Sequential separation of lysozyme, ovomucin, ovotransferrin, and ovalbumin from egg white

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ABSTRACT Ovalbumin, ovotransferrin, ovomucin, and lysozyme are a few of the egg white proteins that can be used as functional components. The objective of this study was to develop a simple, sequential separation method for multiple proteins from egg white. Separated proteins are targeted for human use, and thus any toxic compounds were excluded. The methods for individual components and the sequential separation were practiced in laboratory scale first, and then tested for scale-up. Lysozyme was separated first using FPC3500 cation exchange resin and then ovomucin using isoelectric precipitation. Ovalbumin and ovotransferrin were separated from the lysozyme- and ovomucin-free egg white by precipitating ovotransferrin first using 5.0% (wt/vol) $(NH_4)_2SO_4$ and 2.5% (wt/vol) citric acid combination. After centrifugation, the supernatant (S1) was used for ovalbumin separation and the precipitant was dissolved in water, and reprecipitated using 2.0% ammonium sulfate (wt/vol) and 1.5% citric acid (wt/vol) combination. The precipitant was used as ovotransferrin fraction, and the supernatant (S2) was pooled with the first supernatant (S1), desalted using ultrafiltration, and then heat-treated to remove impurities. The yield of ovomucin and ovalbumen was >98% and that of ovotransferrin and lysozyme was >82% for both laboratory and scale-up preparations. The SDS-PAGE and western blotting of the separated proteins, except for ovomucin, showed >90% purity. The ELISA results indicated that the activities of separated ovalbumin, ovotransferrin, and lysozyme were >96%. The protocol separated 4 major proteins in sequence, and the method was simple and easily scaled up.

Key words: egg white, sequential separation, protein, yield, purity

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INTRODUCTION

Ovalbumin (54%), ovotransferrin (12%), ovomucin (3.5%), and lysozyme (3.4%) are among the major proteins in egg white (Stadelman and Cotterill, 2001). These proteins are known to have unique functions and can be used in food processing and as pharmaceuticals or antimicrobial agents after separation. Ovalbumin is the major egg white protein with a molecular weight of 45 kDa and is one of the first proteins isolated from egg white. It is known as a phosphoglycoprotein composed of 385 amino acids (Huopalahti et al., 2007). Half of the amino acids in ovalbumin are hydrophobic and one-third is charged (Nisbet et al., 1981). Ovalbumin is widely used as a standard protein in protein assays (Huntington and Stein, 2001) and is important in immunological and nutritional studies (Datta et al., 2009). Albumin was first separated using saturated ammonium sulfate and acetic acid (Hopkins, 1900; Chick and Martin, 1913), but no clear record about the purity and yield is available. Recently, ovalbumin was separated using 2-stage polyethersulfone flat disk membranes (Datta et al., 2009), electrophoretic method (Desert et al., 2001), foam fractionation (Ward et al., 2007), or liquid chromatographic method (Awade and Efstahiou, 1999). However, all these methods are difficult to scale up for industrial applications due to their complicated procedures, material costs, or sample handling capacity.

Ovotransferrin is another major protein found in egg white. It is a monomeric glycoprotein with 686 amino acids and 76 kDa molecular weight with 15 disulfide bonds (Williams, 1968). Ovotransferrin is known to bind and transport irons in animal body. Ovotransferrin is present in apo- (iron free) and holo- (iron bound) forms, and the chemical and physical properties of these 2 forms differ significantly (Wu and Acer-Lopez,

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2012). The holo-form of ovotransferrin is more resistant to chemical and physical conditions such as pH and heat than the apo-form (Ko and Ahn, 2008). Ovotransferrin is well known to have a strong iron binding capability, and thus, can be used as an antimicrobial agent, antioxidant, or an iron-supplementing agent (Fraenkel-Conrat and Feeney, 1950; Valenti et al., 1982; Ibrahim et al., 2000; Ko et al., 2009; Wu and Acer-Lopez, 2012). Over the past several years, different techniques have been developed to separate ovotransferrin from chicken egg white, but most of the methods developed are laboratory scale. Anion exchange chromatography was developed by Omana et al. (2010a) and Wu and Acer-Lopez (2012) with 80% yield. It was also separated using CM-Toyopearl 650M cation exchange (Tankrathok et al., 2009) and SDS-PAGE and immunoelectrophoresis (Desert et al., 2001). However, the methods have difficulties in handling and the resins are not appropriate for large-scale separation of ovotransferrin. Ovotransferrin was also separated using ammonium sulfate precipitation (Fraenkel-Conrat and Feeney, 1950), but the amount of ammonium sulfate used was too high for a large-scale method. Recently, ovotransferrin was produced in large scale by ethanol precipitation (Ko and Ahn, 2008). However, using ethanol has some limitations because it denatures the rest of the proteins, which makes it difficult to use the remaining proteins.

