INTRODUCTION

Ovotransferrin, a monomeric glycoprotein consisting of 686 amino acids and 15 disulfide bonds, was first characterized by Schade and Caroline in 1944 and was called Conalbumin. It was renamed as ovotransferrin after the finding that it can bind iron (Williams, 1968) and has the same amino acid sequence as the transferrin in human serum (Wu and Acero-Lopez, 2012). The molecular weight of ovotransferrin is 76 kDa and has an isoelectric point of 6.1 (Ko and Ahn, 2008). One ovotransferrin molecule can bind 2 iron molecules and transports iron into the body (Wu and Acero-Lopez, 2012). Ovotransferrin is found in two main forms: apo- (iron free) and holo- (iron bound). The chemical and physical properties of these two forms of ovotransferrin differ significantly (Wu and Acero-Lopez, 2012). Apo-form (iron free) is colorless, whereas holo-form (iron bound) has a salmon pink color. The holo-form is more resistant to chemical and physical conditions than apo-form. Iron ion (Fe^{3+}) can be easily attached to ovotransferrin at pH > 7.0, but is released at pH < 4.5 (Ko and Ahn, 2008). Ovotransferrin has similar functions to lactoferrin found in milk and both have iron scavenging and iron delivery functions (Abdallah and Chahine, 1999).

Over the past years, different techniques have been developed to separate ovotransferrin from chicken egg white, but most of the methods developed were laboratory scale. Ion exchange chromatography is among the most widely used method, but has limitations for scale up preparation because of the costs involved, and separation speed and yield issues (Awade et al., 1994;...
Croguennec et al., 2001; Guérin-Dubiard et al., 2005). CM-Toyopearl 650M cation exchange was used for ovo-
transferrin separation by Tankrathok et al. (2009), but the yield was low (21%). Anion exchange chromatogra-
phy was developed by Omama et al. (2010) and Wu and Acero-Lopez (2012) with an 80% yield, but the meth-
ods have difficulties in handling of resins and are not appropriate for large-scale separation of ovotransferrin
due to its complexity and use of Q-sepharose chromatography columns.

Recently, a large scale production of ovotransferrin was developed by Ko et al. (2008) who successfully
separated ovotransferrin from egg white. They have used 43% ethanol to precipitate all the proteins in egg
white except for ovotransferrin followed by 59% ethanol to precipitate ovotransferrin from the ovotransferrin-
containing supernatant fraction. Because ovotransferrin is highly susceptible to extreme pH and tempera-
ture conditions, the apo-form of ovotransferrin in egg albumin was converted to holo-form before treatment
with ethanol. After separating the holo-form of ovo-
transferrin, iron was released from ovotransferrin using pH adjustment and the released iron was removed
using AG1-X2 resin. However, the removal of the re-
leased iron from ovotransferrin requires an extra step
and raises the costs. Another common method used to
separate ovotransferrin from egg white is precipi-
tating it with ammonium sulfate (Fraenkel-Conrat and
Feeney, 1950). Using acidic conditions along with high
levels of ammonium sulfate, ovotransferrin was easily
separated. However the level of purity was not reported
and the amount of chemical added was very high, lead-
ing to a high separation cost. Affinity chromatography
(immobilized metal affinity chromatography) has also
been used to isolate ovotransferrin, but this method is
very difficult to scale up (Wu and Acero-Lopez, 2012).
The SDS-PAGE or immunoelectrophoresis can be used
to isolate ovotransferrin, but the main problems with
these methods are scaling up and denaturation of the
protein (Desert et al., 2001). Therefore, developing an
economical method to purify ovotransferrin in its natu-nal form and at a large scale is vital to use ovotrans-
ferin in the food and pharmaceutical industries. The
objective of this work was to develop a simple and easy
separation method of ovotransferrin (apo-form) from
egg white without using organic solvent.

**MATERIALS AND METHODS**

**Materials**

Chicken eggs (<7-d-old white eggs with average
weight of 60 g) were purchased from a local market.
Egg white from 10 eggs was pooled, and used as a rep-
lication. Each replication was prepared using 240 mL
of the pooled egg white, and 3 replications were prepared.
Ammonium sulfate, acetic acid, and citric acid were
purchased from Fisher Scientific (Thermo Fisher Scien-
tific Inc., Waltham, MA). Standard ovotransferrin was
purchased from Sigma-Aldrich (St. Louis, MO), and anti-ovotransferrin chicken antibody was purchased
from MyBioSource (San Diego, CA). All other stan-
dards and rabbit anti-mouse IgG (H+L)-conjugated AP
antibody were purchased from Bio-Rad (Bio-Rad Labo-
ratory Inc., Richmond, CA).

