An Economic and Simple Purification Procedure for the Large-Scale Production of Ovotransferrin from Egg White

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ABSTRACT The objective of this study was to develop a simple and economical protocol for separating ovotransferrin from egg white. Egg white was separated from the yolk and diluted with the same volume of distilled water. To prevent denaturation during the separation process, ovotransferrin in 2×-diluted egg white was converted to its holo-form by adding 20 mM FeCl₃·6H₂O solution (0.25 to 3 mL/100 mL). The pH of egg white was adjusted to pH 7.0, 8.0, or 9.0, and NaHCO₃ and NaCl were added to 50 mM and 0.15 M, respectively (final concentrations) to facilitate iron binding to ovotransferrin. The iron-bound ovotransferrin was separated from the egg white using different concentrations of ethanol (30 to 50%). Ethanol at 43% (final concentration) and pH at 9.0 were the best conditions for separating iron-bound ovotransferrin from 2×-diluted egg white solution. Almost all egg white proteins including ovalbumin were precipitated at 43% ethanol, but most of the iron-bound ovotransferrin remained in the supernatant. Holo-ovotransferrin in the 43% ethanol solution started to precipitate as the concentration of ethanol increased, but the optimal condition for precipitating ovotransferrin was when the ethanol concentration reached 59% (final). The precipitated holo-ovotransferrin was dissolved with distilled water, and AG1-X₂ ion exchange resin (at 3× iron content in ovotransferrin) was used to remove iron bound to ovotransferrin after pH adjustment to 4.7 using 500 mM citric acid. The apo-ovotransferrin obtained using this protocol was >80% in purity and around 99% in yield. The protocol developed is simple, economical, and appropriate for a large-scale production of ovotransferrin from egg white. Also, the isolated ovotransferrin can be applied in human foods, because the only solvent used in this process is ethanol. Furthermore, the AG1-X₂ ion exchange resin and ethanol used in this process can be regenerated and recovered.

Key words: ovotransferrin, separation method, ethanol extraction, egg white

INTRODUCTION

Ovotransferrin is a major avian egg white protein that constitutes 12 to 13% of total egg white protein (Stadelman and Cotterill, 1986). Ovotransferrin is a monomeric glycoprotein consisting of 686 amino acids with 15 disulfide bridges, has a molecular weight of 76,000 D, and isoelectric point of 6.1 (Abola et al., 1982; Stadelman and Cotterill, 1986). Ovotransferrin is composed of a N- and a C-terminal domain and 1 atom of a transition metal such as Fe(III), Cu(III), and Al(III); can bind tightly within the interdomain cleft of each lobe; and shows a strong antimicrobial activity. Therefore, it has a high potential to be used as a natural antimicrobial agent in foods, if a simple, economical, and large-scale separation method can be developed.

A metal-free ovotransferrin (apo-form) is colorless and easily destroyed by physical and chemical treatments, whereas iron-bound ovotransferrin (holo-form) shows a salmon-pink color and is resistant to proteolytic hydrolysis and thermal denaturation (Azari and Feeney, 1958). Because most chemical reagents and denaturing conditions decrease the affinity of iron to ovotransferrin, a particular spatial configuration of native ovotransferrin is needed for the formation of colored iron-bound ovotransferrin (Fraenkel-Conrat, 1950). The binding of iron to ovotransferrin requires 1 molecule of CO₂ as CO₃²⁻ or HCO₃⁻ per atom of Fe³⁺ (Warner and Weber, 1953) to overcome the effect of citrate, which can bind iron (Mason and Heremans, 1968). Reiter et al. (1975), however, reported that bicarbonate rather than pH is a critical factor for bacteriostatic capability of lactoferrin. To release Fe³⁺ from ferric transferrin, a simple anion, such as pyrophosphate, sulfate, and chloride, is required in vitro. The anion-induced Fe³⁺ release is closely related to the opening of a domain in either lobe (Baldwin and de Sousa, 1981; Cheuk et al., 1987; Bailey et al., 1997). At acidic pH, the N-lobe of ovotransferrin displays lower
iron-binding capacity and faster release of Fe$^{3+}$ than the C-lobe. In the absence of NaHCO$_3$, the pH affects the efficiency of citrate-mediated release of Fe$^{3+}$ from ovotransferrin. Citrate-mediated Fe$^{3+}$ release is more efficient at pH 6.8 than at pH 7.4 (Griffiths and Humpherys, 1977). Iron could be removed from iron-transport proteins at low pH (pH 4.5) in the presence of a large excess of citrate (Guo et al., 2003). Cunningham and Lineweaver (1965) reported that when holo-ovotransferrin solution was heated at pH 9, the protein was not altered significantly. However, ovotransferrin was denatured when the proteins were exposed to a pH below 4.2, but the influence of pH was reversible under certain experimental conditions (Phelps and Cann, 1956).

