

Sequential Separation of Lysozyme, Ovomucin, Ovotransferrin and Ovalbumin from Egg White

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Summary and Implications

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 a 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 twice using $(\text{NH}_4)_2\text{SO}_4$ and citric acid combination. After centrifugation, the supernatants were used for ovalbumin separation. The precipitants were used as ovotransferrin fraction, and the supernatant was desalted using ultrafiltration, and then heat-treated to remove impurities. The yield of ovomucin and ovalbumin was > 98% and that of ovotransferrin and lysozyme was > 82% for both laboratory and scale-up preparations. SDS-PAGE and Western Blotting of the separated proteins, except for ovomucin, showed > 90% purity and the activities of separated ovalbumin, ovotransferrin, and lysozyme were > 96%. The protocol separated four major proteins in sequence, and the method was simple and easily scaled-up. The separated proteins can be used as functional components.

Introduction

Ovalbumin (54%), ovotransferrin (12%), ovomucin (3.5%) and lysozyme (3.4%) are among the major proteins in egg white. 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 and is widely used as a standard protein in protein assays, and important in immunological and nutritional studies. Albumin was first separated using saturated ammonium sulfate and acetic acid, but no clear record about the purity and yield is available. Recently, ovalbumin was separated using two-stage polyethersulfone (PES) flat disk membranes, electrophoretic

method, foam fractionation, or liquid chromatographic method. 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 known to bind and transport irons in animal body, and thus can be used as an antimicrobial, antioxidant or an iron supplementing agent. 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. Recently, ovotransferrin was produced in large scale by ethanol precipitation. However, using ethanol has some limitations because it denatures rest of the proteins, which makes it difficult to use the remaining proteins.

Another important protein found in egg white is lysozyme. 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 commonly used to trap lysozyme. However, due to its fine granule sizes, handling is difficult and flow rate is very slow for column chromatography. Lysozyme is one of the first egg white proteins which was isolated and used in industry. It has the ability to control limited spectrum of bacteria and fungi which cause spoilage of food.

Ovomucin is responsible for the gel like properties of thick egg white. Various approaches including isoelectric precipitation, dual-column gel filtration, gel electrophoresis, β -mercaptoethanol, calcium chloride, dual-column gel filtration, gel electrophoresis, and NaCl + pH adjustment also have been tested. 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. Also, the separated lysozyme, ovotransferrin and ovalbumin in sequence had low yield and purity, and could not 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 Method

Separation of lysozyme: Separation of lysozyme was done with cation exchange chromatography. Amberlite FPC 3500 resin was used as the cation exchange resin. The lysozyme trapped to the resin was eluted with 0.1 M glycine-NaOH

buffer, pH 9.3, containing 0.5M NaCl. The eluent was desalted using an ultrafiltration unit and then freeze-dried.

Separation of ovomucin: The lysozyme-free egg white (from the first step) was adjusted to pH 4.75 to precipitate ovomucin and centrifuged. The precipitated ovomucin was homogenized, washed several times and then freeze dried.

Separation of ovotransferrin and ovalbumin: Ovotransferrin and ovalbumin was separated by precipitating ovotransferrin using ammonium sulfate and citric acid combinations twice. After centrifugation, the precipitant and supernatant were collected. The supernatant was used for ovalbumin and precipitant for ovotransferrin separation. The precipitant was dissolved in distilled water, desalted using an ultrafiltration unit, and then lyophilized. The supernatant was desalted and concentrated using an ultrafiltration unit, and heated at 70 °C for 15 min to precipitate impurities. After removing the precipitant by centrifugation the final supernatant containing ovalbumin was lyophilized as above.

Yield and purity calculation: The yields of lysozyme, ovomucin, ovotransferrin and ovalbumin were calculated using their theoretical values in egg white. To check the separation efficiency, SDS-PAGE was conducted under reduced conditions using Mini-Protein II cell. The purity of protein was calculated by converting the density of protein bands in the gel picture using the ImageJ software 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. The activity of the proteins was checked with the ELISA method. As the primary antibody for ovomucin was not available, ELISA was done only for ovalbumin, ovotransferrin and lysozyme.

Statistical analysis: Separation protocol was replicated 3 times and data were analyzed using Microsoft EXCEL2010. Differences in mean values were compared by one-way analysis of variance (ANOVA) using MINITAB 16.0.