**Separation of Ovotransferrin**

Egg white (240 mL) was diluted with 1 volume of dis-
tilled water (DW), homogenized using a hand mixer,
the pH adjusted to 4.5 to 5.0 using 3 N HCl, and centri-
fuged at 3,400 × g for 30 min at 4°C. The supernatant
was collected and treated with different levels of am-
monium sulfate and acid combinations. Four different
levels of ammonium sulfate ranging from 2.5 to 10%
at 2.5% interval and 4 levels of citric acid from 2.0 to
3.5% at 0.5% interval were used. The samples were kept
overnight at 4°C and centrifuged at 3,400 × g for 20 min
at 4°C. The precipitant was measured with weighing
balance, dissolved with 2 volumes of DW, and then de-
salted using an ultrafiltration unit (Hollow fiber, 10,000
Da cut-off-size, Flexstand, GE Healthcare Bio-Sciences
Corp., Piscataway, NJ). Ovotransferrin was precipi-
tated from the solution by again adding various levels
of ammonium sulfate and acid combinations. Four dif-
ferent levels of ammonium sulfate ranging from 1.5 to
3% at 0.5% interval and 4 levels of citric acid from 1.0
to 2.5% at 0.5% interval were used. After storage
overnight in a cold room, the samples were centrifuged
at 3,400 × g for 20 min at 4°C. Samples were analyzed
using SDS-PAGE to check the purity. Western blot was
used to identify ovotransferrin. The whole separation
procedure was replicated 3 times.

**Yield of Ovotransferrin**

The yield obtained by using the final protocol was
calculated because this protocol produced the best
yield and purity. The yield was calculated using the re-
ported value of ovotransferrin in egg white (Stadelman
and Cotterill, 2001). The results of 3 replications were
used for the yield calculation.

**SDS-PAGE**

The optimum level of ammonium sulfate and citric
acid was evaluated using SDS-PAGE. The SDS-PAGE
was conducted under reduced conditions using a Mini-
Protein II cell (Bio-Rad). Ten percent SDS gel and
Coomassie Brilliant Blue R-250 staining were used. A
broad range molecular marker (Bio-Rad) was used as
the marker. Quantification of electrophoreograms and
determination of molecular weight of protein bands
were conducted with a Pharmacia Phast Imagine Gel
Analyzer using AlphaEase FC software (Alpha Inno-
techn Corp., San Leandro, CA).
Western Blot

Western blot was carried out using the method of Xie et al. (2002) with some modifications. After running SDS-PAGE, proteins in the gel were transferred to a nitrocellulose membrane (Bio-Rad), and the membrane was blocked with 5% skim milk solution in PBS with Tween (PBST). Ovotransferrin chicken antibody was added after diluting it to 1:15,000 ratios and incubated overnight at 4°C. The membrane was washed 3 times with PBST solution at 10-min intervals. Rabbit anti-mouse IgG (H+L) conjugated AP12 was diluted 1:20,000 with 5% skim milk dissolved in a PBST, added to the membrane as the secondary antibody, and incubated for 1 h at room temperature. The membrane was washed 3 times with PBST, exposed to ECL Prime and incubated for 1 h at room temperature. The membrane was blocked with 5% skim milk solution in PBS and transferred to a nitrocellulose membrane (Bio-Rad). Purified ovotransferrin was tested for its activity and purity using an ELISA kit (MyBioSource).

Statistical Analysis

The data were analyzed using the Minitab 16.0 statistical software. One-way ANOVA was calculated, and Tukey’s test was employed ($P < 0.05$) to separate means.

RESULTS AND DISCUSSION

Determining Ammonium Sulfate and Acid Conditions for the Separation of Ovotransferrin

The isoelectric point of apo-ovotransferrin is pH 5.8 (Ko and Ahn, 2008). The main principle used in this study is that when the pH value of a protein solution changes, the charges on the amino acid side chains in a protein change. Therefore, the precipitation behavior of a protein in ammonium sulfate solution can be changed. Previous studies used saturated ammonium sulfate at pH 3.0 to precipitate ovotransferrin. However, the purity of ovotransferrin separated was low, and the amount of ammonium sulfate added was very high. Also, the yield was not specified in the original trial completed in 1950 (Wu and Acero-Lopez, 2012). It is known from large-scale separation of protein trios that altering the pH of a solution by acid or alkali can increase the ionic strength of a solution and change polarity of proteins, resulting in easy precipitation of proteins (Price and Nairn, 2009).

