RESEARCH ARTICLE

Extraction of phosvitin by aqueous two-phase system based on modified polyethylene glycol

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Phosvitin (Pv) is one of the most important bioactive components, which is concentrated in the granular fraction of egg yolk. It has interesting antioxidant properties that can be used to preserve food and cosmetics. However, its industrial application is limited because its separation relies on the use of organic solvents and chromatographic techniques, which are expensive and difficult to assimilate in a continuous process. The aqueous two-phase extraction (ATPE) technology has advantages over conventional extraction techniques such as environmental friendliness, low cost, continuous operation, and easy scale-up, which can be effectively used for concentration and purification experiments on biomolecules. To establish an efficient method for the extraction of phosvitin, a polyethylene glycol modified aqueous two-phase system (ATPS) was developed in this study. The optimum extraction conditions were determined according to the extraction rate of ATPS. Under the optimum extraction conditions, the mass fractions of PEG 4000, K_2HPO_4, and PEG-IDA-Fe^{2+} reached 10.5%, 18%, and 3.6%, respectively. The pH was 6.8. The extraction rate of Pv-E was 85.15 ± 0.55%, while the purity reached 91.06 ± 0.47%. The Pv obtained was characterized by infrared spectroscopy, ultraviolet spectroscopy, fluorescence spectroscopy, mass spectrometry, and gel electrophoresis. The results showed that ATPE could improve the recovery and purity of Pv while maintaining the natural structure of the protein and reduce extraction costs. The physiological properties such as hydrophobicity and emulsifying properties were slightly enhanced, which was of great significance for scientific research and industrial application of Pv.

Keywords: aqueous two-phase extraction; phosvitin; SDS–PAGE; polyethylene glycol.

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Introduction

Phosvitin (Pv), first discovered in egg yolk, is the major phosphoprotein containing 60% of the total phosphorus content of egg yolk [1-2]. It is also the protein with the highest degree of phosphorylation found in nature. Taborysk *et al*. discovered Pv with molecular mass of 40 kDa and 36 kDa [3], while Clark *et al*. separated Pv with molecular weights of 34 kDa and 28 kDa by gel column [4]. Abe *et al*. found two components with molecular weights between 18.5 and 60 kDa on the electrophoresis and named them as α and β yolk high phosphorus proteins [5]. Wallace *et al*. also found that the molecular weights of Pv were 13, 15, 18, 33, and 40 kDa with the higher contents being 33 kDa (α-Pv) and 40 kDa (β-Pv), and other small molecular weight Pv [6]. Pv has many functional properties such as thermal stability, metal chelation, antioxidant activity, and emulsifying properties, and has great application prospects in the food industry.

However, the complex extraction method and the drastic operating conditions make it difficult to obtain Pv that retains its natural structure.

Compared with traditional separation processes, aqueous two-phase extraction (ATPE) has good separation efficiency, convenient operation, mild mass transfer, low environmental impact, and easy industrialization, making it widely used in the separation of bioactive molecules. ATPE has been often used in the separation and purification of various biological products such as organelles, proteins, viruses, nucleic acids, *etc*. In addition, ATPE also has good performance in the separation and purification of antibiotics, natural pigments, and other small molecules with good hydrophilicity, as well as the separation and recovery of metal ions. Polyethylene glycol (PEG), a polyether polymer compound, has the properties of good solubility and biocompatibility, which is soluble in tissue fluid and is excreted without causing toxic side effects to the body. Therefore, it has many applications in fields such as chemical engineering, hygiene, and food [7]. PEG is one of the most used phase-forming substances in ATPE. The PEG-sulphate system was used to purify protein and the results suggested that ATPE was a potentially effective alternative to the purification of proteins using conventional chromatography [8]. Jiang *et al*. used ATPE based on PEG 1000/$(NH_4)_2SO_4$ to extract ovalbumin from salted egg white in one step with the highest extraction rate and purity of ovalbumin as 89.25 ± 0.76% and 96.28 ± 0.97%, respectively [9]. The introduction of functional groups by end group modification can expand the application range of PEG.

Conventional protein separation techniques usually require the use of large amounts of organic solvents or the use of liquid chromatography, which have the disadvantages of being environmentally unfriendly, not conducive to continuous operation, and expensive. In this study, a modified PEG was used in combination with ATPE to extract Pv. The PEG complexes modified with ferrous ions were prepared and characterized, and then Pv was extracted based on the strong chelation between Pv and ferrous ions. The modification would improve the selectivity of Pv and reduce the extraction cost, which had a certain theoretical value and practical significance for the scientific research and industrial application of Pv.

