

# Separation of Ovotransferrin and Ovomuroid from Chicken Egg White

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### Summary and Implications

Ovotransferrin and ovomucoid were separated using two methods after extracting ovotransferrin- and ovomucoid-containing fraction from egg white. Diluted egg white (2x) was added with  $\text{Fe}^{3+}$  and treated with 43% ethanol (final conc.). After centrifugation, the supernatant was collected and treated with either a high-level ethanol (61%, final conc.) or an acidic salt combination (2.5% ammonium sulfate and 2.5% citric acid) to separate ovotransferrin and ovomucoid. For the high-level ethanol method, ovotransferrin was precipitated using 61% ethanol. After centrifugation, the precipitant was dissolved in 9 vol. distilled water and the residual ethanol in the solution was removed using ultrafiltration. The supernatant, mainly containing ovomucoid, was diluted with 4 vol. water, ethanol removed, concentrated, and then used as ovomucoid fraction. For the acidic salt precipitation method, the ethanol in the supernatant was removed, first. The ethanol-free solution was concentrated and treated with 2.5% ammonium sulfate and 2.5% citric acid combination. After centrifugation, the precipitant was used as ovotransferrin and the supernatant as ovomucoid fraction. The ovomucoid fraction from both of the protocols was further purified by heating at 65 °C for 20 min and the impurities were removed by centrifugation. The yields of ovomucoid and ovotransferrin were > 96% and > 92%, respectively. The purity of ovomucoid was > 89% and that of the ovotransferrin was > 88% purity. ELISA results confirmed that the activity of the separated ovotransferrin was > 95%. Both of the protocols separated ovotransferrin and ovomucoid effectively and the methods were simple, fast and easy to scale-up.

### Introduction

Ovotransferrin (12%) and ovomucoid (11%) are two major functional proteins found in egg white. Ovotransferrin can be present in two forms, apo- (iron free) and holo- (iron bound), and are significantly differ in their chemical and physical properties. Holo-form is more resistant to chemical and physical conditions than apo-form. Ovotransferrin is known to have a strong iron binding capability, and thus can be used as an antioxidant, antimicrobial or iron supplementing agent. Different approaches have been used

to separate ovotransferrin from egg white: a high-level ammonium sulfate, SDS-PAGE and immunoelectrophoresis, Carboxymethyl (CM)-Toyoperal 650M cation exchange, and anion exchange chromatography have been used in the past. However, these methods were for laboratory scale and were not practical for scale-up processes.

Ovomucoid is the most highly glycosylated protein among the egg white proteins. It is well known as “trypsin inhibitor” and is considered as the main food allergens present in egg white. Ovomuroid binds with trypsin at 1:1 ratio, and its 3-D structure is secured with the 3 disulfide bonds in it. Ovomuroid was separated using various approaches including trichloroacetic acid (TCA) and acetone or TCA and ethanol combination, SDS-PAGE, ethanol, chromatographic method, but the recovery of ovomucoid ranged 25% - 70%. Most of the methods were for the separation of single protein from egg white and were in laboratory scale.

A few other separation protocols for more than one protein from egg white have been developed. However, none of the sequential separation methods were used for the large scale separation of the egg white proteins. The objective of this study was to develop simple, economical and scale up methods to separate ovotransferrin and ovomucoid using the supernatant from the 43% ethanol precipitation of egg white proteins developed by Ko and Ahn (2008).

