence intensity was measured after 15 min incubation with slit width

of 5 nm and excitation and emission wavelengths of 390 and 470 nm, respectively [10].

Determination of emulsifying property

Emulsions were prepared by adding 10% peanut oil (v/v) to aqueous solutions of Pv-F and Pv standards (10 mg/mL) and homogenizing at 10,000 rpm for 6 min. After preparing the emulsion, 10 µL of Pv sample emulsion was mixed with 8 mL of 0.1% SDS (v/v) solution and the absorbance was measured at 500 nm using 0.1% SDS as blank. Emulsion activity index (EAI) was calculated as follows.

$$EAI = 2T \times \frac{A_0 \times N}{C \times (1 - \emptyset) \times 10000}$$

where A_0 was the absorption measured at 500 nm. N was dilution multiple. T was 2.303. C was the concentration of Pv and Pv samples (mg/mL). $\emptyset$ was the volume percentage of the oil phase (10%). Emulsion stability index (ESI) was determined by using the absorbance after 30 min of the emulsion and calculated as below.

$$ESI\ (m^2/g) = \frac{A_t}{A_0} \times 100$$

where A_t was the absorbance at 500 nm after 30 min [11].

Determination of antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity

2 mL aliquots of 5 mg/mL Pv standard and Pv-F solutions were prepared and mixed with 2 mL of 0.1 mmol/L ethanol-diluted 1,1-diphenyl-2-picrylhydrazyl (DPPH), respectively. After incubating at 37℃ in dark for 40 min, absorbance value at 517 nm was obtained. DPPH radical cleansing activity was then calculated as follows.

$$DPPH = \left(1 - \frac{A - A_i}{A_0}\right) \times 100\%$$

where A was sample absorbance. A_i was control absorbance. A_0 was blank absorbance [12].

Determination of free radical scavenging rate of 2,2'-azinobis-3-ethylbenzthiazoline-6-sulfonate

38 mg 2,2'-Azinobis-3-ethylbenzthiazoline-6-sulfonate (ABTS) was dissolved in 10 mL of 0.1 mol/L PBS solution (pH 7.4) containing 7 mg potassium perdisulfide. After storage in dark for 16 h, the prepared solution was adjusted to the absorbance of 0.7 ± 0.02 at 734 nm using the same buffer. 3 mL of ABTS solution was mixed with 50 µL of 5 mg/mL Pv sample. After 5 min of storage in dark at ambient temperature, absorbance was obtained at 734 nm with the sample buffer as the blank. The scavenging rate of ABTS radicals was calculated as below [13].

$$ABTS = \left(1 - \frac{A}{A_0}\right) \times 100\%$$

where A_0 was blank absorbance. A was sample absorbance value.

Statistical analysis

SPSS version 20.0 (IBM, Armonk, New York, USA) was employed for the statistical analysis in this research. All data were expressed as the mean ± SD. Response surface methodology (RSM) was applied to optimize the factors. Regression analysis was performed using Design-Expert 8.0.6 software (Stat-Ease Inc., Minneapolis, Minnesota, USA) to derive equations for the prediction of extraction rate (Y) and purity (P).

Results and discussion

Single-factor variable analysis

The influence of PEG2000 mass fraction on the separation efficiency showed that, for PEG2000 concentration of 35% (w/w), Y and P reached their maximum values. Protein distribution varied between upper and lower phases, which might be influenced by protein characteristics including shape, molecular weight, volume, and surface area [14]. The main drivers of protein distribution in ATPF were their electrostatic, hydrophobic, and saline nature [15]. When PEG mass fraction was increased from 25% to 35%, upper phase hydrophobicity was responsible for