Saturated ammonium sulfate with acidic condition was used to separate egg white proteins in early 1900s (Hopkins, 1900; Chick and Martin, 1913). Therefore, the first approach was testing how ovotransferrin is behaving under different ammonium sulfate and acid conditions. Acetic acid and citric acids are the 2 most common organic acids that can be added in foods, and thus the effects of acetic acid or citric acid and ammonium sulfate combinations in precipitating ovotransferrin in egg white were tested. The amount and purity of proteins precipitated indicated that ammonium sulfate + citric acid performed better than ammonium sulfate + acetic acid in separating ovotransferrin (data not shown). Therefore, citric acid was used as the acid for ovotransferrin separation.

Once the selection of acid was completed, the next step was to determine the optimum levels of ammonium sulfate and the selected acid (citric acid) to improve the separation of ovotransferrin from egg white. The comparison of ovotransferrin separation at different levels of ammonium sulfate and citric acid combinations indicated that increasing ammonium sulfate concentrations higher than 5% did not improve the precipitation of ovotransferrin when citric acid is at or higher than 2.5% (data not shown). In this study, ammonium sulfate granules instead of saturated ammonium sulfate solution were used to minimize the volume increase and the pH of the samples was around pH 3.5 to 4.0 when 2.5% citric acid was used. Figures 1 and 2 showed that 5.0% (wt/vol) ammonium sulfate in combination with 2.5% citric acid produced the best results among the conditions tested. The DC-protein assay (Bio-Rad) results also showed that there was no significant difference among the ammonium sulfate and citric acid combinations tested. Therefore, 5.0% (wt/vol) ammonium sulfate and 2.5% (wt/vol) citric acid combination was selected as the best conditions to isolate ovotransferrin from egg white. Ammonium sulfate has a tendency to precipitate ovalbumin, also. With 5% ammonium sulfate and 2.5% citric acid treatment, some ovalbumin was precipitated along with ovotransferrin. Thus, further purification was needed to remove ovalbumin from the crude ovotransferrin extract.

Purification of Ovotransferrin from the Crude Precipitant

To remove ovalbumin coprecipitated with ovotransferrin, the precipitant was dissolved with 2 volumes of distilled water and then desalted using ultrafiltration. The desalted crude ovotransferrin was treated again with different levels of ammonium sulfate with fixed citric acid at 2.5% (wt/vol). Figure 3 showed that ammonium sulfate at 2.0% (wt/vol) was better than other ammonium sulfate levels in removing ovalbumin from ovotransferrin when 2.5% citric acid was combined. Increasing ammonium sulfate greater than 2% did not increase the yield of ovotransferrin. Figure 4 is the SDS-PAGE result with a fixed level of ammonium sulfate (2.0%, wt/vol) and varying levels of citric acid combinations. As the levels of citric acid were lowered to <1.5%, removal of ovalbumin from ovotransferrin was not as effective as those achieved at concentrations >1.5% citric acid. The DC-protein assay (Bio-Rad) results indicated that 1.5% (wt/vol) citric acid produced the highest protein content among the combinations ($P < 0.05$). Therefore, 1.5% citric acid and 2.0% ammonium sulfate combinations in precipitating ovotransferrin in egg white were tested. The amount and purity of proteins precipitated indicated that ammonium sulfate + citric acid performed better than ammonium sulfate + acetic acid in separating ovotransferrin (data not shown). Therefore, citric acid was used as the acid for ovotransferrin separation.
Monium sulfate combination was selected as the best conditions for purifying ovotransferrin from the crude precipitant.

From this study, 5.0% (wt/vol) ammonium sulfate and 2.5% (wt/vol) citric acid combination at stage I and 2.0% (wt/vol) ammonium sulfate and 1.5% (wt/vol) citric acid combination at stage II were selected as the best conditions for purifying ovotransferrin from chicken egg white. Western blot results confirmed that the protein separated was ovotransferrin (Figure 5).