### Materials and Method

Egg white was manually separated from yolk, diluted with one vol. of distilled water, added with  $\text{Fe}^{3+}$  to saturate ovotransferrin with iron, and then homogenized using a hand mixer for 2 min at high speed. The proteins in the diluted egg white, except for ovotransferrin and ovomucoid, were precipitated by slowly adding 100% ethanol to the final concentration of 43%, and centrifuged at 3,400 x g for 20 min at 4 °C. The precipitant was re-extracted with 2 vol. of 43% ethanol and centrifuged again. The two supernatants containing ovotransferrin and ovomucoid were pooled and used as the starting material for the separation of ovotransferrin and ovomucoid using the following two approaches: The first method was treating the supernatant with 2.5% (w/v) ammonium sulfate and 2.5% (w/v) citric acid combination (pH 3.0). Ammonium sulfate and citric acid combinations tested were from 2.5% to 10% (v/w) at 2.5% interval, and the final concentrations selected were 2.5% (v/w) each. Ethanol in the supernatant was removed using ultrafiltration, and then 2.5% (w/v) ammonium sulfate and 2.5% (w/v) citric acid combination was slowly added to the ethanol-free supernatant to precipitate ovotransferrin. After held overnight in a 4 °C cold room, the sample was

centrifuged at 3,400 x g for 20 min at 4 °C. To collect ovotransferrin, the precipitant was dissolved with 4 vol. of distilled water with adjusting the pH to 9.0 using NaOH, desalted and concentrated using ultrafiltration, and then lyophilized. The supernatant was mainly consisted of ovomucoid, but contained some ovotransferrin. After desalting, the supernatant was adjusted to pH 4.0 using citric acid, heat-treated at 65 °C for 20 min, centrifuged at 3,400 x g for 20 min at 4 °C to remove the denatured ovotransferrin, and then lyophilized. The two protocols were tested for both in laboratory and large scale separation of ovotransferrin and ovomucoid.

The second approach used 43% ethanol to extract ovotransferrin from the white. The ethanol concentration of the extract was increased to 61% (final) by slowly adding 100% ethanol. The solution was kept overnight at 4 °C and then centrifuged at 3,400 x g for 20 min at 4 °C. The precipitant was collected, dissolved with 10 vol. of distilled water, and the residual ethanol was removed using an ultrafiltration unit. The supernatant was subjected to ultrafiltration, heat-treated to remove residual ovotransferrin, and then lyophilized as mentioned above. The yields of ovotransferrin and ovomucoid were calculated by comparing the amount of separated proteins after freeze drying with the calculated values of each protein in egg white. To check the separation efficiency and yields, SDS-PAGE was conducted under reduced conditions using Mini-Protein II cell. To check the purity of ovotransferrin and ovomucoid, gel pictures were taken after distaining. The purity of proteins was calculated by converting the density of protein bands in the gel picture using the ImageJ software. Western Blot was used to confirm ovotransferrin. ELISA assay was used to check the activity of purified ovotransferrin.

### Results and Discussion

*Separation of ovotransferrin and ovomucoid:* The holo-form of ovotransferrin is more stable to chemical and pH changes than the iron-free (apo) form. Earlier, ovotransferrin has been separated from egg white using 43% ethanol after converting apo-ovotransferrin to holo-ovotransferrin. The same strategy was used to separate ovotransferrin from egg white. With 43% of ethanol (final concentration), all the egg white proteins, except for ovotransferrin and ovomucoid, were denatured and (Figure 3, Lane 3) precipitated. After centrifugation, ovotransferrin and ovomucoid in the supernatant fraction was separated using 2 strategies: one with ammonium sulfate and citric acid combination and the other with a high-level ethanol (61%, final conc.). Each of the method has advantages and disadvantages in separating ovotransferrin and ovomucoid from the supernatant. Separation of ovotransferrin using ammonium sulfate and citric acid combination requires an additional step to remove ethanol from the supernatant

before the acidic-salt precipitation, but the purity of the ovotransferrin separated was better than that with the high-level ethanol precipitation (Table 1). Precipitation of ovotransferrin with 61% ethanol denatured some of the ovotransferrin in the supernatant, but the protocol was simpler than the acidic-salt precipitation method. Both protocols took two days to separate the two proteins and both were applicable for scale-up production.