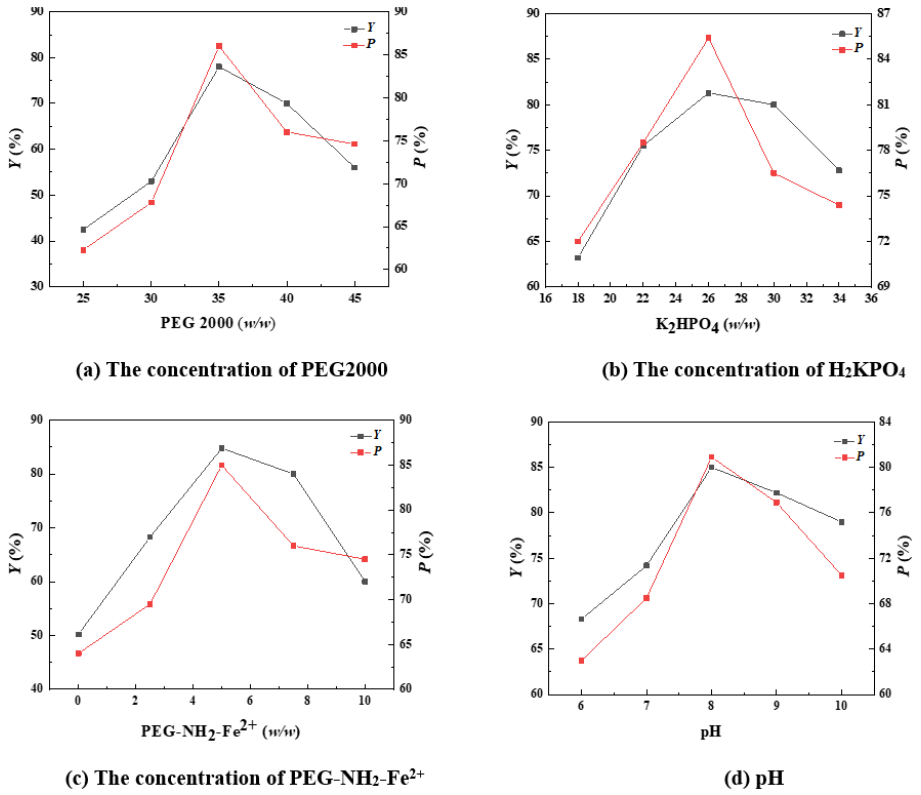

Figure 1. Various factors affecting extraction rate (Y) and purity (P).

PEG concentration variations. Since Pv was highly hydrophobic, it preferentially interacted with PEG, making it easier to separate Pv into the upper phase. If mass percentage was higher than 35%, the affinity of protein to polymer-rich phase was reduced, mainly because spatial repulsion increased with the increase of the concentration of polymer, which increased the resistance of the protein to upper phase (Figure 1a). Consequently, proteins tend to partition into the salt phase. The effect of K_2HPO_4 mass fraction on separation efficiency demonstrated that, if mass percentage was less than 26%, extraction rate and purity were increased with the increase of K_2HPO_4 concentration (Figure 1b). As the salt concentration increased in the lower phase, the number of free water molecules decreased, which could be used for the dissolved proteins in the lower phase, leading to a decrease of protein solubility in the lower phase and the salting effect [16]. Meanwhile, high concentration of salt improved Pv hydrophobicity and hydrophobicity, and salting-out effect played important roles in the distribution of protein between the two phases. Salination effect caused the transfer of hydrophobic Pv from salt to polymer phase with stronger hydrophobicity. When the mass fraction of protein was higher than 26%, the extraction rate and purity of the upper phase decreased. This was probably because the increase in salt concentration led to the formation of interlayer precipitation between the two phases, and the precipitated protein was discarded, affecting the overall separation efficiency. The effect of PEG-NH_2-Fe^{2+} on separation efficiency showed that PEG-NH_2-Fe^{2+} was found to strongly affect Pv-F separation efficiency (Figure 1c). Replacing the affinity ligand increased concentration difference between upper and lower phases of an aqueous two-phase system. The change in the liquid-liquid equilibrium properties of the PEG 2000/K_2HPO_4 system caused by the addition of the affinity ligand was due to the chelation effect of the affinity ligand, which caused the polar groups

