INTRODUCTION

Phosvitin is a principal phosphoprotein present in egg yolk and comprises approximately 7% of egg yolk proteins (Mecham and Olcott, 1949). The molecular weight of phosvitin ranges from 36 to 60 kDa, and the major component is B-phosvitin, with a molecular weight of 45 kDa (Abe et al., 1982). Egg yolk phosvitin contains about 135 phosphoserine residues (Allerton and Perlmann, 1965), and thus has a strong metal-chelating capability (Taborsky, 1963; Byrne et al., 1984). Phosvitin can bind both iron and calcium and could be involved in calcium and iron metabolism during embryonic development. Albright et al. (1984) reported that most iron in egg yolk is bound to phosvitin, but the major functions of phosvitin in egg are not fully understood. The molecular ratio between phosvitin and iron present in egg yolk indicates that the degree of iron saturation in phosvitin is <5% even if all the iron in egg is bound to phosvitin because every 2 phosphate groups in phosvitin can bind 1 iron atom (Ishikawa et al., 2004).

Many separation methods, which use solvent combinations such as methanol and ether, methanol and chloroform, and hexane and ethanol, have been developed to remove lipids from yolk granules (Mecham and Olcott, 1949; Joubert and Cook, 1958; Sundararajan et al., 1960; Tsutsui and Obara, 1982; Wallace and Morgan, 1986; Losso and Nakai, 1994; Choi et al., 2004; Belhomme et al., 2007). Salts such as MgSO₄, ammonium sulfate [(NH₄)₂SO₄], or NaCl are used to precipitate or dissolve phosvitin from egg yolk. Causeret et al. (1991) reported that pH modification or increase in ionic strength resulted in deformation of granular structure and solubilized phosvitin from granules.

ABSTRACT

The objective of this study was to develop a new protocol that could be used for large-scale separation of phosvitin from egg yolk using ethanol and salts. Yolk granules, which contain phosvitin, were precipitated after diluting egg yolk with 9 volumes of distilled water. The pH of the yolk solution was adjusted to pH 4.0 to 8.0 using 6 N HCl or NaOH, and then yolk granules containing phosvitin was separated by centrifugation at 3,220 × g for 30 min. Lipids and phospholipids were removed from the insoluble yolk granules using 85% ethanol. The optimal volumes and concentration of ethanol in removing lipids from the precipitants were determined. After centrifugation, the lipid-free precipitants were homogenized with 9 volumes of ammonium sulfate [(NH₄)₂SO₄] or NaCl to extract phosvitin. The optimal pH and concentration of (NH₄)₂SO₄ or NaCl for the highest recovery rate and purity for phosvitin in final solution were determined. At pH 6.0, all the phosvitin in diluted egg yolk solution was precipitated. Among the (NH₄)₂SO₄ and NaCl conditions tested, 10% (NH₄)₂SO₄ or 10% NaCl at pH 4.0 yielded the greatest phosvitin extraction from the lipid-free precipitants. The recovery rates of phosvitin using (NH₄)₂SO₄ and NaCl were 72 and 97%, respectively, and their purity was approximately 85%. Salt was removed from the extract using ultrafiltration. The salt-free phosvitin solution was concentrated using ultrafiltration, the impurities were removed by centrifugation, and the resulting solution was freeze-dried. The partially purified phosvitin was suitable for human use because ethanol was the only solvent used to remove lipids, (NH₄)₂SO₄ or NaCl was used to extract phosvitin, and ultrafiltration was used to remove salt and concentrate the extract. The developed method was simple and suitable for a large-scale preparation of partially purified phosvitin.

Key words: phosvitin separation, egg yolk, sodium chloride, ethanol, ultrafiltration

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further purification or separation of phosvitin, ion exchange chromatography (Connelly and Taborsky, 1961; Heald and McLachlan, 1963, 1964; Castellani et al., 2003) and gel filtration (Connelly and Taborsky, 1961; Clark, 1970; Abe et al., 1982; Tsutsui and Obara, 1982) have been used. However, gel filtration is not appropriate for large-scale separation of phosvitin, and the application of ion exchange chromatography for purifying phosvitin depends on the precise nature of the ion exchangers (Mok et al., 1966) and is not applicable for commercial-scale methods.