The purities of ovotransferrin and ovomucoid were not changed by increasing ammonium sulfate concentration from 2.5% to 7.5% (Figure 1) and 2.5% (v/w) ammonium sulfate concentration was selected as a salt concentration of the acidic salt method. Figure 1 indicated that the combination of 2.5% (w/v) ammonium sulfate and 2.5% (w/v) citric acid separated ovotransferrin from the supernatant, some ovotransferrin still remained in the supernatant (ovomucoid) fraction. The supernatant with the high-level ethanol precipitation of ovotransferrin also had some ovotransferrin and other impurities. Figures 1 and 2 indicated that crude ovomucoid solution after centrifugation had some ovotransferrin and other impurities (Fig. 1, Lane 3 and Fig. 2, Lane 2). In order to remove the residual proteins, crude ovomucoid solution was heat-treated. Ovomucoid is more heat-stable protein than ovotransferrin, and heating crude ovomucoid solution at 65 °C did not damage ovomucoid. In Figure 2, different heating times (15, 20, 25, and 30 min) at 65 °C were tested and 20 min was selected as a heating condition for both methods to improve the purity of ovomucoid.

The separated ovotransferrin can be used to produce functional peptides with growth-inhibiting effects on human cancer cell lines. Ovomucoid was reported as one of the main egg allergen present in egg white, but separated ovomucoid can be used to produce bioactive peptides that have metal-binding, antioxidant, anti-hypertensive and anti-microbial activities.

*Purity, yield and activity of the separated proteins:* The yield of ovotransferrin in small scale was 98% for ethanol separation and 95% for acid-salt precipitation method, whereas that of large scale was 98% and 92%, respectively. The yield of ovomucoid in small scale was over 100% in small scale in both methods while that in large scale was over 100% for ethanol precipitation protocol and > 96% for acidic ammonium sulfate method (Table 1). The purity of ovomucoid was 90-92% for ethanol precipitation method and 89-91% for acidic ammonium sulfate method, while that of ovotransferrin was 97-98% for ethanol precipitation protocol and 88-90% for acidic ammonium sulfate method (Table 1). The yield was calculated according to the calculated values using the literature values for ovotransferrin and ovomucoid and the actual protein content measured from egg white used in this study. The yield and purity results of our study suggested that the

amount of ovomucoid in egg white could be higher than the reported value. We have seen similar results with ovomucin in our previous study.

This work is different from previous works that used ethanol extraction and ammonium sulfate and citric acid combination, respectively, to separate ovotransferrin from egg white. The previous two methods separated only ovotransferrin while current protocols separated both ovotransferrin and ovomucoid in a single sequence. Compared with the other sequential separation, both of the current protocols produced much higher yields of ovomucoid and ovotransferrin (ovomucoid 21% vs 100% and ovotransferrin 21% vs 97%). Recently, a separation method for lysozyme, ovotransferrin, ovalbumin and ovoflavoprotein with the yield over 90% has been developed, but this method was not easy for scale-up production and is not practical due to sophisticated separation procedure used.

The two protocols (ethanol and acidic ammonium sulfate) were tested for both in laboratory and large scale. Both scales showed good separation of the two proteins. Difference in the yield between laboratory and large scale processes were mainly due to loss of the proteins during ultrafiltration, and showed that the methods can be used for large preparation of ovotransferrin and ovomucoid. Figure 3 shows the proteins during the separation steps of the two methods. According to the Figure 3 both proteins showed similar separation patterns. Figure 4 confirms the separated ovotransferrin with Western Blotting and ovomucoid with SDS-PAGE with relevant standards. The activity of the ovotransferrin separated using both of the protocols indicated that they have over 95%. The activity of ovotransferrin separated using the ethanol protocol showed 95.8% and that separated using the ammonium sulfate and

citric acid combination showed 97.8%, indicating that the ovotransferrin separated using both of the protocols maintained their activity. Even though this value was slightly lower than that of the previous studies the activity reported here are still very high. ELISA of ovomucoid was not working using currently available ovomucoid antibodies.