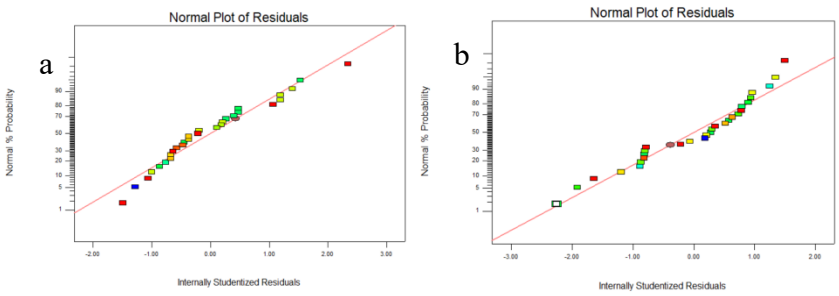

Figure 2. Prediction/actual relationships in the extraction rate (**a**) and purity (**b**) models.

(hydroxyl groups) on the PEG molecules to be partially replaced, reducing the polarity of the PEG molecules, and increasing the hydrophobicity difference between the PEG molecules and the K_2HPO_4 solution, that was, increasing the driving force for phase separation. The influence of system pH on separation efficiency demonstrated that pH significantly affected the separation efficiency of Pv-F. As pH value increased from 6 to 8, extraction rate and purity increased. ATPF protein distribution pattern was also influenced by pH, which might be due to variations in the surface properties of the protein. If the pH was above the isoelectric value (pI), the protein had a negative load on it. Conversely, when pH was below pI, the protein had a positive load, and the protein's net load was zero at the isoelectric point [17]. The pI value of Pv-F was 4.2. Therefore, when pH was higher than 4.2, Pv-F was negatively charged and negatively charged biomolecules tended to the polymer phase in the system with greater pH [18]. Due to the existence of dipole moment, pH values of higher than the pI value of the protein increased the affinity of the protein to the PEG-rich phase. If the pH value of the system was above 8, purity was decreased, meaning that more contaminant proteins entered the upper phase. Both extraction rate and purity peaked at pH 8. Therefore, the best separation performance of the developed ATPF system was obtained when the pH of the system was 8 (Figure 1d). Based on the results, the best values for ATPF parameters were 35% PEG 2000 (w/w), 26% K_2HPO_4 (w/w), 5% PEG-NH_2-Fe^{2+} (w/w), and pH 8.0.

Response surface analysis

(1) Statistical analysis and model fitting

Box-Behnke design (BBD) was adopted for operating in accordance with response surface analysis based on the single factor experimental results. The extraction rate (Y) and purity (P) for Pv-F were calculated as follows.

$$Y = 92.20 - 0.78A - 1.47B + 1.16C + 0.64D + 2.89AB + 2.36AC - 3.91AD - 0.97BC - 1.95BD + 1.23CD - 5.02A^2 - 13.69B^2 - 11.56C^2 - 4.30D^2$$

$$P = 90.36 - 0.19A - 0.48B + 4.93C + 1.86D + 0.12AB - 0.33AC - 0.12AD - 1.86BC - 1.77BD + 1.06CD - 2.39A^2 - 2.39B^2 - 12.13C^2 - 1.61D^2$$

(2) Analysis of variance

Regression model results showed that both extraction rate and purity models were significant ($P < 0.01$), which indicated that the fitting degree of the regression model was very good, and the mathematical model could infer the results of the test. The results showed that the predicted and actual values had good correlation with the correlation coefficient and variation coefficient for extraction rate as 99.99% and 0.033%, respectively, while those for purity were 99.99% and 0.100%, respectively (Figure 2). These results indicated a high-quality fitting and a good correlation between actual and predicted values. Therefore, the model developed in this study was sufficiently accurate and reliable for the analysis and prediction of the optimal conditions for Pv separation.