Another important protein found in egg white is lysozyme. The molecular weight of lysozyme is 14.4 kDa and is consisted of a single polypeptide chain with 129 amino acids (Radziejewska et al., 2008). It is a strongly basic protein with isoelectric point (**pI**) of 10.7 and has 4 disulfide bridges leading to high thermal stability (Huopalahti et al., 2007). Lysozyme was first separated using high levels of ammonium sulfate, but the characteristics of lysozyme were changed by pH and salts (Alderton et al., 1945). The most frequent separation technique used for lysozyme is based on cation exchange chromatography mainly due to its high pI value. Carboxymethyl cellulose (**CMC**) was used in the past to trap lysozyme (Strang, 1984). However, due to its fine granule sizes, handling is difficult and flow rate is very slow for column chromatography. Magnetic cation exchange isolation (Safarik et al., 2007), use of reductants such as β-mercaptoethanol (Chang et al., 2000), and ultrafiltration (Wan et al., 2006) were some other methods used to separate the protein. However, all these methods have problems in scaling up and β-mercaptoethanol cannot be used in the food industry. This is one of the first egg white proteins that was isolated and used in industry. It has the ability to control a limited spectrum of bacteria and fungi that cause spoilage of food (Durance, 1994). Other than that, it is widely used in kimchi pickles, sushi, Chinese noodles, and cheese and wine production (Mine et al., 2004).

Ovomucin is a glycoprotein consisting of 2 subunits (α- and β-subunits) with a molecular weight of 5.5- 8.3 $\times 10^3$ kDa (Omana et al., 2010b), and is responsible for the gel-like properties of thick egg white. α-Ovomucin is composed of 2 subunits called α1 and α2 (Hiidenhovi et al., 1999) and can be solubilized using dissociating agents such as urea, β-mercaptoethanol, SDS, guanidine hydrochloride, and dithiothreitol (Hiidenhovi et al., 1999; Huopalahti et al., 2007). Ovomucin was separated mainly by isoelectric precipitation. MacDonnell et al. (1951) separated ovomucin by bringing down the pH of egg white to 4.5, the pI of ovomucin and centrifugation. Others used various approaches, which include isoelectric precipitation (Donovan et al. 1970; Rabouille et al., 1990; Dubiard et al., 2005; Omana et al., 2010b), dual-column gel filtration (Hiidenhovi et al., 1999), gel electrophoresis (Desert et al., 2001), β-mercaptoethanol (Hiidenhovi et al., 2002), calcium chloride (Omana and Wu, 2009b), and NaCl + pH adjustment (Wang and Wu, 2012). Among the methods, however, isoelectric precipitation was the best way of separating ovomucin.

All the methods discussed above were separating single protein from egg white and rest of the proteins were either denatured during the separation processes or discarded. Separation of more than one protein has been done by a few research groups, but none of them were in large scale. Tankrathok et al. (2009) separated ovalbumin, lysozyme, ovotransferrin, and ovomucoid using Q-Sepharose fast-flow anion exchange chromatography and then with CM-Toyopearl 650M cation exchange chromatography, but the yields were 54, 55, and 21%, respectively. Also, lysozyme, ovotransferrin, and ovalbumin were separated in sequence by Vachier et al. (1995), but the yield of lysozyme was as low as 60%. Both of these methods have low yields and cannot be scaled up for commercial production. The objective of this study was to develop a simple, economical, sequential, and scalable method to separate lysozyme, ovomucin, ovotransferrin, and ovalbumin from egg white.

MATERIALS AND METHODS

Materials

Chicken eggs (large size, less than 3 d old) were purchased from a local market and used within a day. For small scale 280 g and for large scale 3,625 g of egg white was used for the separation of the proteins. Amberlite FPC 3500 (styrene-divinylbenzene, total exchange capacity >2.6 mEq/g, H^+-form), ammonium sulfate, and citric acid were purchased from Fisher Scientific (Waltham, MA). Standard ovalbumin, lysozyme, and ovotransferrin were purchased from Sigma-Aldrich (St. Louis, MO), and rabbit polyclonal to lysozyme, lysozyme marker HRP, anti-ovotransferrin, and anti-ovalbumin antibodies were purchased from Abcam (Cambridge, MA). Rest of the standards and rabbit anti-mouse IgG (H^+L) conjugated AP^{12} antibody from Bio-Rad (Richmond, CA).

Separation of Lysozyme

Separation of lysozyme was done with cation exchange chromatography. Amberlite FPC 3500 resin was

used as the cation exchange resin. Egg white was diluted with an equal volume of distilled water, Amberlite FPC 3500 resin was added (0.5 g/10 mL of diluted egg white), and then the mixture was stirred 12 h in a cold room (4°C) using an overhead stirrer set at the lowest speed (RW20 digital, IKA Works Inc., Wilmington, NC). The solution was filtered through a paper towel (for laboratory scale) or centrifugation for 3,400 × *g* for 20 min at 4°C (for scale-up study), and the resin was collected and washed several times with distilled water, and then once with 0.1 *M* glycine-NaOH buffer, pH 9.3. The lysozyme trapped to the resin was eluted with 0.1 *M* glycine-NaOH buffer, pH 9.3, containing 0.5 *M* NaCl. The eluent was desalted using an ultrafiltration unit equipped with a hollow fiber cartridge (10 kDa cut-off sizes, Quixstand for laboratory scale and Flexstand for scale-up study, GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and lyophilized (Labconco Corp., Kansas City, MO). The freeze dryer conditions were as follows: collector temperature −51°C, vacuum set at 0.002 mbar, and drying chamber temperature set at −15°C.

Separation of Ovomucin

The lysozyme-free egg white (from the first step) was adjusted to pH 4.75 using 3 *N* HCl to precipitate ovomucin and centrifuged at 3,400 × *g* for 30 min at 4°C. The precipitated ovomucin was collected, homogenized, and washed with 10 volumes of distilled water to remove impurities, and the washing was repeated 3 times. The pH of the final homogenate with 10 volumes of distilled water was adjusted to pH 12.0 using 3 *N* NaOH to dissolve the protein.