*Comparison of the two protocols:* Separating ovotransferrin and ovomucoid using ethanol was simple and fast, but the amount of ethanol needed is high. Ammonium sulfate and citric acid combination protocol uses less amount of ethanol and uses low levels of ammonium sulfate and citric acid to precipitate ovotransferrin, but needs an extra step to remove ethanol before the precipitation step. Heating is required for both protocols to increase the purity of ovomucoid. Both protocols produced ovotransferrin and ovomucoid with similar yield and purity but the ovotransferrin produced is “holo” form and should be converted to “apo” form if necessary.

### Conclusion

Ovomucoid and ovotransferrin can be easily separated in sequence from the supernatant of ethanol extraction using a high concentration of ethanol or ammonium sulfate and citric acid combination. The yield of the separated ovotransferrin and ovomucoid were > 92% and > 95%, respectively, the purity of the separated proteins ranged 88-98%, and the activity of the purified ovotransferrin using both of the protocols remained at > 95%. The protocols are simple, easy, and scalable to separate the two proteins in sequence.

**Table 1:** Yield and purity of ovotransferrin and ovomucoid using two sequential separation protocols

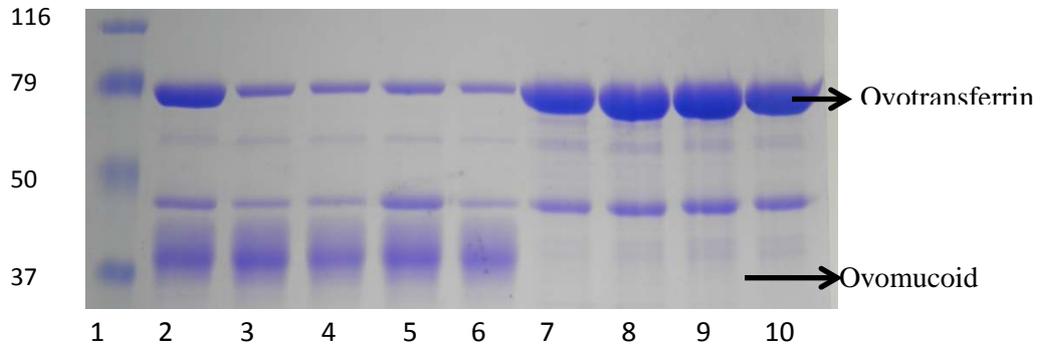
Sample	Small Scale			Large Scale		
	Weight (g)	Yield %	Purity (%)	Weight (g)	Yield %	Purity (%)
Ovotransferrin <sup>1</sup>	4.70	98.12	98.1	38.66	98.07	97.7
Ovomucoid <sup>1</sup>	4.41	100.46	92.3	36.29	100.44	90.16
Ovotransferrin <sup>2</sup>	4.56	95.20	90.25	36.64	92.94	88
Ovomucoid <sup>2</sup>	4.42	100.61	91.4	34.99	96.84	89.29

Calculated amount of egg white proteins in egg white solution (total egg white proteins in egg white was 10.95%). Laboratory scale (365 g of egg white): ovotransferrin, 4.79 g; ovomucoid, 4.39 g. Large scale (3,000 g of egg white): ovotransferrin 39.42 g; ovomucoid, 36.13 g. n=3.

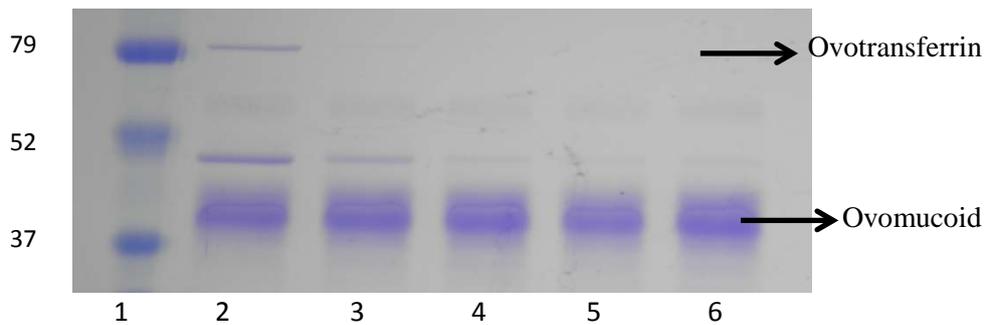
<sup>1</sup>Ovotransferrin and ovomucoid produced using ethanol precipitation protocol.