Materials and methods

Pretreatment of Pv high phosphorus protein

The egg yolks purchased from the commercial market of Harbin, Heilongjiang, China were separated manually with the ultrapure water and stirred using magnetic stirrer ice-water bath (Hangzhou Instrument Motor Co. Hangzhou, Zhejiang, China) until the egg yolks and water were well mixed before centrifugation at 10,000 g for 45 mins at 4°C using SC-3610 low-speed centrifuge (Anhui Zhongke Zhongjia Scientific Instrument Co., Hefei, Anhui, China). The precipitate was collected, and 12% ammonium sulphate at 10 times of the volume was added. The mixture was stirred in an ice water bath again until complete dissolution. After adjusting pH to 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 by using 0.1 mol/L HCl or NaOH, the mixture was centrifuged at 10,000 g for 25 mins. The supernatant was collected and stored at 4°C overnight. After stirred at 80°C in a water bath, the sample was dialyzed using a 13,000 Da MWCO filter (SpectrumLabs, Los Angeles, CA, USA) for 12 h followed by centrifugation for 25 mins. The supernatant was collected, evaporated through N-1100 Rotary Evaporator (RIKEN Instruments Co., Tokyo, Japan) and freeze dried by LyoQuest-85 Plus Freeze Dryer (Telstar, Terrassa, Barcelona, Spain) [10]. The resulting crude extract of Pv was frozen and stored at -80°C in MDF-U53V Ultra Low Temperature Refrigerator (SANYO ELECTRIC CO. Osaka, Japan) for future use.

Total protein determination

The total protein content in the top phase obtained from the aqueous two-phase extraction was determined using the Coomassie Brilliant Blue method. The standard curve was

constructed using bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) with blank controls to avoid interference from substances in the top phase. Three experiments were carried out on each set of samples in parallel.

Preparation of aqueous two-phase extraction system

The stock solutions of different phase forming material were prepared by dissolving PEG (Shanghai Aladdin Biochemical Technology Co., Shanghai, China), modified PEG, and K_2HPO_4 in ultrapure water and mixed PEG stock solution with salt stock solution to form ATPE. Pv crude extracts were added to ATPE, and the mass of the whole system was adjusted by ultrapure water to ensure the mass to be 10 g. 1 mol/L HCl or NaOH was used to adjust the pH of the whole system. After well mixed, the reaction was centrifuged at 2,000 g for 20 min at room temperature. The top and bottom phase volumes were measured, and both phases were collected separately for further experiments. The Pv extracted by the ATPE system was denoted as Pv-E.

Selection of salt and polymer

Salt commonly used in the ATPE includes phosphates, carbonates, citrate salts, and sulphates. According to previous studies, PEG was suitable for protein extraction, and then was selected to combine with various salts to form ATPE for the extraction of Pv in this study. 15% (w/w) of PEG 4000 was set as a fixed factor and 20% (w/w) of different types of salts were set as variables to form different aqueous two-phase systems for Pv-E extraction. Alternatively, 20% (w/w) K_2HPO_4 was set as a fixed factor and 25% (w/w) PEG 1000, PEG 2000, and PEG 4000 were set as variables for Pv-E extraction.

Single factor experiments of aqueous two-phase extraction

Four sets of one-way experiment were performed to explore the effects of PEG mass fractions of 10, 12, 14, 16, and 18%, PEG-IDA-Fe^{2+} mass fractions of 1, 3, 5, 7, and 9%, K_2HPO_4 mass fractions of 14, 16, 18, 20, and 22%, and the pH values of 5.0, 6.0, 7.0, 8.0, and 9.0 on the extraction rate (K) and purity (P) of Pv in aqueous two-phase extraction systems. The protein extraction rate (Y) was defined as the ratio of the ovalbumin content in the top phase to the total ovalbumin content added to the system (equation 1).

$$Y = \frac{C \times V_{top}}{m_{total}} \times 100\% \quad (1)$$

where C was the top phase protein concentration (mg/mL). V was the top phase volume (mL). m was the total mass (g) of added crude extract of Pv.

Characterization of Pv

The Pv-E samples were multiple dialyzed in dialysis bags with molecular cut off 8,000 -14,000 against distilled water until no Fe^{2+} could be found in the dialysate. The samples were concentrated and lyophilized for subsequent experiments.