(3) Interactive analysis

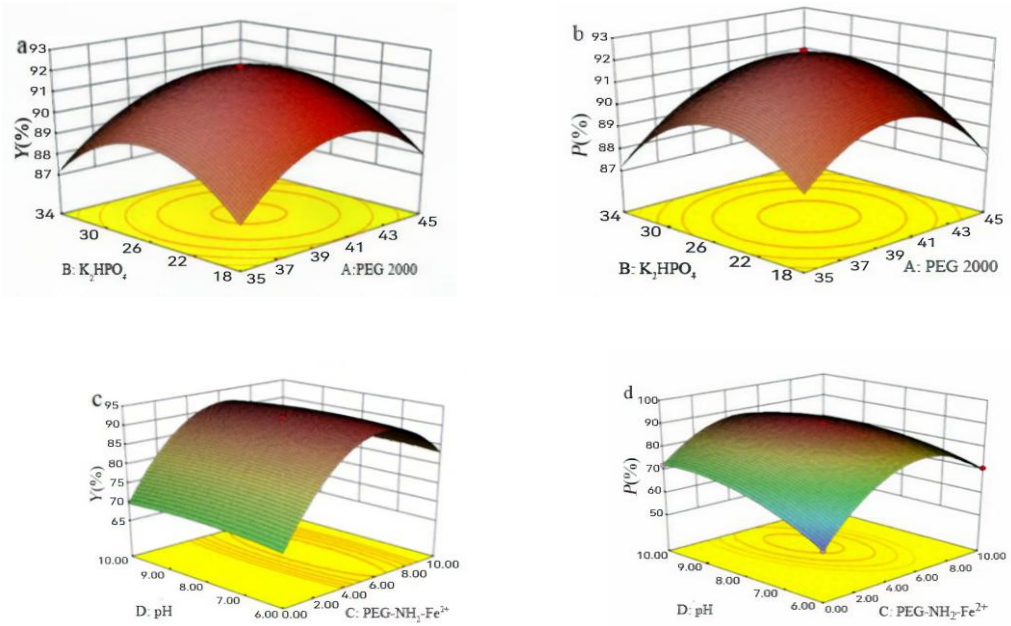

Figure 3. The response surface plots for extraction rate (**a** and **c**) and purity (**b** and **d**) of Pv.

Response value relationships and variables could be represented by a three-dimensional (3D) response surface plot and the interaction between the two variables could be more intuitively represented by a 3D plot [19]. The factors affecting extraction rate and purity of Pv showed that, in ATPF, the main reason for protein distribution might be salting out and hydrophobic interaction. The lower mass percentages of K_2HPO_4 and PEG2000 did not promote the transfer of Pv to the upper phase. With the increase of the amounts of K_2HPO_4 and PEG2000, the ionic strength of the lower phase gradually increased, exposing the hydrophobic part of the protein to the face of the molecule and increasing the hydrophobicity of the protein, which was easier to be adsorbed on the surface of the bubble and transferred to the upper phase with the bubble (Figures 3a and 3b). However, increase of the concentration of the polymer increased the hydrophobicity of the upper phase and hydrophobic Pv-F tended to hydrophobic polymer phase, increasing extraction rate and purity. Furthermore, with the continuous increase of the mass percentage of K_2HPO_4, salting out effect was gradually enhanced, causing more proteins to move up with bubbles. However, excessive PEG content could increase PEG concentration in the upper phase, viscosity, the resistance of bubbles entering the upper phase, and bubble rupture time, thus affecting the process by which the bubbles carried the protein to the upper phase. Meanwhile, excessive salting out effect and ionic strength might cause protein precipitation [20]. Both effects decreased the selectivity of the upper phase, which decreased both extraction rate and purity. pH and PEG-NH_2-Fe^{2+} mass fraction did not affect the distribution of Pv to the upper phase (Figures 3c and 3d). With the increase of pH and PEG-NH_2-Fe^{2+} mass fraction, ionic strength gradually increased, so that more hydrophobic parts of the proteins were exposed to molecule surface, driving more Pv into the hydrophobic upper phase. However, excessive ionic strength and strong alkalinity decreased purity and upper phase selectivity for Pv. Therefore, appropriate affinity ligand concentration and pH should be selected in ATPF.

