Sequential separation of immunoglobulin Y and phosvitin from chicken egg yolk without using organic solvents

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ABSTRACT A study was conducted to develop a simple sequential separation protocol to separate phosvitin and IgY from egg yolk without using organic solvents. Egg yolk was diluted with 2 volumes of distilled water (DW), homogenized, and centrifuged. The precipitant was collected and homogenized with 4 volumes of 10% NaCl (wt/vol) in 0.05 N NaOH solution to extract phosvitin. The pH of the homogenate was adjusted to 4.0 and the precipitate was removed by centrifugation. The supernatant was collected and then heat-treated at 70°C for 30 min and centrifuged to remove impurities. The supernatant containing phosvitin was collected, had salts removed, and was concentrated and then freeze-dried. The supernatant from the centrifugation of diluted egg yolk was diluted again with 3 volumes of DW, and the precipitate was removed by centrifugation. The resulting supernatant was concentrated using ultrafiltration and then IgY was precipitated using 20% saturated (NH₄)₂SO₄ + 15% NaCl (wt/vol). The precipitant was collected after centrifugation at 3,400 × g for 30 min at 4°C and dissolved with DW, had salts removed, and then was freeze-dried. The purity of separated phosvitin and IgY was checked using SDS-PAGE and the proteins were verified using Western blotting. The purity of phosvitin and IgY was 97.2 and 98.7%, and the yield was 98.7 and 80.9%, respectively. The ELISA results indicated that the activities of separated IgY and phosvitin were 96.3 and 98.3%, respectively. This study proved that both phosvitin and IgY can be separated in sequence from egg yolk without using an organic solvent. Also, the method is very simple and has a high potential for scale-up processing.

Key words: phosvitin, immunoglobulin Y, sequential separation, egg yolk

INTRODUCTION

Specific antibodies produced from immunized chickens have very high potentials to cure various animal and human diseases, and for immunodiagnostic and pathogen control in food products (Reilly et al., 1997; Kovacs-Nolan and Mine, 2004; Chalghoumi et al., 2009; Vega et al., 2011). The amount of IgY in chicken serum is approximately 5 to 7 mg/mL and around 20 eggs can be produced from one hen per month, which is equivalent to 300 mL of serum (1.5–2.1 g of IgY, Shimizu et al., 1994). This productivity represents a reduction in animal numbers and improved animal welfare because invasive blood collection from animals can be avoided. Also, direct use of egg yolk as an antibody source will be simple and cost less. Therefore, chicken has a greater potential than mammals for antibody production (Fay-er and Jenkins, 1992; Svendsen et al., 1995). However, its application and efficiency have not been fully appreciated (Hatta et al., 1993). Although egg yolk from immunized chickens can be directly used to cure various diseases in animals, the efficiency and application can be increased dramatically if the isolation and purification methods of immunoglobulin in egg yolk can be improved.

Several published antibody purification methods from egg yolk are available, but most of them are for laboratory scale and are complicated (Jensenius et al., 1981; Polson et al., 1985; Akita and Nakai, 1992; Dong et al., 2008). One of the main difficulties in separating IgY from egg yolk is its high lipid content (Hansen et al., 1998). To remove lipids and improve extraction and separation efficiencies of IgY from egg yolk, various...
methods such as adding xanthan (Hatta et al., 1988) or carrageenan gum (Hatta and Kim, 1990), and dilution with acidic water (Akita and Nakai, 1993) were tested. Also, precipitation of IgY from egg yolk extracts using polyethylene glycol (Polson et al., 1985), pectin (Chang et al., 2000), dextran sulfate (Jensenius et al., 1981), and ammonium sulfate (Akita and Nakai, 1993) had been tested. However, many of these approaches have limitations for the commercial production of IgY due to low yield, incompatibility for human use, and complicated procedures.