(1) Fourier-transform infrared spectroscopy

2 mg of lyophilized Pv-E and Pv standard (Sigma-Aldrich, St. Louis, MO, USA) were mixed with 200 mg of spectroscopically pure KBr (Sinopharm Chemical Reagent Co., Beijing, China), respectively. The mixtures were ground well and then pressed using blank KBr as background. A Fourier transform infrared (FTIR) spectrometer (Bruker, Billerica, Massachusetts, USA) was used to analyze the samples following manufacturer's instructions. The measurement parameters were set as resolution 4/cm, scan times 32, and wave number scanning range from 400-4000/cm. The data were analyzed using Peakfit v4.12 software (Grafiti LLC, Palo Alto, CA, USA) [11].

(2) Ultraviolet spectrophotometer

An UV-2550 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) was used to perform spectral scans of Pv-E and Pv standards solutions in the range of 250 nm to 360 nm following manufacturer's instructions.

(3) Fluorescence spectrometer

A PerkinElmer LS55 fluorescence spectrometer (PerkinElmer Inc., Waltham, MA, USA) was used

to measure the fluorescence spectra of Pv-E and Pv standards following manufacturer's instructions. Protein samples were dissolved in 10 mmol/L phosphate buffer (pH 7.2) (Zhongke Ruitai (Beijing) Biotechnology Co., Beijing, China) and diluted to an adequate concentration to record the fluorescence spectrum at 280 nm excitation wavelength. The scanning range of the emission spectrum was 315-415 nm, and the widths of the emission and excitation slits were both 5 nm. The sample buffer solution was used as a blank control for baseline subtraction of the fluorescence spectrum [12].

(4) SDS-PAGE electrophoresis

SDS-PAGE reagent kit (Beijing Boao Toda Technology Co., Beijing, China) was used to prepare a 12% separating gel and a 5% concentrated gel. After loading 10 µL of each sample onto the gel, the electrophoresis was performed using a Mini-PROTEAN electrophoresis apparatus (Bio-Rad, Hercules, CA, USA) with the pre-colored protein marker from 14.4 - 116.0 kDa. The voltage for concentrated gel was 80 V, while the voltage for the separated gel was 120 V. The results were observed by dying the gel with Coomassie brilliant blue R-250 for 30 mins [13].

(5) Liquid chromatography-nanoelectrospray ionization mass spectrometry

The electrophoretic lane of Pv-E was isolated and digested with trypsin (Promega, Madison, Wisconsin, USA) in 100 mM ammonium bicarbonate digestion buffer (pH 8.5). The obtained peptides were extracted with acetonitrile and completely dried in a SpeedVac unit [13]. The dried sample was then dissolved in a solution containing 2% acetonitrile, 97.5% ultrapure water, and 0.5% formic acid. A Nano LC-ESI-MS/MS mass spectrometer and an Agilent C18 column (75 µm × 8 cm, 3 µm) were used for peptide identification. Mobile phase A consisted of 97.5% water, 2% acetonitrile and 0.5% formic acid, while mobile phase B consisted of 9.5% water, 90% acetonitrile, and 0.5% formic acid. Mobile phase B changed from 2% to 90% within 0-60 min. Sample loading time was 20 min. Column wash time was 20 min. The flow rate was 0.8 µL/min. The sample size was 3 µL. The mass spectral conditions were set as 1.5-1.8 kV of spray voltage, 100°C of the capillary temperature, and 33% of the collision energy. The microscan quality range was 350 - 1650 amu. The mass spectrometry data were identified using the UniProt protein database (https://www.uniprot.org/) and analyzed using ProtQuest software suite (ProtTech, Philadelphia, Pennsylvania, USA).

(6) Determination of surface hydrophobicity

Pv standard and Pv-E sample were prepared with concentrations ranging from 0.1 to 0.5 mg/mL. 3 mL of different concentrations of Pv standard and Pv-E samples were mixed with 12.5 µL of 8 mmol/L 8-aniline-1-naphthalenesulphonic acid (ANS) solution prepared with 10 mmol/L phosphate buffer (pH 7.0), respectively. The fluorescence intensity of the sample was measured after 15 mins under the excitation wavelength of 390 nm, emission wavelength of 470 nm, and the slit width of 5 nm. The concentration of the protein was plotted as horizontal coordinate (X) and the intensity of the fluorescence was plotted as the vertical coordinate (Y). The size of the slope was the surface hydrophobicity of the protein [14].