Ovotransferrin can be separated from egg white by aqueous and ethanol fractionation procedures (Bain and Deutsch, 1948; Warner and Weber, 1951), fractional precipitation with ammonium sulfate, or coagulating ovalbumin (Warner, 1954; Azari and Baugh, 1967). However, the drawback of these techniques such as ammonium sulfate precipitation at acidic conditions and ethanol precipitation for purification of ovotransferrin is that they denature ovotransferrin and the purity of the resulting products is relatively low (Vachier et al., 1995). To overcome these drawbacks from ethanol or ammonium sulfate precipitation, cation exchange chromatography such as carboxymethyl cellulose (Rhodes et al., 1958; Azari and Baugh, 1967), diethylaminoethyl (DEAE) cellulose anion exchange chromatography (Mandeles, 1960), and Q-Sepharose Fast Flow column (Vachier et al., 1995) were employed for purification of ovotransferrin from egg albumin. Guerin and Brule (1992) used a cation exchange (Duolite C464 and C476) chromatography for industrial-scale production of ovotransferrin from egg white devoid of lysozyme. Al-Mashikh and Nakai (1987) used a single-step chromatographic method using an immobilized metal affinity chromatography (a copper-loaded Sepharose 6B column) to separate ovotransferrin from undiluted, blended egg white. Chung et al. (1991) used a bifunctional dye-ligand chromatography using DEAE Affigel Blue as the first step to fractionate egg white proteins. The fraction containing ovotransferrin obtained from

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**Figure 1.** Schematic diagram for the isolation of ovotransferrin from egg white.

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Dilution of egg white with 1 volume distilled water

Add NaHCO$_3$, NaCl, FeCl$_3$ and adjust pH to 9.0

Stir for 30 min, add ethanol (43%, final), and centrifuge at 3,220 × g for 20 min

Supernatant (1st)

Precipitate

Centrifugation at 3,220 × g, 20 min

Supernatant (2nd)

Re-extract with 43% ethanol

Add ethanol (supernatant: ethanol = 5:2) and centrifuge

Dissolve precipitant with distilled water

pH adjustment to 4.7 with 50 mM citric acid

Add 0.6 g of AG1-X$_2$ resin to 100 mL holo-ovotransferrin solution

Stir for 1 h and filter through Whatman #1 paper

Repeat AG1-X$_2$ resin treatment by the same method as above

Apo-ovotransferrin
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DEAE Affi-Gel Blue then was further purified using Fast Flow liquid chromatography. A 2-step chromatographic procedure involving gel permeation on a Superose-6 Prep grade column and anion-exchange chromatography on a Q-Superose Fast Flow was also employed (Awade et al., 1994).

Recently, Ahlborn et al. (2006) separated ovotransferrin from egg white using Econo-Pac High S-Cation and High Q-Anion Exchange cartridges. Even though ion-exchange chromatography or immobilized metal affinity chromatography have been developed on a laboratory scale, the application of these techniques on a pilot-scale is difficult, because these methods are labor-intensive and very expensive (Awade, 1996).

The objective of this study was to develop a simple and rapid procedure for an economical, large-scale production of ovotransferrin from egg white.

### Materials and Methods

#### Materials

Chicken eggs were purchased from a local store. Egg white (500 mL) was separated from the eggs and diluted with the same volume of distilled water. One liter of 2×-diluted egg white solution was blended for 2 min using an electric blender and was used as a starting material for ovotransferrin separation. The work was replicated 3 times.