(4) Validating the optimal conditions for extraction and purity

Based on BBD results, when the concentrations of PEG2000, K_2HPO_4, and PEG-NH_2-Fe^{2+}, as well as

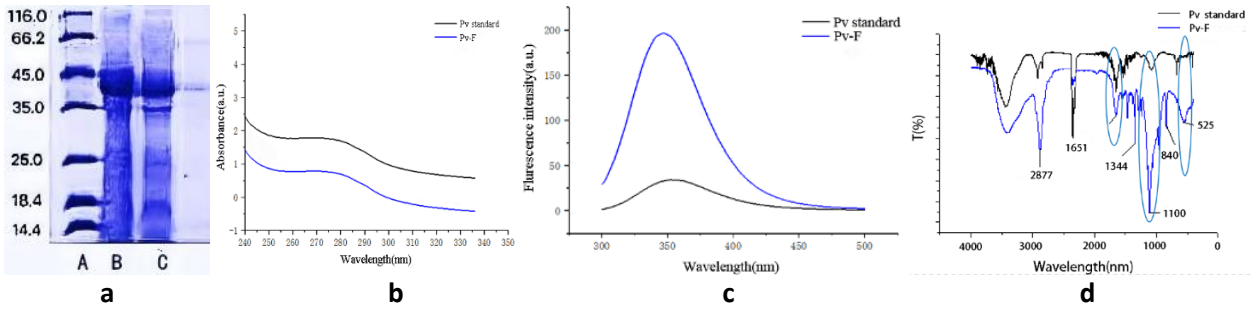

Figure 4. Pv standard and Pv-F. **a.** SDS–PAGE electrophoretogram (lane A: molecular standards. Lane B: 10 µg Pv standard. Lane C: 20 µg ATPF sample). **b.** UV spectra. **c.** fluorescence spectrum. **d.** FTIR spectra.

pH were 39.88%, 24.8%, 6.13%, and 8.5, respectively, extraction rate was 92.71% and Pv purity was 91.40%. The predicted optimal condition to facilitate practical operation was 40% PEG2000, 25% K_2HPO_4, 6.1% PEG-NH_2-Fe^{2+}, pH 8.5. The experimental results showed that the actual extraction rate was 92.55 ± 0.35% and the purity was 91.72 ± 0.43%. No significant differences were observed between experimental and prediction results, which verified the reliability of the model and effectiveness of experimental optimization.

Characterization of Pv structure

The electrophoretogram of Pv-F showed one band with a molecular weight of about 40 kDa, indicating that single main protein was separated from the upper phase and its molecular weight was close to that of Pv standard (Figure 4a). The results indicated that ATPF was able to separate and purify Pv. The UV spectra of Pv standard and Pv-F showed that the maximum absorption of Pv-F occurred at about 280 nm, which was essentially the same as that of standard Pv, proving that the spatial structure of Pv was not significantly changed during separation (Figure 4b). The endogenous fluorescence spectra of standard Pv and Pv-F demonstrated that maximum absorption of Pv-F was basically consistent with that of Pv standard (Figure 4c). Pv itself contained fluorescent chromophores such as tyrosine (Tyr), tryptophan (Trp), and phenylalanine (Phe). Pv-F had strong fluorescence enhancement effects, possibly because Pv-F was more readily enriched in ATPF upper phase and tended to accumulate more due to the upward pressure of the bubbles. However, no red or blue shifts were observed in the maximum value of Pv-F relative to that of standard Pv, indicating the occurrence of no significant change in Pv structure. Fourier transform infrared (FT-IR) measurement results showed that there was an asymmetric strain peak of the vibration of PO_4^{3-} at 1,079/cm. Furthermore, the characteristic absorption peak of Pv band was in the range of 1,600 - 1,700/cm, while the spectrum of Pv-F showed a new peak at about 525/cm (Figure 4d), which was the bending vibration peak of PO_4^{3-} at 648/cm that was sifted to 525/cm and might be due to the condensation of Pv and ferrous ions [18]. The findings revealed that carboxyl in Pv-F reacted with ferrous ion. The spectrum of protein sample contained the characteristic peak of Pv, indicating that the natural structure of the protein had not been altered. There was a minor secondary structure difference between Pv standard and Pv-F (Table 1), which might be due to the fact that metal ions were not present during the extraction of Pv, and Pv was more inclined to require β-type structures.