Results and Discussion

Lysozyme separation: Amberlite FPC 3500, a cation exchange chromatography resin was used to separate lysozyme from egg white. Batch method was used because it is easy, fast and compatible for large scale production of lysozyme from egg white. The resin was directly added to the diluted egg white solution. The yield of lysozyme from egg white using Amberlite FPC 3500 was around 90% and the purity over 95% purity (Table 1). No pre-treatment, except for 1:1 dilution of egg white solution was used because the pH of fresh egg white is around 9.0-9.3 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,

CMC chromatography, β -mercaptoethanol with thermal treatment, ultrafiltration, and magnetic cation exchange chromatography, the use of Amberlite FPC 3500 resin was much easier, simpler and more efficient. Since 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.

Separation and solubilization of ovomucin: Ovomucin has a tendency to bind with other proteins specially lysozyme. Therefore, removal of lysozyme from egg white solution at the first step can help increasing the purity of ovomucin. In our sequential separation protocol, we have used iso-electric 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 x g for 30 minutes and did not require high-speed centrifugation. After separating ovomucin from egg white, the remaining supernatant was used to separate other proteins in the subsequent steps.

Separation of ovotransferrin and ovalbumin: 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 rest of the egg white proteins including ovalbumin soluble. 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.

The supernatants collected were pooled and subjected to heat treatment after desalting because ovalbumin has higher thermal resistance than ovotransferrin, the major impurities remaining in the supernatant. The heat treatment results indicated that heating the pooled, desalted supernatant at 70 °C for 15 min removed most of the impurities (Figure 1, lane 11), indicating that a two-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 much smaller amount of ammonium sulfate compared with other's works. Western Blot results in Figure 2 confirmed the proteins separated as ovalbumin, lysozyme and ovotransferrin.

Yield and purity: In both laboratory scale and scale up preparations, the yield of ovalbumin 98-99%, lysozyme was 91-92%, ovomucin > 100%, and ovotransferrin 82-83% (Table 1), which was much higher than that of the other's. 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%. There had been several separation methods developed for individual as well as multiple proteins from egg white, but this protocol is the first one that showed the separation of the four major egg white proteins in sequence is possible in both laboratory and large scale processes. Lysozyme and ovomucin could be separated within 2 days and ovotransferrin and ovalbumin within next two days. Therefore, all four proteins could be separated within 4 days even in large scale preparation.

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 four 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.

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

Sample	Laboratory Scale			Large Scale			
	Weight (g)	Yield (%)	Purity (%)	Weight (g)	Yield (%)	Purity (%)	Activity (10 µg/ml)
Ovalbumin	16.24	98.54	94.2	213.23	99.99	88.2	9.85
Lysozyme	0.96	89.72	96.5	12.27	88.72	96.2	9.86
Ovotransferrin	3.07	83.39	96.2	39.36	83.01	94.3	9.77
Ovomucin	1.08	100.93	82.2	13.95	100.87	82.2	ND 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, 16.48g; 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.
¹Not determined

Figure 1: 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.

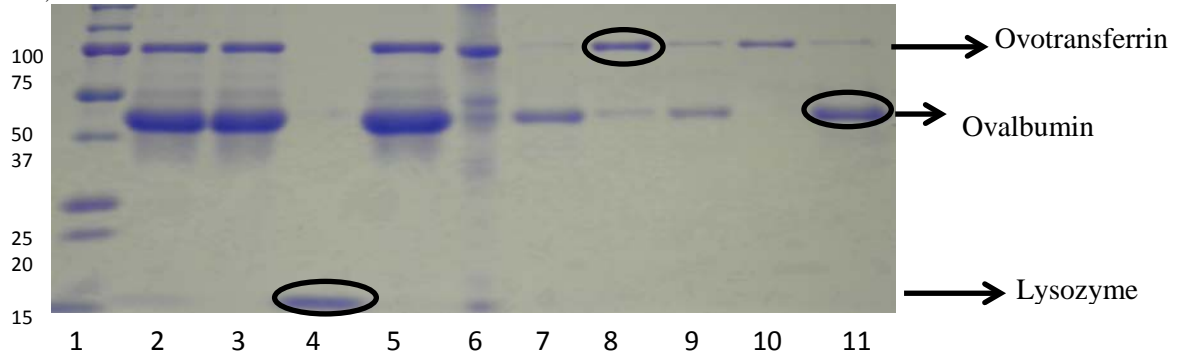


Figure 2: Western blot pictures of ovotransferrin, ovalbumin and lysozyme, M = Marker, S = Purified protein, Std = Standard protein