The most popular strategy for separating IgY from egg yolk is extracting IgY from yolk using 10 volumes of acidic water and then precipitating IgY using (NH₄)₂SO₄ or sodium sulfate (Akita and Nakai, 1992, 1993). However, extreme volume increase makes it difficult for handling. Ultrafiltration had been used to reduce the volume of IgY extract (Hernandez-Campos et al., 2010; Liu et al., 2011) but the efficacy of ultrafiltration were significantly affected by the amount of lipids in the extract. To improve the purity of IgY separated, affinity chromatography or ion-exchange chromatography is often employed (Ko and Ahn, 2007; Dong et al., 2008).

Phosvitin is a major protein in egg yolk and contains a very large number of phosphate groups in its structure (Mecham and Olcott, 1949). About 57% of amino acids in phosvitin are serine residues and almost all of them are phosphorylated. Therefore, phosvitin has very strong metal-chelating capacities, which makes it an excellent candidate for natural antioxidant or antibacterial agent (Khan et al., 2000; Ishikawa et al., 2004).

Over the past 60 yr, various separation methods for phosvitin from egg yolk had been developed primarily using a 3-step process: lipid removal using organic solvents such as methanol, ether, chloroform, hexane, and ethanol from yolk, extraction of phosvitin from the lipid-free fraction using NaCl, and then precipitation of extracted phosvitin using MgSO₄, (NH₄)₂SO₄, or NaCl (Joubert and Cook, 1958; Losso and Nakai, 1994; Castellani et al., 2003; Ko et al., 2011). For further purification of extracted phosvitin, anion exchange and affinity chromatography methods have been commonly used (Connelly and Taborsky, 1961; Lei and Wu, 2012; Zhang et al., 2012). As indicated earlier, most of the current methods for separating phosvitin use non-food-grade solvents such as chloroform, methanol, and ether to remove lipids in granules from egg yolk, and thus the phosvitin cannot be accepted for human use. Also, the solvents caused denaturation and modification of phosvitin structure, which led to low phosvitin recovery and loss of functions (Castellani et al., 2003). Recently, several separation methods for phosvitin without using toxic solvents, which include polyethylene glycol, heat treatment, and carbonate-bicarbonate buffer, have been published (Zhang et al., 2011; Lei and Wu, 2012). However, all these methods used anion exchange chromatography or dialysis for further purification, which has limitations to apply for industry scale production.

Moreover, all the separation methods developed were for a single component, not for multiple components from egg yolk. The objective of this study was to develop a simple and easy separation method for IgY and phosvitin in sequence without using toxic compounds or solvents. Also, the developed method is aimed for large-scale production and appropriate for human use with high yield and purity.

**MATERIALS AND METHODS**

**Materials**

Chicken eggs were purchased from a local market. Ammonium sulfate, sodium chloride, and sodium hydroxide were purchased from Fisher Scientific (Waltham, MA). Standard of IgY was purchased from MP Biomedicals, LLC (Santa Ana, CA), and anti-IgY horseradish peroxidase (HRP)-conjugated antibody was from United States Biological (Swampscott, MA). Anti-chicken IgY alkaline phosphatase conjugated antibody and phosvitin standard was purchased from Sigma-Aldrich (St. Louis, MO). Phosvitin primary antibody (mouse monoclonal IgG₂a) and secondary antibody (goat anti-mouse IgG-horseradish peroxidase) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Amersham Enhanced Chemiluminescence (ECL) Prime Western Blotting Detection Reagents were from GE Healthcare Life Sciences (Pittsburgh, PA). 3-Ethylbenzothiazoline-6-sulphonic acid (ABTS) substrate for HRP was from Amersco (Solon, OH) and p-nitrophenyl phosphate (PNPP) substrate for alkaline phosphatase was from Invitrogen (Camarillo, CA). The Detergent Compatable (DC) Protein Assay kit was from Bio-Rad (Hercules, CA). Bovine serum albumin was purchased from Sigma-Aldrich.

**Sequential Separation of IgY and Phosvitin**

Egg yolk was separated from egg white, diluted with 2 volumes of cold (4°C) distilled water (DW) and homogenized for 1 min using a hand blender (Kitchen Aid) at high speed (set at 9). After centrifugation at 3,400 × g for 30 min at 4°C, the supernatant was used to separate IgY and the precipitant for phosvitin separation.