Comparison with the Ethanol Precipitation Method

The activity level of ovotransferrin was compared with that from the ethanol precipitation method (Ko and Ahn, 2008) because it is the latest large-scale separation method for ovotransferrin. The ELISA results of the ovotransferrin purified using the current method was $25.88 \pm 3.71$ ng/mL, whereas that separated using the ethanol method (Ko and Ahn, 2008) was $24.67 \pm 1094$ ABEYRATHNE ET AL.

Figure 1. Effect of ammonium sulfate on the separation of ovotransferrin from egg white. Lane 1 = marker; lane 2 = diluted egg white; lanes 3 to 6 = supernatant obtained from 2.5, 5.0, 7.5, and 10.0% ammonium sulfate, respectively, with a fixed level (5%) of citric acid; lanes 7 to 10 = dissolved precipitant with 2.5, 5.0, 7.5, and 10.0% ammonium sulfate, respectively, with 5% citric acid combinations.

Figure 2. Effect of citric acid on the separation of ovotransferrin from egg white. Lane 1 = marker; lane 2 = diluted egg white; lanes 3 to 6 = supernatant obtained from 2.0, 2.5, 3.0, and 3.5% citric acid, respectively, with a fixed level (5%) of ammonium sulfate; lanes 7 to 10 = dissolved precipitant from 2.0, 2.5, 3.0, and 3.5% citric acid, respectively, with 5% ammonium sulfate combinations.
4.79 ng/mL when a 25 ng/mL sample was used (n = 3), indicating there was no difference in activity and purity between the 2 separation methods.

Table 1 indicated that the yield of ovotransferrin separated using the current method was 83% (n = 3). In the ethanol precipitation method for purifying ovotransferrin from egg white (Ko and Ahn, 2008), addition of FeCl₃ is a critical step. However, the current method does not require the addition of iron, and low levels of ammonium sulfate and citric acid are needed. Therefore, this method uses fewer steps to purify ovotransferrin from egg white. In addition, apo-ovotransferrin was not denatured using our method, and conversion of ovotransferrin from the apo-form to the holo-form, and difficulties of removing released iron from ovotransferrin were not necessary. Even though the yield of ovotransferrin from the protocol developed in this study was lower than that of the ethanol method

Figure 3. Effect of ammonium sulfate on the separation of ovalbumin from ovotransferrin. Lane 1 = marker; lane 2 = diluted egg white; lanes 3 to 6 = supernatant obtained from 1.5, 2.0, 2.5, and 3.0% ammonium sulfate, respectively, with a fixed level (2.5%) of citric acid; lanes 7 to 10 = dissolved precipitant from 1.5, 2.0, 2.5, and 3.0% ammonium sulfate, respectively, with 2.5% citric acid combinations.

Figure 4. Effect of citric acid on the separation of ovalbumin from ovotransferrin. Lane 1 = marker; lane 2 = diluted egg white; lanes 3 to 6 = supernatant obtained from 1.0, 1.5, 2.0, and 2.5% citric acid, respectively, with a fixed level (2.0%) of ammonium sulfate; lanes 7 to 10 = dissolved precipitant from 1.0, 1.5, 2.0, and 2.5% citric acid, respectively, with 2% ammonium sulfate combinations.
(99%), this large-scale method does not use any solvent and can be an alternative method to purify ovotransferrin from egg albumin.

Also, this method produced better apo-ovotransferrin yield than most of the published methods and can be scaled up easily. The highest yield of ovotransferrin reported so far was 96 to 98% when immobilized metal affinity chromatography (Cu-Sepharose 6B) was used, but this method is difficult to scale up (Wu and Acero-Lopez, 2012). Vachier et al. (1995) used ion exchange chromatography to separate ovotransferrin from egg white, but with the same scale-up problem.

The final protocol is shown in Figure 6.

**Conclusion**

Ovotransferrin can be separated from egg white without using solvents. The best combinations for purifying ovotransferrin from chicken egg white was a 2-stage precipitation of ovotransferrin using 5.0% (wt/vol) ammonium sulfate and 2.5% (wt/vol) citric acid combination in step I and a 2.0% (wt/vol) ammonium sulfate and 1.5% (wt/vol) citric acid combination in step II. The yield of the ovotransferrin was 83%, and the purity was greater than 85%.

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REFERENCES


Figure 6. Schematic diagram for the separation of ovotransferrin from egg white. DW = distilled water. Color version available in the online PDF.