Most of the current methods for separating phosvitin from egg yolk use non-food-grade solvent combinations to delipidate soluble or insoluble granules, and the methods are complicated and time consuming. In addition, the structure of phosvitin is denatured or modified because of the solvents used in the separation processes, which results in low phosvitin recovery and limits application in humans (Yasui et al., 1990; Castellani et al., 2003). The objective of this research was to develop a simple and efficient method to prepare partially purified phosvitin from egg yolk using ethanol and salts. The process should be applicable for scale-up and the products should be acceptable for human use.

MATERIALS AND METHODS

Materials

Fresh chicken eggs not more than 7 d old were used. Egg yolk (500 g) was collected and then 2 volumes of distilled water (1,000 mL) were added to the egg yolk. Phosvitin standard was purchased from Sigma-Aldrich Inc. (St. Louis, MO); NaCl, (NH₄)₂SO₄, and ethanol were from Fisher (Thermo Fisher Scientific Inc., Waltham, MA) and were ACS-certified grade.

Protocol for Phosvitin Purification

Egg yolk was separated from egg white and homogenized with 2 volumes of distilled water. The pH of the diluted egg yolk solution was adjusted to pH 4.0, 5.0, 6.0, 7.0, or 8.0 using 6 N HCl and centrifuged at 3,220 × g for 30 min at 4°C. The precipitate containing yolk granules was collected, and lipids and phospholipids were extracted from the egg yolk granules using 4 volumes of 85% ethanol (final concentration). The supernatant containing lipids was discarded after centrifugation at 3,220 × g for 10 min. The precipitant was re-extracted using 4 volumes of 85% ethanol and centrifuged at the same speed and time, and then the precipitant was collected. The fat-free precipitant was homogenized with 9 volumes of 10% NaCl or (NH₄)₂SO₄ solution. After adjusting the pH to 4.0 to 9.0 using 6 N HCl or NaOH, the homogenate was centrifuged at 3,220 × g for 30 min at 4°C. The supernatant was filtered through Whatman No. 1 filter paper to remove floating particles, and (NH₄)₂SO₄ or NaCl in the solution was removed and concentrated using an ultrafiltration unit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), and then lyophilized using a freeze-dryer (Labconco Corp., Kansas City, MO).

Determination of Phosphorus Content

To determine the concentration of phosvitin in samples after ultrafiltration, the content of phosphorus was determined using AOAC (2000) method 986.24. The supernatant solution (3 mL) was heated on a hot plate at 700 to 800°C for 12 h, diluted, and reacted with molybdovanadate reagent. The color strength of solution was measured using a spectrophotometer (Cary 50 Bio, Varian Analytic Instruments, Palo Alto, CA) at 420 nm.

PAGE

A polyacrylamide gel (10 cm wide × 8 cm tall × 1.5 mm thick; Bio-Rad Laboratory Inc., Richmond, CA) consisting of a 10% separating gel [33% acrylamide:N,N-bis-methylene acrylamide = 30%:0.8%, 0.1% (wt/vol) SDS, 0.5% tetramethylethylenediamine (TEMED), 0.05% ammonium persulfate (APS), and 0.38 M Tris-HCl, pH 8.8] and a 4% stacking gel (13.4% acrylamide:N,N-bis-methyene acrylamide = 30%:0.8%, 0.1% SDS, 0.1% TEMED, 0.05% APS, and 0.125 M Tris-HCl, pH 6.8) was used to separate proteins in the final phosvitin solution.

Figure 1. Protocol for purification of phosvitin from egg yolks.
**SDS-PAGE Conditions and Purity Measurement of Phosvitin**

Prepared phosvitin solutions were diluted with deionized water to solutions of 1 mg of protein/mL, and 10 μL of each sample was combined with 40 to 50 μL of sample buffer solution (containing 2% SDS, 10% glycerol, 0.00125% bromophenol blue, and 5% β-mercaptoethanol in 62.5 mM Tris-HCl, pH 8.0), heated at 95°C in a block heater for 5 min, and then loaded on the Protean III cell (Bio-Rad Laboratory Inc.). A broad-range SDS-PAGE molecular weight standard (45 to 200 kDa; Bio-Rad Laboratory Inc.) was used as a marker. The running buffer was composed of 25 mM Tris, 192 mM glycine, and 0.1% gel (10 μL per sample). Electrophoresis was run at 200 V and 100 mA for 40 min. The proteins in the gel were stained using the modified Coomassie Blue method (Hegenauer et al., 1977) that uses 0.1 M aluminum nitrate for proteins containing phosphorous. The gels were destained overnight in 10% acetic acid solution. The purity of phosvitin was estimated using a densitometer (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