For IgY separation, the collected supernatant was diluted again with 3 volumes of cold (4°C) DW, kept in a cold room overnight to precipitate phospholipids and lipoproteins, and then centrifuged at 3,400 × g for 30 min at 4°C. The resulting supernatant was concentrated using ultrafiltration (membrane filter cut-off size: 50 kDa, GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Immunoglobulin Y in the concentrated solution was precipitated using 20% saturated ammonium sulfate (final concentration) and 15% NaCl (wt/vol, final concentration) combination. The precipitant was collected after centrifugation at 3,400 × g for 30 min at 4°C, dissolved with 9 volumes of DW, and then...
precipitated again with the same (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} + NaCl combination to improve purity. The precipitant was dissolved with 9 volumes of DW, desalted using ultrafiltration, and then freeze-dried (FreeZone Freeze Dryer, Labconco Corp., Kansas City, MO).

For phosvitin separation, the precipitant collected from the 3x-diluted egg yolk was homogenized with 4 volumes of 10% NaCl (wt/vol) in 0.05 N NaOH solution for 2 min using a Polytron (Type PT 10/35, Brinkman Instrument Inc., Westbury, NY) at 28,000 rpm to extract phosvitin. The homogenate was diluted with equal volume of distilled water, adjusted the pH to 4.0 using 6 N HCl, centrifuged at 3,400 \( \times g \) for 30 min at 4°C, and collected both supernatant and precipitant. The precipitant was reextracted using 4 volumes of 10% NaCl (wt/vol) in 0.05 N NaOH solutions, pH adjusted to 4.0, and centrifuged at 3,400 \( \times g \) for 30 min at 4°C. The supernatants from the 2 extractions were pooled and then desalted and concentrated using an ultrafiltration unit (membrane filter cut-off size: 10 kD, GE Healthcare Bio-Sciences Corp.). The concentrated solution was heat-treated at 70°C for 30 min, centrifuged, and the resulting supernatant was freeze-dried (Labconco Corp.).

**Protein Assay for IgY and Phosvitin**

The DC protein assay was carried out using a DC Protein Assay kit (Bio-Rad). The assay reaction is similar to the Lowry’s protein assay (Lowry et al., 1951) but with some improved modifications. A Microplate Assay Protocol is adopted from the manual and standard curve was prepared with BSA. The absorbance of blue color developed was measured on a microplate reader (xMark, Bio-Rad) at 750 nm.

**SDS-PAGE for IgY and Phosvitin**

The SDS-PAGE of IgY was carried out using a Mini-Protein Tetra cell (Bio-Rad) under nonreducing conditions. Ten percent SDS-PAGE gel and Coomassie Brilliant Blue R-250 staining were used. After destaining the gel, the protein bands in the gel were checked using the Gel Doc (Bio-Rad). The SDS-PAGE of phosvitin was also done under the same conditions as for the IgY, but the gel was stained using Coomassie Brilliant Blue R-250 containing 0.1 M aluminum nitrate (Hegenauer et al., 1977). The SDS-PAGE was used to estimate the purity of IgY and phosvitin, and the purity of protein was calculated by converting the density of protein bands in the gel picture using the ImageJ software (National Institutes of Health, Bethesda, MD) as the percentage of the total gel density.

**Western Blot of IgY and Phosvitin**

Western blot was used to confirm the separated IgY and phosvitin (Xie et al., 2002). After running the SDS-PAGE, proteins were transferred onto a nitrocellulose membrane (Bio-Rad) at 90 V for 2 h under controlled temperature at 4°C. The transferred membrane was blocked with 5% skim milk powder solution dissolved in phosphate-buffered saline with Tween-20 (PBST). To identify IgY, the membrane was treated with anti-chicken IgY (HRP; United States Biological, after 1:15,000 dilution as a primary antibody) and kept overnight at 4°C with shaking. The membrane was washed 3 times with the PBST solution at 10-min intervals and exposed to Amersham ECL Prime (GE Healthcare) for 5 min, and then analyzed using a Chemidoc (Bio-Rad).