(7) Determination of emulsification characteristics

The emulsion was prepared by adding 10% (v/v) peanut oil to the Pv standard and Pv-E aqueous solution (10 mg/mL) and homogenizing for 6 mins using a high-speed homogenizer at 10,000 rpm [15]. After the emulsion was prepared, 10 µL of Pv standard and Pv-E sample were transferred to the bottom with a syringe, respectively, and mixed with 8 mL of 0.1% (v/v) SDS solution. The absorbance values were then measured at 500 nm with 0.1% SDS solution as a blank. The emulsifying activity index (EAI) was then calculated using equation 2.

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

where A_0 was the absorbance value measured at 500 nm. N was the number of dilutions. T was 2.303. C was the concentration of Pv and Pv-E samples (g/mL). Ø was the volume percentage of 10% conjugated linoleic acid. After allowing the emulsion to stand for 30 mins, the absorbance at 500 nm was measured again. The emulsion stability index (ESI) was calculated as follows.

$$ESI\ (m^2/g) = \frac{A_t}{A_0} \times 100 \quad (3)$$

Statistical analysis

All experiments were carried out in triplicate. The data were expressed as the mean ± standard deviation (SD). SPSS version 20.0 (IBM Corp., Armonk, NY, USA) was used to analyze the data. Duncan's multiple range test was employed to exam the difference between data groups with *P* value less than 0.05 as the significant difference.

Results and discussion

Selection of salt types and polymers

The results showed that ATPE composed of K_2HPO_4 solution as the salt phase demonstrated a better selectivity for Pv-E compared to that of $(NH_4)_2SO_4$ and NaCl as the salt phases (Figure 1). Therefore, K_2HPO_4 solution was used as the salt phase for the extraction of Pv-E from ATPE. In addition, ATPE composed of PEG 2000 as the polymer phase showed a better selectivity for Pv compared to that of PEG 1000 and PEG 4000 as the polymer phases (Figure 2). Therefore, K_2HPO_4 solution was applied with the high polymer phase of ATPE to extract Pv-E.

Single factors experiment on aqueous two-phase extraction of Pv

Pv crude extract was used as raw material for separation and purification of Pv-E using ATPE composed of PEG 4000, PEG-IDA-Fe^{2+}, and K_2HPO_4. By investigating the effects of single factors including the mass fractions of PEG 4000, PEG-IDA-Fe^{2+}, K_2HPO_4 and pH on the extraction rate (K) and purity (P) of Pv, the optimal extraction conditions were determined.

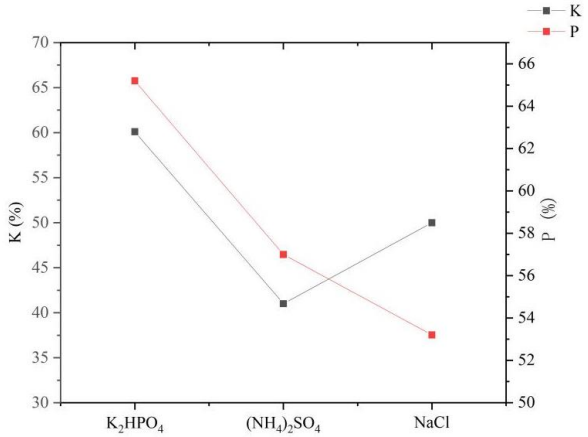

Figure 1. Effect of different kinds of salt on extraction.

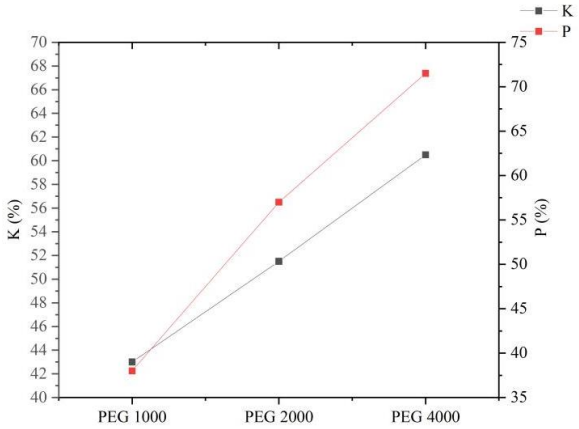

Figure 2. Effect of the weight of different molecular of PEG on extraction.