**Western Blotting**

Proteins in the SDS-PAGE gel was transferred to pure nitrocellulose membranes (Trans-Blot Transfer medium; Bio-Rad Laboratory Inc.) using a Mini Trans-Blot Cell (Bio-Rad Laboratory Inc.) at a constant voltage of 20 V for 14 h. The transfer buffer was composed of 25 mM Tris, 157 mM glycine, and 15% (vol/vol) methanol. The transfer buffer was maintained at 4°C using Bio-Ice cooling unit (Bio-Rad Laboratory Inc.). After transfer, the membranes was blocked in a solution (PBS-Tween) composed of 80 mM disodium hydrogen orthophosphate, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride, 0.1% polyoxyethylene sorbitan monolaurate (Tweens-20), and 5% nonfat dry milk (wt/vol) for 1 h at room temperature. After blocking, a membrane was placed in its respective primary antibody diluted in PBS-Tween. The phosvitin primary antibody (mouse monoclonal IgG₂b, Santa Cruz Biotechnology Inc.) was diluted to 1:2,000 ratio with PBS-Tween and incubated with the blots overnight at 4°C. The membrane was washed 3 times (10 min/wash) using PBS-Tween at room temperature and incubated at room temperature for 1 h with a secondary antibody (goat anti-mouse IgG-horseradish peroxidase, Santa Cruz Biotechnology Inc.) diluted to 1:5,000 with PBS-Tween. After completion of the secondary antibody incubation, the membrane was washed 3 times (10 min/wash). Detection was initiated using premixed reagents (ECL Plus Western Blotting Detection Reagents, RPN2132, Amersham Bioscience UK, Ltd., Little Chalfont, UK). Chemiluminescence was detected using a 16-bit megapixel CCD camera FluorChem 8800 and FluorChem IS-800 software (Alpha Innotech Corp., San Leandro, CA). Densitometric measurement was completed using the AlphaEaseFC software (Amersham Bioscience UK).

**Recovery Rate of Phosvitin**

Phosvitin contains about 10% phosphorus (Mecham and Olcott, 1949), and the amount of phosvitin in final solution was obtained by multiplying the value of phosphorus content by 10. The recovery rate was calculated under the assumption that 1 g of egg yolk contains 12 mg of phosvitin (Juneja and Kim, 1997).

**Statistical Analysis**

Samples were replicated 3 times and data were analyzed using the JMP software (version 5.1.1; SAS Institute., Cary, NC). Differences in the mean values were compared by Tukey honestly significant difference test, and mean values and standard deviations were reported (Kuehl, 2000).

**RESULTS AND DISCUSSION**

**Effects of pH on Precipitation of Egg Yolk Granules in Diluted Egg Yolk**

This step was to determine the optimal pH conditions of egg yolk solution to yield the highest recovery rate of phosvitin from diluted egg yolk. Unlike other commonly used methods, we used a 3× dilution of egg yolk with distilled water to remove most of the phospholipids and water-soluble proteins from egg yolk plasma and to recover as much yolk granule as possible. As shown in Figure 2, a higher pH (pH 6, 7, and 8) of yolk solution was better than lower pH (pH 4

![Figure 2](image_url)
and 5) in terms of phosvitin yield. However, phosvitin recovery rates did not differ significantly among solutions at pH 6, 7, and 8. The yolk granules consist of 3 proteins: low-density lipoprotein, lipovitellin, and phosvitin. Phosvitin is present inside the micelles of yolk granules and is not in direct contact with the aqueous environment. However, pH modifications (pH 4.0 and 5.0) of diluted egg yolk solution result in deformation of granule structure, solubilize some of the micelle proteins, and release phosvitin (Causeret et al., 1991). The phosvitin released seems to exist as a random coil at near neutral pH in solution because of a discernible lack of secondary structure. However, negative charges on the phosphoserines are neutralized at acidic pH or in less polar solvents so that phosvitin molecules undergo a conformational transition to β-sheet structure (Taborsky, 1970; Perlmann and Grizzuti, 1971). Most of the yolk granules containing phosvitin were not damaged and precipitated easily at neutral pH. Fortunately, as the original pH of diluted egg yolk solution was near neutral pH, around 6.5, it was not necessary to adjust the pH of the initial egg yolk solution before centrifugation. Through the Western blotting study, we confirmed that little phosvitin was present in the supernatant obtained after centrifugation. This indicated that almost all egg yolk granules in diluted egg yolk solution were intact at neutral pH and could be separated by low speed centrifugation.