For phosvitin, the transferred cellulose membrane was placed in the phosvitin primary antibody solution (mouse monoclonal IgG2a, Santa Cruz Biotechnology Inc.), which was diluted to a 1:1,000 ratio with 5% skim milk (wt/vol) dissolved in PBST and incubated overnight at 4°C. The membrane was washed 3 times with PBST solution at 10-min intervals at room temperature (22°C) and then incubated with a secondary antibody (goat anti-mouse IgG-HRP, Santa Cruz Biotechnology Inc.) for 1 h at room temperature. The secondary antibody was diluted at 1:5,000 with 5% skim milk (wt/vol) dissolved in PBST. After completion of the secondary antibody incubation, the membrane was washed 3 times with PBST solution at 10-min intervals, exposed to ECL Prime (GE Healthcare) for 5 min, and detected using a Chemidoc (Bio-Rad).

**ELISA Assay of Separated Proteins**

The ELISA assays were carried out to check the purity of the purified intact proteins after freeze drying with the ELISA using the method of Ko and Ahn (2007). Standard curves were prepared using the IgY and phosvitin standards (IgY from MP Biomedicals and phosvitin from Sigma-Aldrich) with serial dilutions. Each of the standard and purified proteins were dissolved in a 50 mM carbonate buffer (pH 9.6) and diluted to the final protein concentration of 10 \( \mu \)g/mL. One hundred microliters of the diluted standard and sample were coated onto a microplate and incubated overnight at 4°C.

For IgY activity, the wells were washed with PBST (200 \( \mu \)L) and blocked with 1% BSA solution in PBS (200 \( \mu \)L) for 2 h at room temperature. The wells were washed with PBST 3 times and incubated with 100 \( \mu \)L of primary anti-chicken IgG (1:35,000 solution diluted with 1% BSA-conjugated alkaline phosphatase) for 2 h at room temperature in the dark. After washing with PBST 3 times, 100 \( \mu \)L of PNPP substrate (p-nitrophenyld phosphate) was added to each well and the samples were incubated at room temperature for 30 min in the dark. The enzyme reaction was stopped by adding 3 \( N \) NaOH, and the color developed was read on the microplate reader (xMark, Bio-Rad) at 405 nm.

For phosvitin, 5% skim milk in PBST was used as a blocking solution. Both primary and secondary anti-
bodies were used. Phosvitin primary antibody (mouse monoclonal IgG2a, Santa Cruz Biotechnology Inc.) was diluted to 1:1,000 with 5% skim milk (wt/vol) dissolved in PBST and the secondary antibody (goat anti-mouse IgG-horseradish peroxidase, Santa Cruz Biotechnology Inc.) was diluted to 1:5,000 with 5% skim milk (wt/vol) dissolved in PBST solution before use. An ABTS substrate for HRP was used for the enzyme reaction and 0.1 M citric acid was used as a stop solution, and the absorbance was measured at 416 nm.

Yield of IgY and Phosvitin

The weights of freeze-dried IgY and phosvitin and the theoretical amounts of IgY and phosvitin in chicken egg yolk (Stadelman and Cotterill, 2001) were used to calculate the yield of the 2 separated proteins. For yield measurement, the sequential separation process was repeated 3 times.

Statistical Analysis

The data were analyzed using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA). Differences in mean values were compared by 1-way ANOVA using JMP pro software (Release 10.0.2, SAS Institute Inc., Cary, NC). The separation method was replicated 3 times.