Separation of Ovotransferrin and Ovalbumin

Ovotransferrin and ovalbumin was separated by precipitating ovotransferrin using ammonium sulfate and citric acid combinations. The ovomucin- and lysozyme-free egg white solution (from the second step) was added with 5.0% (wt/vol) ammonium sulfate and 2.5% (wt/vol) citric acid combination (final pH 2.90), and held at 4°C for 12 h to precipitate ovotransferrin. After centrifugation at 3,000 × *g* for 30 min at 4°C, the precipitant and supernatant (S1) were collected. The supernatant was used for ovalbumin and precipitant for ovotransferrin separation. The precipitant was dissolved with 4 volumes of distilled water and then reprecipitated using a 2.0% ammonium sulfate and 1.5% citric acid combination (final pH 3.35). After centrifugation (3,400 × *g* for 30 min at 4°C), the precipitant was collected and dissolved in distilled water, desalted using an ultrafiltration unit equipped with a hollow fiber cartridge (30 kDa cut-off size, Quickstand for laboratory scale; Flexstand for scale-up study, GE Healthcare Bio-Sciences Corp.) at 22°C, and then lyophilized (Labconco Corp.). The supernatant from the second precipitation (S2) was pooled with the first supernatant (S1), desalted and concentrated using an ultrafiltration unit equipped with a hollow fiber cartridge (30 kDa cut-off size, Quixstand for laboratory scale; Flexstand for scale-up study, GE Healthcare Bio-Sciences Corp.) at 22°C, and heated at 70°C for 15 min to precipitate impurities. After removing the precipitant by centrifugation at 3,000 × *g* for 30 min at 4°C, the final supernatant containing ovalbumin was lyophilized as above (Labconco Corp.).

Yield and Purity Calculation

The yields of lysozyme, ovomucin, ovotransferrin, and ovalbumin were calculated using their theoretical values in egg white. Yield was calculated with the ratio between lyophilized protein and the theoretical value could be presented. To check the separation efficiency, SDS-PAGE (Ko and Ahn, 2008) was conducted under reduced conditions using Mini-Protein II cell (Bio-Rad). Ten percent SDS gel and Coomassie brilliant blue R-250 (Sigma-Aldrich) staining were used. The purity of protein was calculated by converting the density of protein bands in the gel picture using the ImageJ software (NIH, Bethesda, MD) as the percent of the total gel density. The actual protein content (10.95%) obtained from the egg white sample was used to calculate the yields.

Western blot was also used to confirm lysozyme, ovotransferrin, and ovalbumin. Western blot was carried out using the method of Xie et al. (2002) with some modifications. After running the SDS-PAGE, proteins were transferred onto a nitrocellulose membrane (Bio-Rad) at 90 V for 2 h under controlled temperature. The transferred membrane was blocked with 5% skim milk powder dissolved in PBS with Tween 20 (**PBST**).

To identify lysozyme, the membrane was treated with rabbit polyclonal antibody to lysozyme (Abcam, after 1:10,000 dilution) and kept overnight at 4°C with shaking. The membrane was washed 3 times with PBST solution at 15-min intervals, exposed to Amersham ECL Prime (GE Healthcare, after 1:1 dilution) for 5 min, and then analyzed using a Chemidoc (Bio-Rad).

For ovalbumin, anti-ovalbumin antibody (Abcam) was used after diluting 1:15,000 with distilled water and incubating overnight at 4°C. For ovotransferrin, ovotransferrin chicken antibody was used after diluting 1:15,000. Rabbit anti-mouse IgG ($H^{+}L$) conjugated AP[12] (Abcam) was used after diluting 1:20,000 as the secondary antibody, incubated 1 h at room temperature, and analyzed.

ELISA Assay for the Activity of Purified Proteins

The activity of the proteins was checked with the ELISA method of Vidal et al. (2005) with some modifications. Because the primary antibody for ovomucin was not available, ELISA was done only for ovalbumin, ovotransferrin, and lysozyme. Standard curves were prepared using the standards purchased (lyso-

zyme from Sigma-Aldrich, ovotransferrin and ovalbumin from Abcam). Each of the standards and separated proteins were dissolved in a carbonate buffer (pH 9.6) and diluted to the final concentration of 10 μg/mL. For each protein, 100 μL of the diluted standard or sample were coated onto a microplate and incubated overnight at 4°C. The microplate wells were washed with PBS Tween 20 (200 μL) twice and blocked with 5% skim milk (200 μL) for 2 h at room temperature. The wells were washed again with PBS Tween 20 twice, incubated with 100 μL of primary antibody (1:10,000, Abcam) dissolved in 5% skim milk, incubated for 2 h at room temperature in the dark, and then washed with PBST 4 times. After removing incubation solution, 100 μL of substrate (ABTS substrate) was added and the samples were kept at room temperature for 30 min in the dark. The reaction was stopped by adding 0.1 *M* citric acid and the absorbance was measured at 416 nm using a microplate reader (xMark, Bio-Rad). A secondary antibody (rabbit anti-mouse IgG (H^+L) conjugated AP[12] (Abcam) was used for ovotransferrin and ovalbumin after diluting 1:10,000 ratio.

Statistical Analysis

The separation protocol was replicated 3 times, and data were analyzed using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA). Differences in mean values were compared by 1-way ANOVA using Minitab 16.0 (State College, PA).