Determination of the properties of Pv

The endogenous fluorescence of tyrosine and tryptophan residues was very sensitive to the microenvironment polarity of protein conformation, meaning that this method could be applied to reflect Pv hydrophobic characteristics. The hydrophobicity of Pv standard was 485.9 ± 1.88 and that of Pv-F

Table 1. Secondary structure contents of phosvitin standard and Pv-F.

Name	β-fold (%)	β-corner (%)	Irregular curl (%)
Pv standard	20.54	17.39	30.66
Pv-F	19.19	22.92	28.12

sample was 475.43 ± 1.53. The results indicated that the hydrophobicity of Pv-F and Pv standard was not significantly changed, which indicated that the structure of Pv did not change through the extraction process using ATPF system and the functional characteristics of the protein were maintained unchanged. The emulsion stability index (ESI) and emulsion activity index (EAI) results of Pv standard and Pv-F demonstrated that the ESI of the Pv-F sample extracted using ATPF system was slightly higher and its EAI was slightly lower than the corresponding values for Pv standard (Figure 5), which might be that Pv-F protein was separated and purified by the aqueous two-phase system that led to protein aggregation. Unaggregated Pv had better emulsifying activity than aggregated Pv, while aggregated Pv had better emulsifying stability due to its stronger hydrophobic interaction [21].

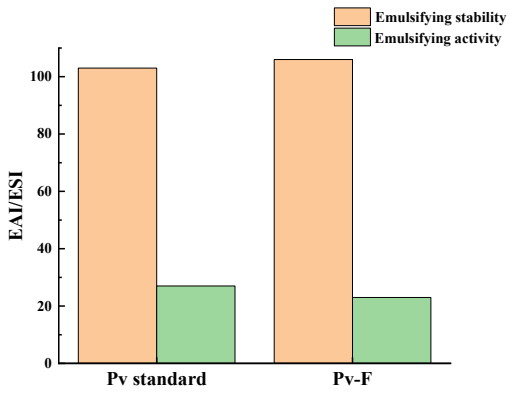

Figure 5. ESI and EAI of Pv standard and Pv-F.

The activities of Pv standard and Pv-F on DPPH and ABTS free radical scavenging showed that Pv-F had significantly higher antioxidant activity than that of Pv standard (Figure 6), which might be that the antioxidant activity of Pv affected protein concentration, temperature, and pH in the extraction process. Under the condition of controlling the concentration and temperature of the protein, increase of pH value could effectively prevent the reaction of the system to generate hydroxyl radical (-OH). Therefore, the antioxidant activity of the protein was significantly increased [22].

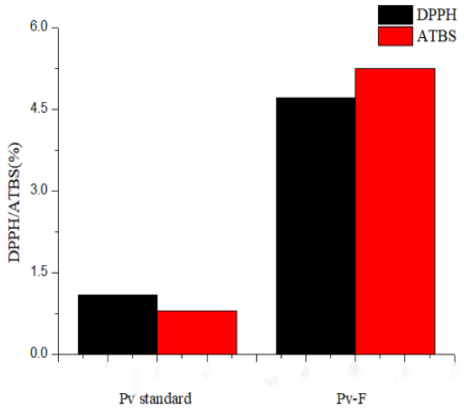

Figure 6. DPPH and ABTS free radical scavenging ability.

Conclusion

This research demonstrated a successful method for Pv extraction from egg yolk using a one-step process through ATPF containing PEG2000/K_2HPO_4. The optimal extraction conditions were determined as 40% PEG2000, 25% K_2HPO_4, 6.1% PEG-NH_2-Fe^{2+}, and pH 8.5. Under the optimal conditions, the extraction rate and purity of Pv-F were 92.55 ± 0.35% and 91.72 ± 0.43%, respectively. The validation experimental results confirmed that Pv separation and purification by the proposed ATPF was effective. The surface hydrophobicity, ESI, EAI of Pv-F were not significantly different to those of Pv standard, except that the antioxidant properties of Pv-F were remarkably enhanced. The results further confirmed that Pv separation

by ATPF was effective and feasible. Therefore, ATPF could be considered as a valuable method for the separation of Pv from egg yolk and might decrease pollution and improve utilization in an effective and sustainable way.

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