RESULTS AND DISCUSSION

Purification of IgY from Egg Yolk

After concentrating the crude extract of egg yolk (supernatant) using ultrafiltration, 2 salt combinations were tested to precipitate IgY effectively. Our preliminary trials indicated that 20% saturated (NH4)2SO4 or 15% NaCl (wt/vol) was the best condition to precipitate IgY. When (NH4)2SO4 or NaCl was used alone, large amounts of IgY were still remaining in the supernatant. Kim and Nakai (1998) reported that addition

![Figure 1. Effect of (NH4)2SO4 and NaCl combination on the separation of IgY from egg yolk. a) Lanes 1 to 6: precipitant obtained from 5.0, 10.0, 12.5, 15.0, 17.5, 20.0% (saturated) (NH4)2SO4 with a fixed level (15%) of NaCl (wt/vol); lanes 7 to 12: supernatant obtained from 5.0, 10.0, 12.5, 15.0, 17.5, and 20.0% saturated (NH4)2SO4 with a fixed level (15%) of NaCl (wt/vol). b) Lanes 1 to 6: precipitant obtained from 5.0, 10.0, 12.5, 15.0, 17.5, 20.0% NaCl (wt/vol) with a fixed level [20% saturated (NH4)2SO4]; lanes 7 to 12: supernatant obtained from 5.0, 10.0, 12.5, 15.0, 17.5, 20.0% NaCl (wt/vol) with a fixed level (20% saturated ammonium sulfate).]
of NaCl polymerized IgY and facilitated the isolation of IgY from other proteins. Ko and Ahn (2007) suggested 2 times precipitation of IgY using 40% (NH₄)₂SO₄. In the current study, the combination of fixed level of (NH₄)₂SO₄ (20% saturation) with varying levels of NaCl and fixed level of NaCl (15%, wt/vol) with varying levels of (NH₄)₂SO₄ were tested. Figure 1a shows the comparison of IgY separation at different concentrations of (NH₄)₂SO₄ with fixed level 15% NaCl (wt/vol). The result showed that increasing (NH₄)₂SO₄ > 15% saturation did not improve the precipitation of IgY.

Figure 1b does not show much differences in the separation of IgY when 10 to 17.5% of NaCl was combined with the fixed level (20% saturated) of (NH₄)₂SO₄. However, 5% NaCl (wt/vol) was not enough to precipitate the IgY in the crude extract of egg yolk and more than 17.5% NaCl (wt/vol) did not help to precipitate IgY effectively. The DC protein assay results indicated that the largest amount of IgY was obtained when 15.0% NaCl (wt/vol) and 20% saturated (NH₄)₂SO₄ combination was used (data not shown). From Figures 1a and 1b, the 2 best salt combinations were compared with confirm the final conditions to isolate IgY. The SDS-PAGE and DC protein assay results showed that the 15% NaCl (wt/vol) + 20% saturated (NH₄)₂SO₄ combination precipitated IgY more effectively than 15% saturated (NH₄)₂SO₄ + 15% NaCl (wt/vol) conditions. Therefore, 20% saturated (NH₄)₂SO₄ and 15% NaCl combination was selected as the final conditions for separating IgY from the crude extract of egg yolk. The egg yolk solutions for each step obtained by salt combination treatment are shown in Figure 2 and the purified IgY was confirmed by the Western blot.

Recently, Marcet et al. (2011) and Pauly et al. (2011) used multi-step polyethylene glycol (PEG) precipitation to separate IgY from egg yolk. They precipitated IgY 3 times using different levels of PEG and then further purified using anion exchange chromatography. However, it was difficult to remove PEG using dialysis or ultrafiltration because the molecular size of PEG greatly increased by binding hundreds of water molecules. Also, the SDS-PAGE showed high levels of impurities in the precipitant. Therefore, using salt instead of PEG to precipitate IgY will be easier and better for the separation and scale-up process of IgY from egg yolk.