(1) The influence of K_2HPO_4

The result of the influence of K_2HPO_4 mass fraction on the separation efficiency showed that, when the mass fraction was less than 18%, both K and P of Pv-E increased with the increasing of K_2HPO_4 concentration (Figure 3). As the salt concentration in the current phase increased, the amount of free water molecules available to dissolve proteins in the bottom phase decreased, leading to a decrease in the solubility of proteins in the bottom phase and the salt out effect. Meanwhile, high salt concentrations increased the hydrophobicity of proteins, and hydrophobicity and the salt-out effect played a major role in the distribution of proteins between the two phases. The salt-out effect drove the

transfer of hydrophobic Pv-E from salting to the more hydrophobic polymer phase. When the mass fraction was higher than 18%, the K and P values of Pv-E in the top phase decreased, which might be due to the increase in salt concentration which led to the formation of the protein to form an interlayer interface precipitation between the two stages. The discarded precipitated protein affected the overall separation efficiency.

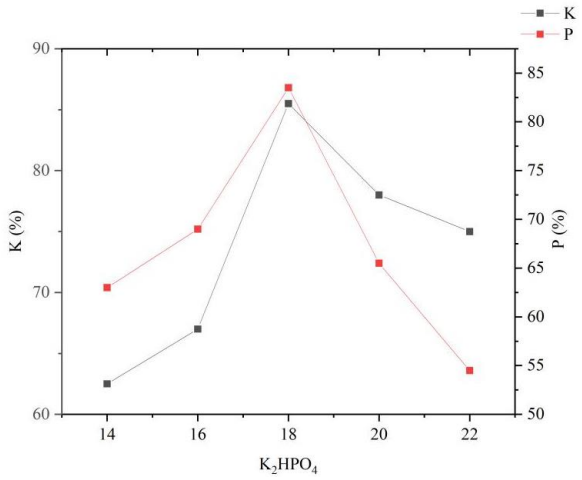

Figure 3. Effect of the concentration of K_2HPO_4 on the separation of Pv-E in ATPE.

(2) The impact of PEG 4000 content

The results showed that, at a mass fraction of PEG 4000 of 14% (w/w), the K and P of Pv-E reached the maximum values (Figure 4). The differences in the distribution of proteins between the top and bottom phases might be due to the differences in the characteristics of the proteins such as appearance, molecular mass, size, and superficial area. The electrostatic interaction, hydrophobicity, and salt precipitation effect were the major driving forces for protein distribution in ATPE. As the mass fraction of PEG 4000 increased from 10 to 14%, the change in PEG 4000 concentrations affected the hydrophobicity of the top phase. The more hydrophobic Pv-E might have preferential interaction with the more hydrophobic PEG phase, making it easier for Pv-E to separate from the top phase. When the mass fraction was higher than 14%, the affinity of proteins for the high polymer phase decreased, which mainly due to the increase in polymer concentration leading to increases of spatial repulsion and resistance of proteins to enter the top phase. Therefore, proteins tended to partition into the PEG phase.

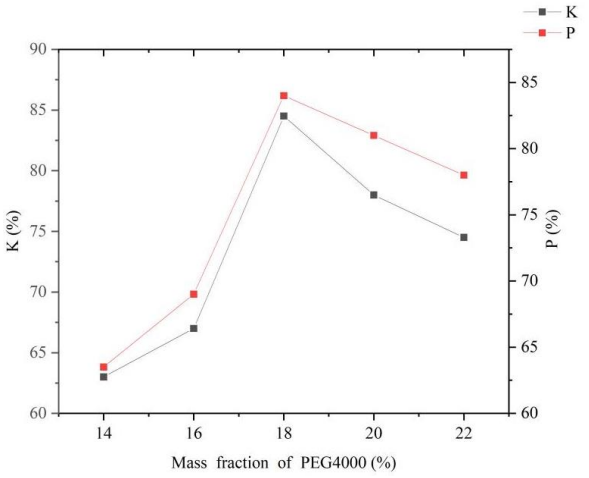

Figure 4. Effect of the concentration of PEG 4000 on the separation of Pv-E in ATPE.

(3) The influence of PEG-IDA-Fe^{2+} mass fraction

The separation efficiency of Pv was strongly influenced by PEG-IDA-Fe^{2+}. After the addition of PEG-IDA-Fe^{2+}, the K and P values of Pv-E increased significantly (Figure 5). The substitution of affinity ligands led to an increase of the concentration difference in concentrations between the top and bottom phases of the aqueous two-phase system. The main reason for the change in the liquid-liquid equilibrium properties of the PEG 4000/K_2HPO_4 system caused by the addition of the affinity ligands was that the polar groups (hydroxyl groups) on the PEG molecules were partially replaced due to the chelation of affinity ligands, resulting in a decrease in the polarity of the PEG molecules. As the difference in hydrophobicity between the aqueous PEG solution and the aqueous K_2HPO_4 solution increased, the driving force for phase separation increased. The results suggested that the optimum single factor terms for ATPE extraction of Pv-E were 14% (w/w) of PEG 4000,

18% (w/w) of K_2HPO_4, and 3% (w/w) of PEG-IDA-Fe^{2+}.