<sup>2</sup>Ovotransferrin and ovomucoid produced using acidic ammonium sulfate protocol.

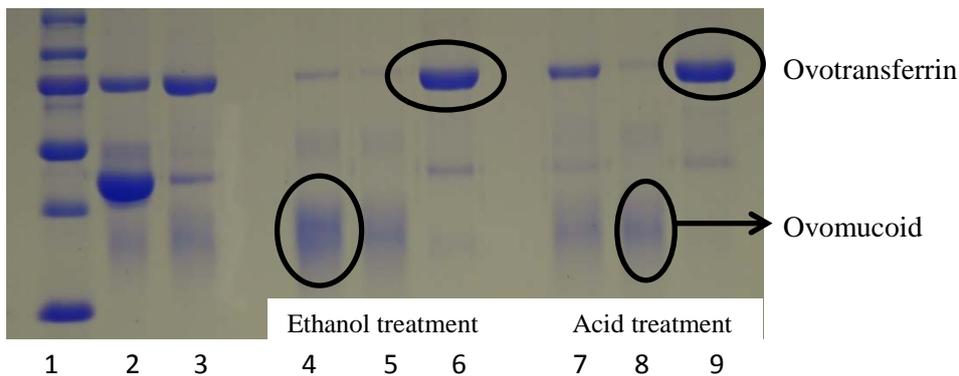
**Figure 1:** SDS-PAGE picture of the lower levels of ammonium sulfate (AS) and citric acid (CA) treatments for purifying ovotransferrin from ovomucoid. Lane 1 = Marker, Lane 2 = Crude ovomucoid and ovotransferrin, Lane 3 = Supernatant of AS 2.5 and CA 2.5, Lane 4 = Supernatant of AS 5.0 and CA 2.5, Lane 5 = Supernatant of AS 7.5 and CA 2.5, Lane 6= Supernatant of AS 10.0 and CA 2.5, Lane 7= Precipitant of AS 2.5 and CA 2.5, Lane 8 = Precipitant of AS 5.0 and CA 2.5, Lane 9 = Precipitant of AS 7.5 and CA 2.5, Lane 10 = Precipitant of AS 10.0 and CA 2.5



**Figure 2:** SDS-PAGE picture of the heating treatment to remove residual proteins at 65 °C temperature. Lane 1 = Marker, Lane 2 = Crude ovomucoid, Lane 3 = Supernatant after heating for 15 min, Lane 4 = Supernatant after heating for 20 min, Lane 5 = Supernatant after heating for 25 min, Lane 6= Supernatant after heating for 30 min



**Figure 3:** SDS-PAGE of egg white proteins collected over the sequential separation steps. Lane 1 = Marker, Lane 2 = Diluted egg white, Lane 3 = Supernatant after 43% ethanol addition, Lane 4 = Supernatant after 61% ethanol before heat treatment, Lane 5 = Ovomuroid from ethanol treatment, Lane 6 = Ovotransferrin from ethanol treatment, Lane 7 = Supernatant after 2.5% ammonium sulfate and citric acid and before heat treatment, Lane 8 = Ovomuroid after heat treatment, Lane 9 = Ovotransferrin after 2.5% ammonium sulfate and citric acid.



**Figure 4:** Western blot pictures of ovotransferrin and SDS-PAGE picture of ovomucoid. Mr = Marker, OTA = Ovotransferrin separated with ammonium sulfate and citric acid, OTE= Ovotransferrin separated with ethanol, St = Standard protein, OME= Ovomuroid separated using ethanol precipitation method, OMA= Ovomuroid from ammonium sulfate and citric acid precipitation method.