**Optimal Concentrations of NaCl and (NH₄)₂SO₄ for Phosvitin Extraction**

Our preliminary studies indicated that lipids and phospholipids that formed the yolk micelle structure could be removed using 85% ethanol (final concentration). Removal of phospholipids by 85% ethanol twice disintegrated the yolk micelle structure completely and precipitated all the proteins. To extract phosvitin from the ethanol precipitant, 9 volumes of 10% NaCl or 9 volumes of 5, 10, 15, 20, or 25% (NH₄)₂SO₄ was used. The results indicated that addition of 5% (NH₄)₂SO₄

![Figure 3](image1.png)

**Figure 3.** Effect of (NH₄)₂SO₄ concentration on phosvitin extraction from lipid-free precipitants (n = 3). Concentration of phosvitin (mg/mL) was estimated by conversion of the phosphorus content contained in each solution. Means with different letters are significantly different (P < 0.05).

![Figure 4](image2.png)

**Figure 4.** Effect of (NH₄)₂SO₄ concentration on the purity of phosvitin extracted. Lane 1: molecular weight marker; lane 2: phosvitin standard (Sigma-Aldrich, St. Louis, MO); lane 3: empty; lane 4: solution prepared by addition of distilled water to the fat-free precipitant; lanes 5 to 9: supernatant obtained after extracting phosvitin from the fat-free precipitant using 5, 10, 15, 20, and 25% (NH₄)₂SO₄.
produced the lowest recovery rate, whereas addition of 10% (NH₄)₂SO₄ resulted in the highest recovery rate of phosvitin among the ammonium sulfate treatments (Figure 3). At concentrations higher than 15% (NH₄)₂SO₄, a part of the phosvitin extracted seemed to become precipitated again. Ingraham et al. (1985) reported that high concentrations of (NH₄)₂SO₄ induce multiple conformational states of phosvitin and promote partial separation of polypeptides, which results in low solubility and yield of phosvitin.

The SDS-PAGE results indicated that the major protein extracted from the precipitant with (NH₄)₂SO₄ was phosvitin and only a few other minor protein bands were detected. Among the minor bands, protein with a molecular weight of 66 kDa gradually disappeared as the concentration of (NH₄)₂SO₄ increased. Phosvitin samples extracted with 20 and 25% (NH₄)₂SO₄ seemed to have greater purity than those extracted with lower concentrations of (NH₄)₂SO₄, but differences on SDS gel patterns was not clear (Figure 4). Considering the recovery rate and purity, 10% (NH₄)₂SO₄ was determined to be the best concentration to extract phosvitin from the lipid-free yolk precipitants.

Extraction of phosvitin using 10% NaCl produced a higher recovery rate than that using 10% (NH₄)₂SO₄. Even though the volumes of supernatant obtained after extraction with 10% (NH₄)₂SO₄ and 10% NaCl were the same, the phosvitin concentration was greater with salt extraction than with (NH₄)₂SO₄ extraction (Table 1). The recovery rate of phosvitin in final solution obtained with 10% (NH₄)₂SO₄ was around 72%, whereas that with 10% NaCl was approximately 97% (Table 1). Western blotting results indicated that the phosvitin in the fat-free yolk precipitants was not completely extracted when 10% (NH₄)₂SO₄ was used.

In several studies of phosvitin purification, 0.45 M MgSO₄ (corresponding to 11% MgSO₄) was used to precipitate phosvitin from egg yolk. However, the isolated solution appeared to be a crude mixture of proteins containing high levels of impurities and insoluble materials (Joubert and Cook, 1958; Bernardi and Cook, 1960; Castellani et al., 2003). Causseret et al. (1991) reported that at least 0.58 M NaCl was required to disintegrate granules composed of high-density lipoprotein and phosvitin. However, Belhomme et al. (2007) used 1.74 M NaCl (corresponding to around 10%) solution (wt/vol) to dissolve phosvitin from insoluble granules at pH 7.25.