**Extraction and Purification of Phosvitin from Egg Yolk**

The egg yolk solutions for each step for phosvitin separation are shown in Figure 3. For the efficient preparation of phosvitin from egg yolk, phosvitin should be extracted from egg yolk. Radomski and Cook (1964) reported that phosvitin is present inside the micelle structure of egg yolk granules and complexed with lipovitellin through phosphocalcic bridges. Therefore, phosvitin in the micelles should be released first and then the phosphocalcic bridges removed. To disrupt the granule structure and extract phosvitin, 10% NaCl
(wt/vol) was commonly used after removing phospho-
lipids from the egg yolk granules using ethanol and hex-
ane (Losso and Nakai, 1994; Ko et al., 2011). Lei and 
Wu (2012) studied granule solubilation by changing 
the pH of egg yolk solution from pH 5 to 12 and found 
that egg granules could be completely dissolved in 0.05 
M carbonate-bicarbonate buffer at pH 9.6. However, 
most phosvitin was still remaining in the granules after 
2 extractions using 0.05 M carbonate-bicarbonate buf-
fer, pH 9.6. Although further purification method using 
anion exchange chromatography improved the purity 
of phosvitin, the recovery rate of phosvitin was very 
low (35.4%). In this study, however, high ionic strength 
and high pH using NaCl and NaOH combinations were 
used to disrupt the granules (precipitant) and solubilize 
phosvitin because lipovitellin is soluble at alkaline pH 
(Burley and Cook, 1961; Anton and Gandemer, 1997).

Figure 4 shows the effect of 10% NaCl (wt/vol) in 
alkaline conditions or 10% NaCl + heating at 70°C for 
30 min on the separation of phosvitin from egg yolk. 
Extraction of phosvitin from the granules using 10% 
NaCl (wt/vol) in 0.05 N NaOH was better than that 
with higher concentrations of NaOH (0.75 and 0.1 N) in 
terms of phosvitin yield in the extract (Figure 4, lanes 
2, 3, and 4). Extraction of phosvitin using 10% NaCl + 
heating at 70°C for 30 min extracted phosvitin, but the 
yield of phosvitin was much lower than that with 10% 
NaCl (wt/vol) in 0.05 N NaOH solution (Figure 4, lane 
5). Heating the homogenate of 10% NaCl (wt/vol) in 
NaOH solutions (0.05, 0.075, and 0.1 N NaOH) at 70°C for 30 min either destroyed phosvitin or coprecipitated 
it with other yolk proteins (data not shown). High ionic 
strength (10% NaCl) + alkaline condition (0.05–0.075 
N NaOH) + heating (70°C for 30 min) combination 
was too harsh to extract phosvitin from egg yolk gran-
ules, and the results were not reproducible. Thus, heating 
was eliminated from the extraction parameter, but 
used as a tool to remove impurities after the initial 
extraction of phosvitin from egg yolk. Yang and Cotter-

![Figure 3](image1.png)

**Figure 3.** The SDS-PAGE patterns of egg yolk solutions at each separation step. Lane 1: marker; lane 2: phosvitin standard; lane 3: diluted egg yolk; lane 4: precipitant prepared by centrifuge after addition of distilled water; lane 5: supernatant of first and second extraction combined from 10% NaCl in 0.05 N NaOH solution; lane 6: precipitant of first extraction obtained from 10% NaCl in 0.05 N NaOH solution; lane 7: supernatant obtained 10% NaCl (wt/vol) in 0.05 N NaOH solution with heat treatment at 70°C for 30 min.

![Figure 4](image2.png)

**Figure 4.** Effect of 10% NaCl (wt/vol) in alkaline conditions or heating on the separation of phosvitin from egg yolk. Lane 1: phosvitin standard; lane 2: 10% NaCl in 0.05 N NaOH solution; lane 3: 10% NaCl in 0.075 N NaOH solution; lane 4: 10% NaCl in 0.1 N NaOH solution; lane 5: 10% NaCl with heat treatment 70°C for 30 min.
ill (1989) reported that low-density lipoprotein (LDL) and high-density lipoprotein (HDL) started to denature at 70°C and form gels at 75°C, whereas phosvitin was less sensitive to thermo-coagulation than LDL. Under the high pH conditions with 0.05 or 0.075 N NaOH (pH 11.0–12.0), however, phosvitin denaturation should have occurred easily even at lower temperature conditions, and almost all the phosvitin was precipitated with LDL and HDL. Liu et al. (2011) used 0.5 M NaCl to dissolve granule portion of egg yolk and then heated at 80°C for 30 min to extract phosvitin. However, the purity of phosvitin in the SDS-PAGE was not clear and the majority of phosvitin band was shown at around 35 kDa, which is smaller than the natural phosvitin at around 45 kDa.