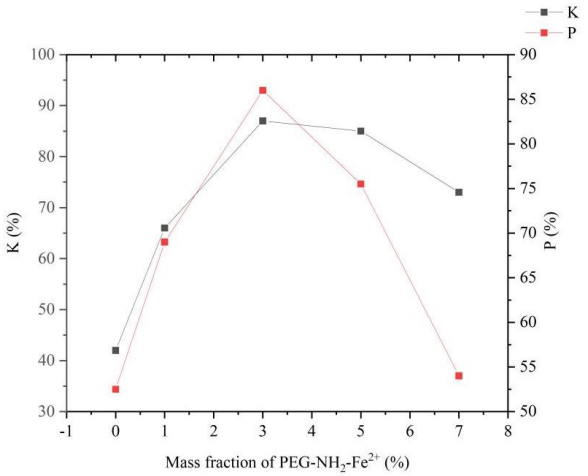

Figure 5. Effect of PEG-IDA-Fe^{2+} on the separation of Pv-E in ATPE.

(4) The impact of pH

The protein separation efficiency was strongly affected by pH values. Both K and P of Pv-E increased as the pH increased from 5 to 8 (Figure 6). Proteins distributed in ATPE were influenced by pH because of the changes in the properties of the surface and the cost of the proteins. If the pH is above the isoelectric point (pI), proteins are negatively charged. Conversely, if the pH was below the isoelectric point, proteins carry positive charges and the net charge of proteins of isoelectric point is zero [16]. The isoelectric point of Pv-E was 4.0. When pH was above 4.0, Pv-E carried a negative charge, which was more likely to enter the polymeric phase in higher pH systems. Due to the presence of positive dipole moments, pH values above the isoelectric point of proteins could induce an increase in protein affinity for PEG-rich phases [17]. When the pH of the system was higher than 8, P decreased, indicating that more impurity proteins entered the top phase. At pH 8, both K and P of Pv-E reached their maximum values while maintaining the biological activity of the protein [15]. Therefore, the ATPE system achieved the best separation efficiency at pH 8.

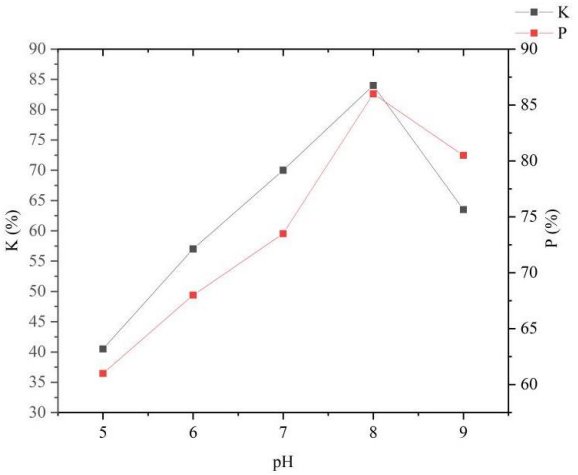

Figure 6. Effect of pH value on the separation of Pv-E in ATPE.

Characterization of Pv structure

(1) Gel electrophoresis analysis

The Pv-E on SDS-PAGE analysis showed a single band at 45 kDa, which indicated that just one important protein had been derived to the top phase and the position of this band was similar to that of the Pv standard (Figure 7). The results confirmed that ATPE could separate and purify Pv.

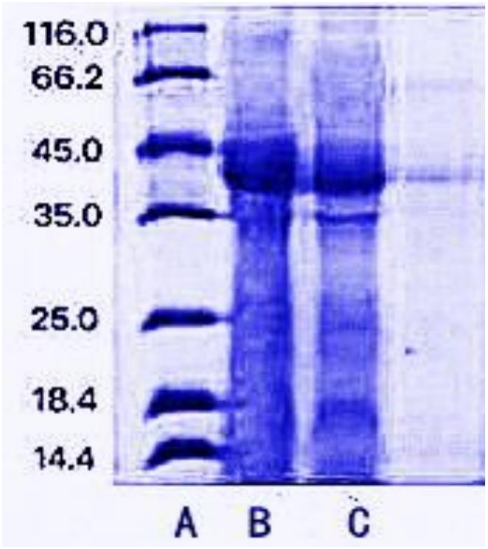

Figure 7. SDS–PAGE profiles of Pv extracted by the system. **A.** Marker. **B.** Pv standard. **C.** Pv-E.