RESULTS AND DISCUSSION

Lysozyme Separation

Carboxymethyl cellulose was used first by Strang (1984) to separate lysozyme from egg white. However, because of its fine granule sizes and low efficiency, separation of lysozyme from egg white using CMC was not easy especially in large scale. Therefore, Amberlite FPC 3500, a cation exchange chromatography resin with different granule size and ion exchange capacity, was used to separate lysozyme from egg white. When Amberlite FPC3500 resin was used, either batch or column method could be used to separate lysozyme from egg, but batch method was used in this study. For the batch system, the resin was directly added to the diluted egg white solution. Batch method was easy, fast, and compatible for large-scale production of lysozyme from egg white. The yield of lysozyme from egg white using Amberlite FPC 3500 was around 90% and the purity over 95% purity (Table 1). No pretreatment, except for 1:1 dilution of egg white solution, was used because the pH of fresh egg white is around 9.0 to 9.3, which was similar to the optimal pH conditions for trapping lysozyme from egg white using the Amberlite FPC 3500 resin. Compared with the ammonium sulfate separation (Alderton et al., 1945), CMC chromatography (Strang, 1984), β-mercaptoethanol with thermal treatment (Chang et al., 2000), ultrafiltration (Wan et al., 2006), and magnetic cation exchange chromatography (Safarik et al., 2007), the use of Amberlite FPC 3500 resin was much easier, simpler, and more efficient than egg white. Amberlite FPC 3500 performed better than CMC: with CMC, only part of the lysozyme in the egg white was trapped and a significant amount of lysozyme was still remaining in egg white solution (data not shown). Another associated issue of CMC resin was that the fine particles of CMC floated on the surface of the liquid and resulted in loss of the resin during the equilibration and washing steps for scale-up study. The Amberlite FPC 3500 resin had larger granules (0.3–1.18 mm vs. 25–60 μm in diameter), which made it easier to handle, had higher ion exchange capability (>2.6 vs. 1.0 mEq/mL), and lower price than CM cellulose resin. CM-Toyopearl 650M cation exchange resin also had small bead size and had much lower ion exchange capacity but much higher price than Amberlite FPC 3500 resin (0.24 vs. 2.6 mEq/mL). Thus, Amberlite FPC 3500 is superior cation exchange resin for lysozyme separation from egg white to other resins.

Because no chemical was added to egg white, the proteins in the lysozyme-free egg white solution had no physical or chemical changes. Thus, separation of lysozyme using FPC 3500 resins was selected as the first step for the sequential separation of multiple egg white proteins. After removing lysozyme, the resin could be easily regenerated by washing the resin 2 to 3 times with 5 volumes of 3% HCl, rinsing 2 to 3 times with 10 volumes of distilled water, and then equilibrating with 0.1 *M* glycine-NaOH buffer, pH 9.0 to 9.3, before using it again.

Table 1. Yield and purity of ovalbumin, lysozyme, ovotransferrin, and ovomucin using the sequential separation method[1]

	Laboratory scale			Large scale			
Sample	Weight (g)	Yield (%)	Purity (%)	Weight (g)	Yield (%)	Purity (%)	Activity (10 μg/mL)
Ovalbumin	16.240 ± 0.75	98.54 ± 4.47	94.2	213.23 ± 2.67	99.99 ± 1.24	88.2	9.85 ± 0.11
Lysozyme	0.96 ± 0.02	89.72 ± 1.93	96.5	12.27 ± 0.50	88.72 ± 3.61	96.2	9.86 ± 0.55
Ovotransferrin	3.07 ± 0.17	83.39 ± 5.33	96.2	39.36 ± 1.29	83.01 ± 2.69	94.3	9.77 ± 0.23
Ovomucin	1.08 ± 0.05	100.93 ± 2.25	82.2	13.95 ± 0.14	100.87 ± 2.69	82.2	ND[2]

[1]The calculated amount of egg white proteins in egg white solution (total egg white proteins in egg white was 10.95%). Laboratory scale (280 g of egg white): ovalbumin, 16.48 g; ovotransferrin: 3.66 g; lysozyme, 1.07 g; ovomucin, 1.07 g. Large scale (3,625 g of egg white): ovalbumin, 213.38 g; ovotransferrin, 47.42 g; lysozyme, 13.83 g; ovomucin, 13.83 g). n = 3.

[2]Not determined.

Separation and Solublization of Ovomucin

Ovomucin has a tendency to bind with other proteins, especially lysozyme. Therefore, removal of lysozyme from egg white solution at the first step can help increasing the purity of ovomucin. Centrifugation with different buffers and pH conditions was practiced by many researchers to separate ovomucin from egg white. The main reason for using pH adjustment and centrifugation to separate ovomucin is that it can be easily precipitated by adjusting pH close to its pI ranges (pH 4.5–5.0). Robinson and Monsey (1975) used isoelectric precipitation of ovomucin with Tris-HCl buffer and centrifugation at 35,000 × *g* and others (Omana and Wu, 2009a) used a combination of low concentration NaCl (100 m*M*) and high-speed centrifugation (11,200 × *g*).

In our sequential separation protocol, we have used isoelectric precipitation of ovomucin by adjusting the pH of egg white to 4.75. Brining the pH of egg white down to pH 4.5 to 5.0 neutralized most of the charges of ovomucin and facilitated the separation of ovomucin from the rest of the proteins. The precipitated ovomucin was easily separated by centrifugation at 3,400 × *g* for 30 min at 4°C and did not require high-speed centrifugation. Although the pI of ovalbumin (pH 4.5) is similar to that of the ovomucin, only a minimal amount of ovalbumin was precipitated along with ovomucin. On the other hand, ovomucin is easily separated due to its large molecular weight once it reaches its pI value. Most of the coprecipitated ovalbumin and other proteins trapped in ovomucin coagulates were washed off by homogenizing and centrifuging the ovomucin precipitant.