### Table 1. Recovery of phosvitin from final solution obtained by the developed protocol (n = 4)

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>Supernatant¹ (g)</th>
<th>Phosvitin² (mg/mL)</th>
<th>Vol. × Conc.³ (mg/g)</th>
<th>Yield⁴ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂ SO₄</td>
<td>96.8 ± 0.26</td>
<td>3.36 ± 0.19</td>
<td>324.8 ± 18.10</td>
<td>72.4 ± 4.18</td>
</tr>
<tr>
<td>NaCl</td>
<td>96.2 ± 0.28</td>
<td>4.35 ± 0.21</td>
<td>418.3 ± 19.58</td>
<td>96.54 ± 4.52</td>
</tr>
</tbody>
</table>

¹The amount (g) of supernatant collected from 10 g of precipitant after 10% ammonium sulfate or 10% NaCl extraction.
²Phosvitin concentration in the supernatant after salt extraction and filtration.
³Amount of supernatant × phosvitin.
⁴Calculated on the basis that 1 g of egg yolk contains 12 mg of phosvitin (7% of yolk protein, 17% protein in yolk).

### Optimal pH Conditions for Phosvitin Extraction Using 10% (NH₄)₂SO₄ or 10% NaCl

The pH of 10% (NH₄)₂SO₄ solutions during phosvitin extraction significantly influenced the recovery rate of phosvitin (Figure 5). The recovery of phosvitin using 10% (NH₄)₂SO₄ extraction was the highest when the pH of the solution was adjusted to pH 4.0, and the recovery rates decreased as the pH of extraction solution increased. In SDS-PAGE, however, the effect of pH on the purity of phosvitin was not clear (Figure 6).

In alkaline solutions (pH >8), phosphate groups are di-anionic and exhibit 1 negative charge per amino acid residue, whereas in acidic conditions (pH 3 to 5), they are singly charged and exhibit one-third negative charge per amino acid residue (Allerton and Perlmann, 1965). A coil conformation of phosvitin was transformed to a β-structure conformation when the pH of solution became <3.0 due to partial neutralization of its negatively charged parts (Taborsky, 1968; Renugopalakrishnan et al., 1985; Yasui et al., 1990). Castellani et al. (2003) suggest that the pH of the final extraction solution should be <1.5 to increase phosvitin yield. Therefore, the optimal pH for phosvitin extraction is pH 4.0.
fore, we tested again to determine if a lower pH (<pH 4.0) was better than pH 4.0, but found that pH 4.0 was the optimal condition for extracting phosvitin from the salt-free granules, as shown in Figure 7. The samples extracted with 10% NaCl or 10% (NH₄)₂SO₄ solution after adjusting the pH to 2.0 had lower phosvitin yields than those extracted at pH 4.0. Extraction of phosvitin using 10% NaCl after adjusting the solution pH to 4.0 resulted in a higher recovery rate than that with pH 3.0. Extracting phosvitin using 10% (NH₄)₂SO₄, however, did not show a significant difference in the recovery rate between pH 3.0 and 4.0. Therefore, we suggest that adjusting the pH of 10% (NH₄)₂SO₄ or NaCl solution to 4.0 before extracting phosvitin from delipidated egg yolk granules would produce the highest recovery for phosvitin.

Belhomme et al. (2007) isolated phosvitin from insoluble granules using 1.74 M NaCl at neutral pH. Because phosvitin molecules undergo a conformational transition to a β-sheet structure at acidic pH by neutralization of the negative charges on the phosphoserines (Taborsky, 1970; Perlmann and Grizzuti, 1971), an acidic pH seemed to be more efficient for phosvitin dissolution than a neutral pH. The isolated phosvitin was confirmed using the Western blot (Figure 8).

**Yield and Purity of Purified Phosvitin**

The electropherogram of proteins in the delipidated egg yolk granules (Figure 9, lane 4) showed a greater number of proteins than those reported by Shantz and Dawson (1976), who observed 18 proteins with molecular weights ranging from 12 to 120 kDa on SDS-PAGE electropherograms. As the pH of extraction solution increased, a greater number and intensity of protein bands other than phosvitin started to show up in the
electropherogram (Figure 6, lanes 5–9). The final protein separated using 10% (NH₄)₂SO₄ solution had 2 phosvitin bands, a major and minor phosvitin, whereas the solution obtained by 10% NaCl contained a few more impurities, consisting of proteins with a small molecular weight, in addition to the 2 phosvitin bands (Figure 9, lanes 7 and 8).