(2) FTIR and Fluorescence spectra

The FTIRT results demonstrated that there were many overlapping peaks in the infrared spectra of Pv-Standard and Pv-E (Figure 8). For example, 1,079/cm was the asymmetric stretching vibration peak of PO_4^{3-}, while 1,600 – 1,700/cm was the characteristic absorption peak of the amide I band. Compared to the standard sample, Pv-E generated a new peak at 525/cm, and the bending vibration peak of PO_4^{3-} at 648/cm shifted to 525/cm, possibly due to the condensation reaction between Pv and ferrous ions [17]. The results indicated that the carboxyl group of Pv-E underwent condensation reaction with ferrous ions, but the protein samples all contained characteristic absorption peaks of Pv, and the natural structure of the protein remained unchanged. The endogenous fluorescence spectra of Pv standard and Pv-E were shown in Figure 9. The maximum absorption peak of Pv obtained by the ATPE process was essentially the same as that of Pv standard. The protein itself had fluorescent chromophores such as tyrosine (Tyr), tryptophan (Trp), and phenylalanine (Phe), and Pv-E had a fluorescence enhancing effect. However, the maximum absorbance peak of the protein did not show any red or blue shift compared to that of the standard, indicating that the natural structure of the extracted protein itself had not been significantly altered.

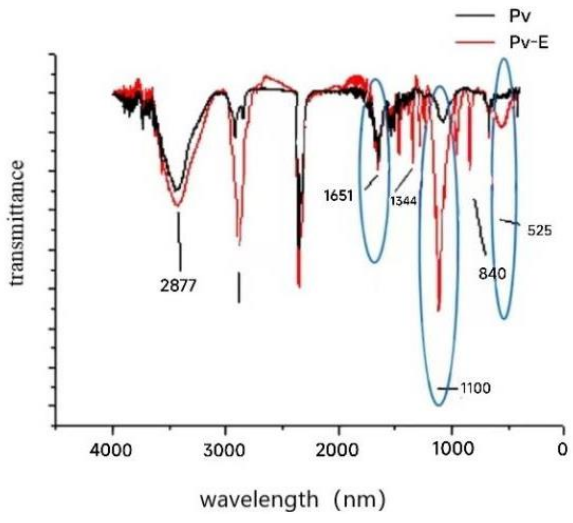

Figure 8. FTIR spectra of Pv and Pv-E.

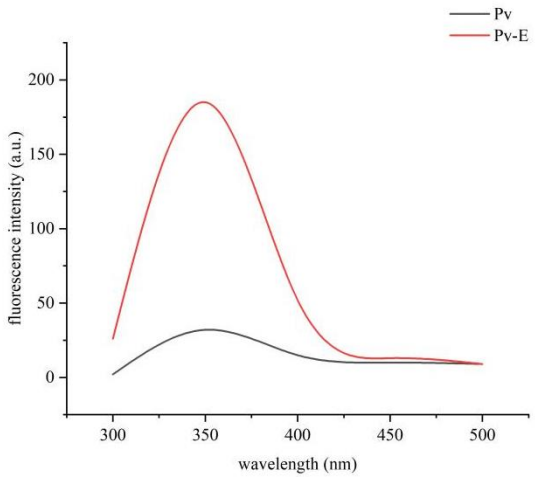

Figure 9. Fluorescence Spectroscopys of Pv and Pv-E.

(3) UV spectra

The UV spectra of Pv standard and Pv-E were shown in Figure 10. The results showed that the maximum absorbing peak of Pv-E was around 280 nm, which was basically the same as the Pv standard, suggesting that, during the separation process, the spatial structure of Pv had not changed and still retained the properties of Pv itself.

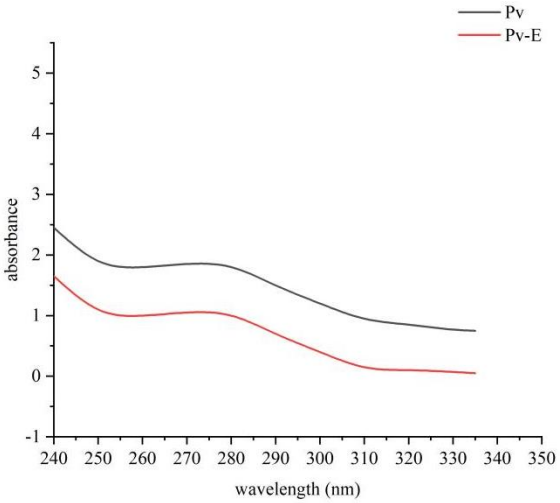

Figure 10. UV-vis spectroscopy of Pv standard and Pv-E.