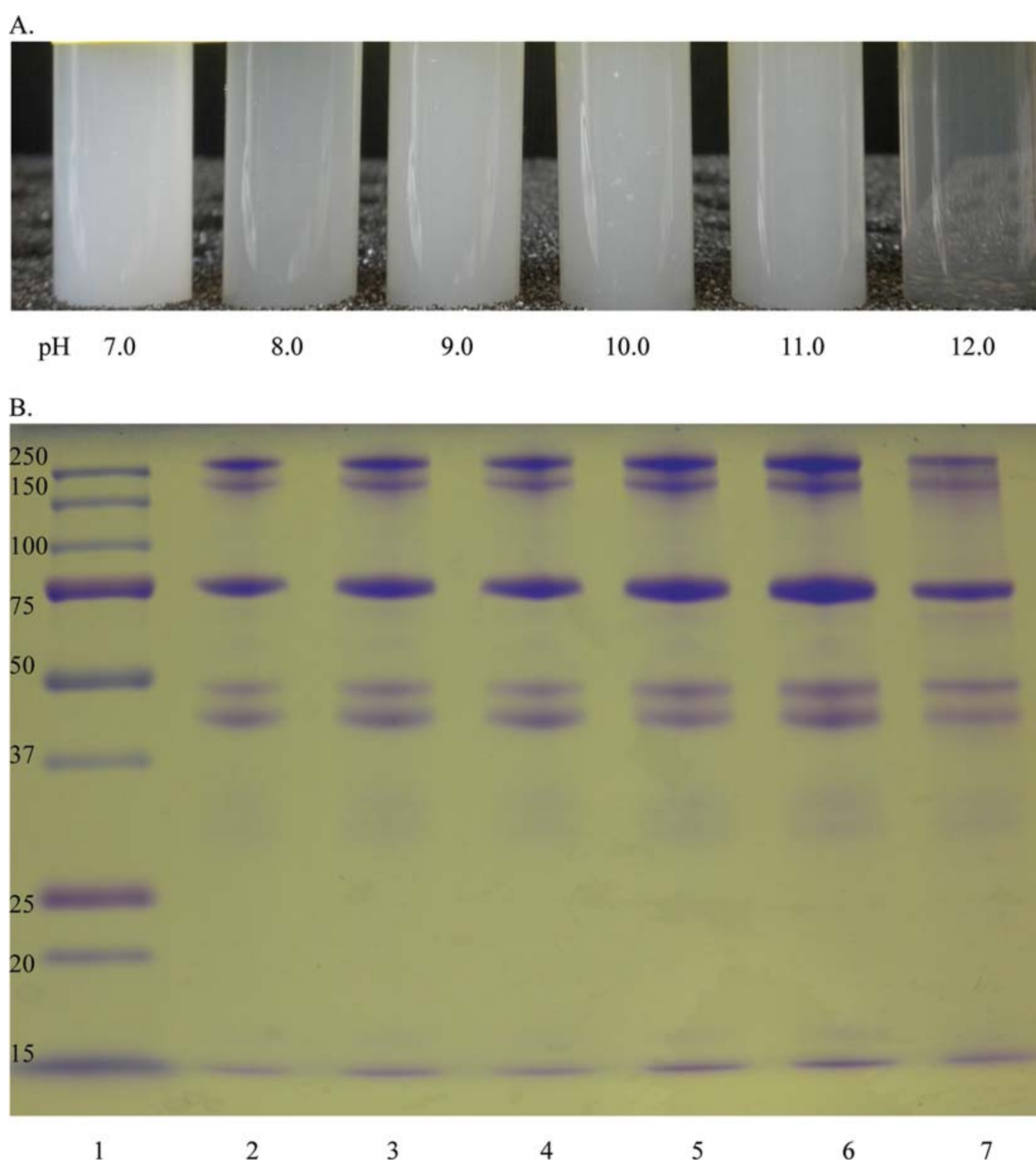

Figure 1. The SDS-PAGE of egg white proteins collected over the sequential separation steps. Lane 1 = marker, lane 2 = egg white, lane 3 = egg white after removing lysozyme, lane 4 = lysozyme separated, lane 5 = egg white after removing lysozyme and ovomucin, lane 6 = ovomucin dissolve at pH 12.0, lane 7 = supernatant after ammonium sulfate and citric acid precipitation, lane 8 = separated crude ovotransferrin, lane 9 = supernatant after removing ovotransferrin, lane 10 = purified ovotransferrin, lane 11 = purified ovalbumin after heat treatment. Color version available in the online PDF.