As shown in Figure 4 (lane 2), extraction of phosvitin using 10% NaCl (wt/vol) in 0.05 N NaOH was the best among the treatments. However, it also had large amounts of impurities, mainly lipoproteins. Figure 5 (lanes 1 and 3) showed that the pH adjustment of the homogenate to pH 4.0 and centrifugation removed most of the impurities, but some lipoproteins were still remaining in the extract. Castellani et al. (2003) indicated that adjusting the pH of phosvitin extract to 5.0 or lower (pH 3.6 and 2.5) precipitated lipovitellin to eliminate the contamination of HDL. As the final step to improve the purity of extracted phosvitin, heat treatment was used. Different temperature (70 to 90°C at 5°C interval) and heating time (30 to 60 min at 10-min intervals) combinations were tested. The result showed that there was no significant difference among all treatments, indicating that the minimum temperature and time is enough to eliminate the impurities. The final heating of phosvitin extract at 70°C for 30 min was used.

![Figure 5. Effect of additional heating at 70°C for 30 min on the extraction of phosvitin using 10% NaCl (wt/vol) in 0.05 N NaOH solution after removing impurities by pH adjustment (4.0), centrifugation, and desalting. Lanes 1 and 3: Supernatant after centrifugation; lanes 2 and 4: precipitant after centrifugation; lane 5: supernatant obtained from additional heating and centrifugation.]

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**Table 1.** Yield of IgY and phosvitin using the reported value in egg yolk

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight (g)</th>
<th>Yield (%)</th>
<th>Purity (%)</th>
<th>Activity (10 μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgY²</td>
<td>0.81 ± 0.01</td>
<td>80.94 ± 0.01</td>
<td>98.7</td>
<td>9.63 ± 0.97</td>
</tr>
<tr>
<td>Phosvitin³</td>
<td>2.05 ± 0.04</td>
<td>98.70 ± 0.04</td>
<td>97.2</td>
<td>9.83 ± 0.14</td>
</tr>
</tbody>
</table>

¹The theoretical amount of IgY and phosvitin in egg yolk (IgY is 0.6% and phosvitin is 1.25% of total egg yolk, Stadelman and Cotterill, 2001) was used to calculate the yields. The original amount of egg yolk was 165 g per replication; n = 3.

²IgY was produced using a (NH₄)₂SO₄ and NaCl combination.

³Phosvitin was produced using 0.05 N NaOH in 10% NaCl (wt/vol) and heating at 70°C for 30 min.
Figure 6: Western blot of phosvitin and IgY. Lane 1: marker; lane 2: standard phosvitin (1 mg/mL); lanes 3 and 4: purified phosvitin; lane 5: 10 times diluted purified IgY; lanes 6 and 7: purified IgY; lane 8: standard IgY (400 ng/mL); lane 9: marker.

Figure 7. Schematic diagram of the sequential separation of IgY and phosvitin from egg yolk. DW = distilled water. Color version available in the online PDF.
min produced the highest purity and yield (Figure 5, lane 5), which was also confirmed by the Western blot (Figure 6A).

The purity of IgY analyzed by ImageJ software was 98.7% and that of phosvitin was 97.2%. The activities measured by ELISA were 96.3% for IgY and 98.3% for phosvitin. The yield of IgY and phosvitin using the current protocols was 80.9 and 98.7%, respectively (Table 1), indicating that the methods developed are highly efficient and a very high possibility of scale-up. The protocols also do not use any solvents to separate them from egg yolk in a sequence (Figure 7).

Conclusion

This study showed that a simple, effective, and scalable sequential separation method for IgY and phosvitin can be developed. The methods separated IgY and phosvitin from egg yolk without using any solvents or harmful chemicals. The methods are efficient and the compounds separated had high purities, yields, and activities. The separation protocols are simple, and the separation methods are environmentally friendly and open up the possibility of using these value-added proteins (IgY and phosvitin) for various food and nonfood applications.

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REFERENCES