(4) Liquid phase secondary mass spectrometry analysis

Table 1. Nano LC-ESI-MS/MS detection results of Pv-E.

	Protein molecular weight	Number of peptides	Sequence	Relative abundance
1	206,755.42	101	P02845	53.1%
2	212,643.75	85	P87498	37.9%
3	71,865.52	5	P19121	1.1%
4	28,258.73	4	P02752	7.7%
5	43,181.49	2	P01012	0.6%

The test results of phosvitin sample by Nano LC-ESI-MS/MS were shown in Table 1. After searching the database, five proteins were identified from this study including P02845, P87498, P19121, P02752, P01012. The relative abundances of P02845 and P87498 were 53.1% and 37.9%, respectively, which suggested that Pv was the main protein in the experimental samples (Table 2).

Table 2. The test results of phosvitin sample by Nano LC-ESI-MS/MS.

	Pv-E	Pv database
1110		NRPSKKGNTV
1120		LAEFGTEPDA
1130	TSSSSSSAS	KTSSSSSSAS
1140	STATSSSSSS	STATSSSSSS
1150	ASSPNRKKPM	ASSPNRKKPM
1160	DEEENDQVK	DEEENDQVKQ
1170		ARNKDASSSS
1180		RSSKSSNSSK
1190		RSSSKSSNSS
1200		KRSSSSSSSS
1210		SSSSRSSSSS
1220		SSSSSNSKSS
1230		SSSSKSSSSS
1240		SRSRSSSKSS
1250		SSSSSSSSSS
1260		SSKSSSSRSS
1270		SSSSKSSSHH
1280		SHSHHSGHLN
1290		GSSSSSSSSR
1300		SVSHHSHEHH
1310		SGHLEDDSSS
1320		SSSSSVLSKI
1330		WGRHEIYQYR

Pv property determination

(1) Surface hydrophobicity analysis of Pv

Tyrosine and tryptophan residues endogenous fluorescence, which are very sensitive to the polarity of the microenvironment of the protein conformation, so that it can be used to react to Pv's hydrophobic properties [18]. The surface hydrophobicity of the Pv standard was 485.9 ± 1.88 and that of the Pv-E sample was 476.76 ± 1.41. There was no significant change in hydrophobicity through Pv-E compared to that of Pv standard, which indicated that the structure of the egg yolk high phosphorus protein had not changed during the extraction process [19].

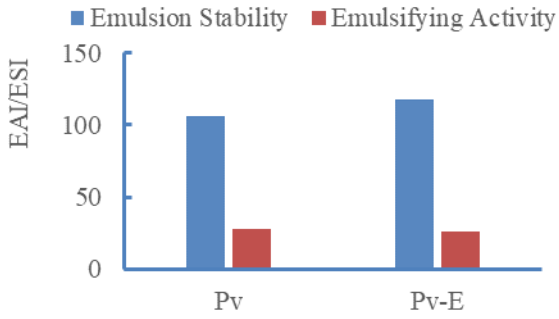

Figure 11. ESI and EAI of Pv standard and Pv and Pv-E.

(2) Analysis of emulsification characteristics

The emulsification stability index and emulsification activity index of Pv samples extracted from Pv standards and proposed systems showed that the Pv samples extracted by the two systems had a slightly higher emulsification stability index and a slightly bottom emulsification activity index compared to that of the Pv standard (Figure 11). The possible reason for this result might be that the protein of Pv-E was isolated and purified in a two-phase aqueous system, leading to protein aggregation

[20]. Non-aggregated Pv possessed better emulsification activity than aggregated Pv, but aggregated Pv had better emulsifying stability because of its stronger hydrophobic interactions [21].

Conclusions

ATPS based on PEG 4000, PEG-IDA-Fe^{2+}, and K_2HPO_4 was used to extract Pv from eggs. Under the optimum conditions, Pv was successfully separated from eggs with a yield of 65.08 ± 0.02% and a purity of 75.41 ± 1.52%. The Pv obtained was characterized by several tests. The results showed that there was no significant difference in structure and most properties between Pv-E and Pv standard. The results confirmed that the proposed method could be an efficient and sustainable way to reduce the impact on the environment and improve the efficiency of phosvitin utilization. However, further studies on purification methods are still needed.

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