Thus, the separation of ovomucin from egg white can be done in large scale as well as in laboratory scale because only pH adjustment and a low-speed centrifugation is required. Ovomucin is not soluble in water, but it could be solubilized by adjusting the pH to alkaline conditions (pH 12.0, Figure 1a). Burgess and Deutscher (2009) reported that alkaline conditions increased the solubility of ovomucin by interacting with disulfide bonds. Even though ovomucin bands were seen in all SDS-PAGE gel tested (pH 7–12), the ovomucin was not completely soluble until the pH reached to 12.0 (Figure 1a and 1b). Because there is no commercially available ovomucin standard and anti-ovomucin antibody to identify ovomucin using western blotting, only SDS-PAGE results were shown. Previous studies reported 6 to 8 bands on a SDS-PAGE gel when ovomucin was dissolved in alkaline conditions (Huopalahti et al., 2007). Figure 1b showed 6 bands in SDS-PAGE as reported by Huopalahti et al. (2007). The molecular weight of α-ovomucin varies from 150 to 220 kDa, whereas β-ovomucin varies from 400 to 523 kDa (Omana et al., 2010b). The multiple bands shown in SDS-PAGE gel should be the products of the 2 subunits during the sample preparation, which uses β-mercaptoethanol for the SDS-PAGE. Previous studies showed that ovomucin can also be dissolved using urea and SDS (Omana et al., 2010b). After separating ovomucin from egg white, the remaining supernatant was used to separate other proteins in the subsequent steps.

Separation of Ovotransferrin and Ovalbumin

Hopkins (1900) used high levels of ammonium sulfate under acidic conditions to precipitate ovalbumin and separate it from egg white. Our protocol also uses ammonium sulfate in acidic conditions, but our approach is different from that of Hopkins. Instead of using high levels of ammonium sulfate (saturated) in acidic conditions to precipitate ovalbumin, we have used low levels of ammonium sulfate and citric acid combination to precipitate ovotransferrin and leave the rest of the egg white proteins including ovalbumin soluble. The addition of citric acid helped precipitation of ovotransferrin by ammonium sulfate probably because citric acid lowered the pH of egg white solution toward the pI values (6.0) of ovotransferrin. Our previous studies showed that a 5.0% (wt/vol) ammonium sulfate and 2.5% (wt/vol) citric acid combination showed an excellent capability in precipitating ovotransferrin from egg white solution (Abeyrathne et al., 2013). However, along with the ovotransferrin, a significant amount of ovalbumin (about 35% of total precipitated proteins) was also precipitated. To increase the purity of ovotransferrin and to recover some of the ovalbumin coprecipitated with ovotransferrin, the precipitant mainly containing ovotransferrin was dissolved with water and reprecipitated using a lower level of ammonium sulfate (2.0% wt/vol) and citric acid (1.5% wt/vol) combination.

The supernatants collected from the second ovotransferrin precipitation (S2) was pooled with the first supernatant (S1) from the 5.0% (wt/vol) ammonium sulfate and 2.5% (wt/vol) citric acid precipitation and used for ovalbumin separation. The pooled supernatant was mainly composed of ovalbumin, but some impurities remained in the solution. To remove the impurities, the pooled supernatant was subjected to heat treatment after desalting because ovalbumin has higher thermal resistance than ovotransferrin, the major impurities remaining in the supernatant (Stadelman and Cotterill, 2001). The heat treatment results indicated that heating the pooled, desalted supernatant at 70°C for 15 min removed most of the impurities (Figure 2, lane 11), indicating that 2-step, low concentrations of ammonium sulfate and citric acid combinations efficiently separated ovalbumin and ovotransferrin from the lysozyme-free and ovomucin-free egg white solution. This method used a much smaller amount of ammonium sulfate compared with the Hopkins (1900) method. Western blot results in Figure 3 confirmed the proteins separated as ovalbumin, lysozyme, and ovotransferrin.

Yield and Purity of Lysozyme, Ovomucin, Ovotransferrin, and Ovalbumin

In both laboratory scale and scale-up preparations, the yield of ovalbumin 98 to 99%, lysozyme was 91 to 92%, ovomucin >100%, and ovotransferrin 82 to 83% (Table 1), which was much higher than that of the Tankrathok et al. (2009; lysozyme 55%, ovotransferrin 21%, and ovalbumin 54%). However, no separation method can produce 100% yield even though there are some impurities in each of the separated proteins. The exceptionally high yield for ovalbumin and >100% yield for ovomucin could be attributed to the following: our proximate analysis of egg white (moisture 86.53%, protein 10.95%, carbohydrate 1.77%, and ash 0.75%) indicated that the carbohydrate content was about 1% higher than the reported value (Stadelman and Cotterill, 2001). All egg white proteins are glycoproteins, but the content of carbohydrate in ovomucin was higher than other egg white proteins. Also, the sum of individual protein content in egg white used for calculation in this study was 95.8% (Stadelman and Cotterill, 2001), indicating that protein content, especially that of ovalbumin and ovomucin could have been underestimated. The purity of lysozyme and ovotransferrin from the laboratory-scale preparation analyzed using the ImageJ software was >96% and that of ovalbumin was over 94%. The purity of lysozyme in large-scale preparation remained at 96%, but that of the ovotransferrin was 94%. The purity of ovalbumin with scale-up preparation was 88%, which were slightly lower than that with laboratory-scale preparation. The purity of ovomucin for both small- and large-scale preparations was 85%. Although a sequential separation protocol for ovalbumin, ovomucin, ovotransferrin, and lysozyme from egg