Castellani et al. (2003) reported that the purity and composition of final phosvitin products depended upon the isolation methods used. Mecham and Olcott (1949) reported that isolated phosvitin was electrophoretically heterogeneous in the PAGE electropherogram, showing 2 components designated as α- and β-phosvitin, and Taborsky and Mok (1967) estimated the molecular masses of the major and minor phosvitin to be 36 and 40 kDa, respectively. Clark (1970) reported that phosvitin isolated by gel filtration was composed of 2 components, a major protein of 34 kDa and a minor one of 28 kDa. On the other hand, McBee and Cotterill (1979) observed that native egg yolk has 3 bands in the area of phosvitin. Abe et al. (1982) reported that hen egg yolk phosvitin was composed of several polypeptides with molecular weights ranging from 36 to 60 kDa, and the major component was β-phosvitin, with a molecular weight of 45 kDa on SDS-PAGE. Tsutsui and Obara (1984) reported that phosvitin isolated using chloroform-methanol and gel filtration or ion exchange chromatography was composed of several subcomponents with molecular weights of 27 to 38 kDa.

The phosvitin isolated using 10% (NH₄)₂SO₄ solution had greater purity than that isolated with NaCl. The phosvitin obtained from 10% NaCl extraction exhibited some impurities just below the main phosvitin band in a low molecular weight area (Figure 9, lane 8). However, some of those bands could be produced by self-digestion of phosvitin during processing and storage in a refrigerator because those bands are not seen

![Figure 8](image_url) Western blotting of phosvitin separated. Lane 1: standard phosvitin; lane 2: empty; lane 3: final solution (supernatant) after filtering and dialysis.

![Figure 9](image_url) The electrophoresis result of phosvitin separated using (NH₄)₂SO₄ or NaCl extraction. Lane 1: molecular marker; lane 2: standard phosvitin; lane 3: empty; lane 4: 3× diluted egg yolk solution; lane 5: supernatant obtained after centrifugation of diluted egg yolk solution; lane 6: egg yolk granules before phosvitin extraction; lane 7: supernatant gained after 10% (NH₄)₂SO₄ extraction; lane 8: supernatant after 10% NaCl extraction.
in the original lipid-free egg yolk granules (Figure 9, lane 4) or the granule solution before salt extraction (Figure 9, lane 6). Wallace and Morgan (1986) reported that chicken phosvitin contained phosphoproteins with molecular weights of 40, 33, 18, 15, and 13 kDa, and the protein bands with low molecular weight besides the 2 major bands were assumed to be a part of phosvitin. We observed that the standard phosvitin molecule was easily digested during storage at a low temperature (−20°C), and phosvitin subcomponents often appeared as several minor bands on SDS-PAGE (data not shown). If these polypeptides were produced by self-digestion at low temperature conditions, the purity of isolated phosvitin separated using 10% NaCl may be comparable to that using 10% (NH₄)₂SO₄. So, both 10% (NH₄)₂SO₄ and 10% NaCl solution are recommended for extracting phosvitin from egg yolk granules after removing lipids and phospholipids using 85% ethanol. The purity of phosvitin can be improved by adjusting the pH of the extraction solution to pH 4.0. However, the recovery rate would be higher with NaCl than with (NH₄)₂SO₄ extraction. Adjusting the pH of the salt solution from egg yolk uses low speed centrifugation, ethanol, and 10% NaCl solution are recommended for extracting phosvitin from lipid-free yolk granules. How- ever, NaCl extraction produced a greater yield than (NH₄)₂SO₄ extraction. Connelly, C., and G. Taborsky. 1961. Chromatographic fractionation of phosvitin. J. Biol. Chem. 236:1364–1368.

In conclusion, egg yolk granules containing phosvitin could be separated after dilution of egg yolk with 2 volumes of distilled water and low speed centrifugation. Natural egg yolk pH (pH 6.0 to 7.0) was better than a lower pH for the precipitation of yolk granules. Most of the lipids and phospholipids in granules could be removed by 85% ethanol alone, and both 10% (NH₄)₂SO₄ and 10% NaCl solutions could be used to extract phosvitin from lipid-free yolk granules. However, NaCl extraction produced a greater yield than (NH₄)₂SO₄ extraction. Adjusting the pH of the salt solutions to pH 4.0 improved phosvitin recovery rate and purity. The protocol developed for phosvitin separation from egg yolk uses low speed centrifugation, ethanol, (NH₄)₂SO₄ or NaCl, and ultrafiltration, and is simple and appropriate for a large-scale production of phosvitin. Moreover, the isolated phosvitin can be applicable for food products and human use because only ethanol and NaCl or (NH₄)₂SO₄ are used to separate phosvitin, and ethanol and salts used could be removed easily at the final stage by ultrafiltration.

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