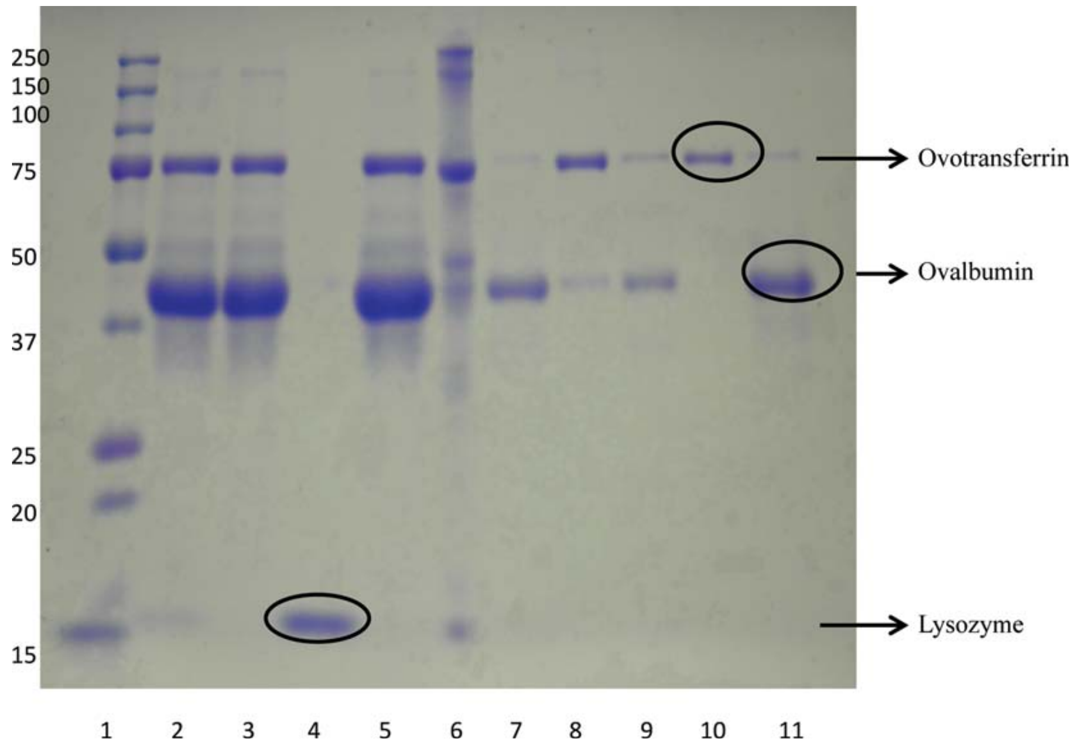

Figure 2. a) Effect of pH on the solubility of ovomucin. Tube 1 = pH 7.0, tube 2 = pH 8.0, tube 3 = pH 9.0, tube 4 = pH 10.0, tube 5 = pH 11.0, and tube 6 = pH 12.0. b) Effect of pH on the SDS-PAGE of ovomucin. Lane 1 = marker, lane 2 = pH 7.0, lane 3 = pH 8.0, lane 4 = pH 9.0, lane 5 = pH 10.0, lane 6 = pH 11.0, and lane 7 = pH 12.0. Color version available in the online PDF.

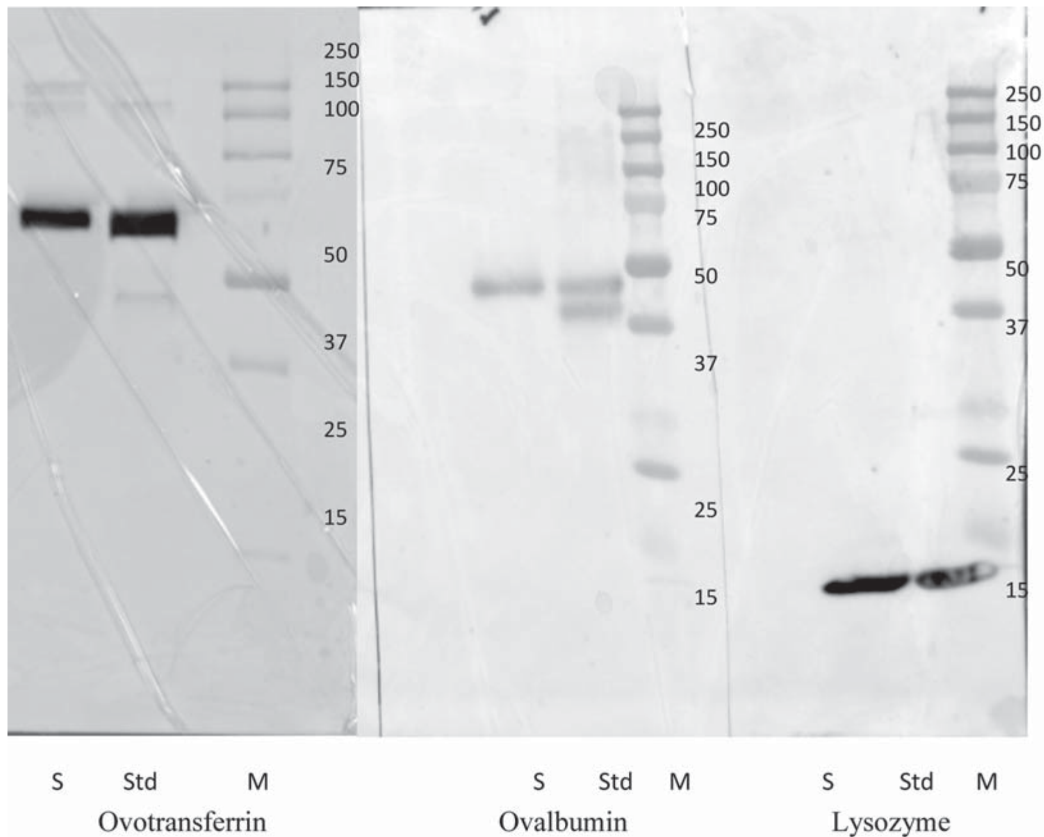

Figure 3. Western blot pictures of ovotransferrin, ovalbumin, and lysozyme. M = marker, S = purified protein, Std = standard protein.

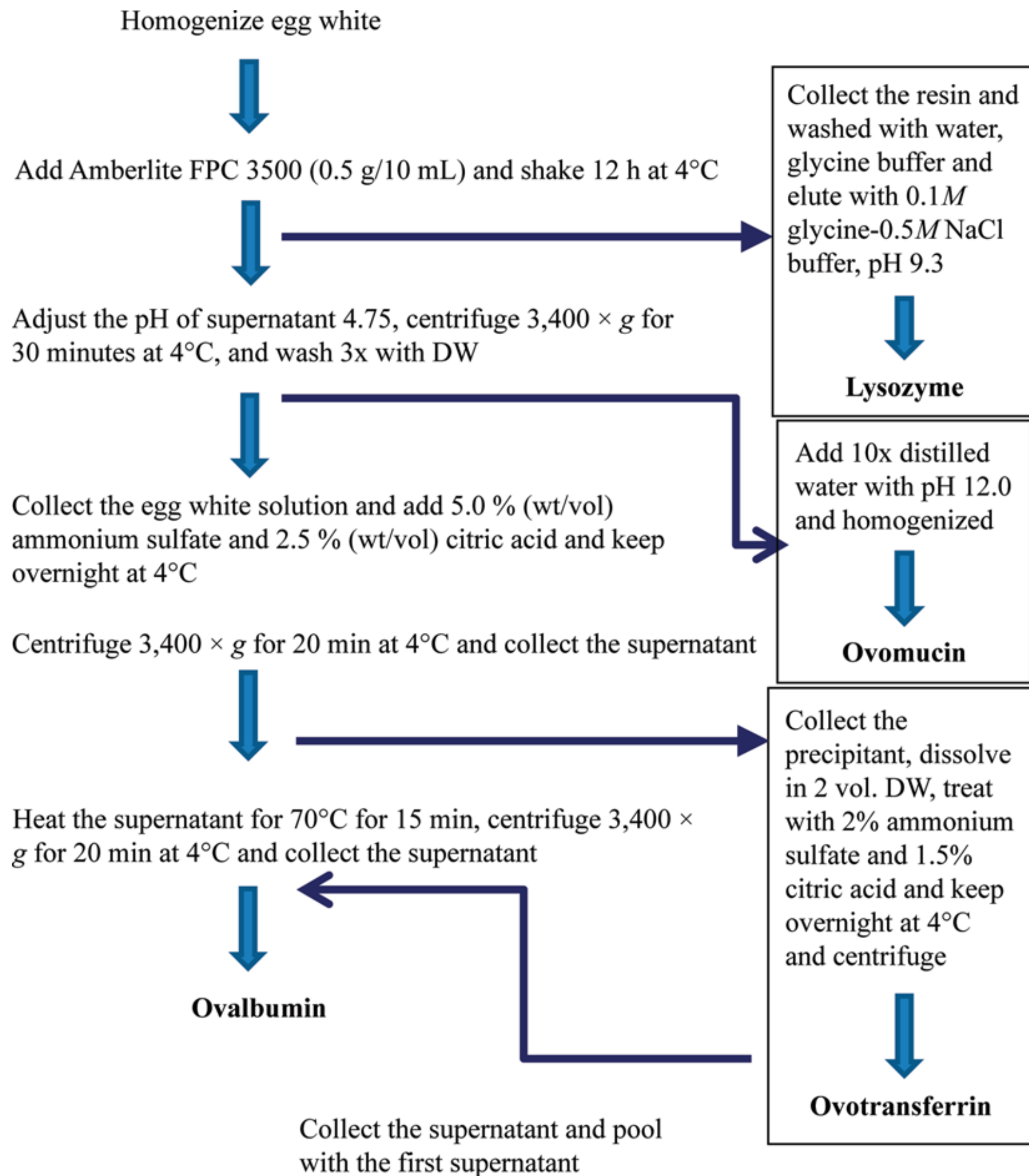

Figure 4. Schematic diagram for the separation of lysozyme, ovotransferrin, ovomucin, and ovalbumin from egg white. DW = distilled water. Color version available in the online PDF.

white using counter-current chromatography has been reported (Shibusawa et al., 1998, 2001), scaling up was not practical. There had been several separation methods developed for individual as well as multiple proteins from egg white (Vachier et al., 1995; Tankrathok et al., 2009; Geng et al., 2010), but this protocol is the first one that showed the separation of the 4 major egg white proteins in sequence is possible in both laboratory and large-scale processes. Figure 4 is the final protocol for the sequential separation of lysozyme, ovomucin, ovotransferrin, and ovalbumin from egg white. Lysozyme and ovomucin could be separated within 2 d and ovotransferrin and ovalbumin within the next 2 d. Therefore, all 4 proteins could be separated within 4 d even in large-scale preparation.

In conclusion, lysozyme, ovomucin, ovalbumin, and ovotransferrin can be separated in sequence using a combination of cation exchange chromatography, isoelectric precipitation, ammonium sulfate and citric acid precipitation, and heat treatment. This protocol separated the 4 major egg white proteins with >98% yield and >85% purity of ovalbumin; >88% yield and >95% purity of lysozyme; >80% yield and >90% purity of ovotransferrin, and >99% yield and >80% purity of ovomucin. Also, ovotransferrin, ovalbumin, and lysozyme showed over 95% activity.

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