#### Separation of Ovotransferrin from Egg White

Because iron-saturated, holo-ovotransferrin is more stable to chemicals such as ethanol than the apo-form,

#### Table 1. Effect of pH on the yield of ovotransferrin from 2×-diluted egg white solution

<table>
<thead>
<tr>
<th>pH</th>
<th>First supernatant Concentration (mg/mL)</th>
<th>Volume (mL)</th>
<th>Second supernatant Concentration (mg/mL)</th>
<th>Volume (mL)</th>
<th>Volume × concentration (mg)</th>
<th>Yield (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>9.40a ± 0.15</td>
<td>61.3a ± 1.26</td>
<td>2.48b ± 0.62</td>
<td>14.0ab ± 0.25</td>
<td>610.7ab ± 25.9</td>
<td>87.18 ± 3.6</td>
</tr>
<tr>
<td>8</td>
<td>8.94b ± 0.82</td>
<td>61.7a ± 2.08</td>
<td>2.82b ± 1.00</td>
<td>13.7b ± 0.14</td>
<td>581.4b ± 32.2</td>
<td>83.07 ± 4.5</td>
</tr>
<tr>
<td>9</td>
<td>9.69b ± 0.08</td>
<td>62.3a ± 0.29</td>
<td>6.62a ± 0.62</td>
<td>14.3a ± 0.14</td>
<td>761.5b ± 0.58</td>
<td>95.88 ± 0.2</td>
</tr>
</tbody>
</table>

4bMean values with different superscript letters are significantly different \((P<0.05); \ n=3\).

1The solution obtained from the first extraction with 43% ethanol.

2The solution obtained from the second extraction with 43% ethanol.

3The yields were calculated based on the theoretical value (7.0 mg) of ovotransferrin in 2×-diluted egg white solution.
the iron-free ovotransferrin in egg white solution was converted to the iron-bound form using FeCl₃ solution. The pH of 2×-diluted egg white solution was adjusted to pH 7.0, 8.0, or 9.0 first, and then NaHCO₃ and NaCl were added to reach concentrations in the egg white solution to 50 mM and 0.15 M, respectively, to help iron binding, and then 0.25, 0.5, 1.0, 2.0, or 3.0 mL of 20 mM FeCl₃·6H₂O solution was added per 100 mL of 2×-diluted egg white solution. The egg white solution was stirred for 30 min at room temperature, and then appropriate amounts of 100% cold ethanol (to make 20, 33, 43, and 50%, final concentrations) were added to precipitate egg white proteins except for the holo-form of ovotransferrin. Holo-ovotransferrin was separated from the precipitated egg white proteins by centrifugation at 3,220 × g for 20 min. The precipitant was reextracted with the same concentration of ethanol and centrifuged at 3,220 × g for 20 min. The supernatants were pooled and filtered through a
Whatman No.1 filter paper to remove floating materials. After filtering, cold ethanol (100%) was slowly added to aliquots of supernatant to determine the optimal ethanol concentration for precipitating iron-bound ovotransferrin in the supernatant. The precipitated holo-ovotransferrin was collected after centrifugation at 3,220 × g for 20 min, dissolved in 10 volumes of distilled water, and then the degree of iron saturation in each solution was estimated by measuring the absorbance at 468 nm.

**Removal of Iron Using AG1-X2 Resin**

AG1-X2 resin (chloride form, Bio-Rad, Richmond, CA) was used to remove iron from the iron-saturated ovotransferrin solution. To facilitate the release of the iron from holo-ovotransferrin, the pH of iron-saturated ovotransferrin solution was adjusted to pH 4.7 using 0.5 M citric acid based on the suggestion of Guo et al. (2003): iron bound to iron-transport proteins such as transferrin and lactoferrin can be removed by forming ferric citrate in the presence of a large excess of citrate. To know the suitable amount of AG1-X2 resin added to the final iron-bound ovotransferrin solution, 0.1, 0.2, 0.3, 0.6, or 0.9 g of AG1-X2 resin was added to 100 mL of iron-bound ovotransferrin solution containing around 6 mg/mL of ovotransferrin. The mixture was gently stirred for 1 h until the yellow color of ferric citrate completely disappeared. The mixture was filtered through a Whatman No.1 filter paper. After releasing iron, the residual iron in the ovotransferrin solution was estimated by the Ferrozine test (Carter, 1971), and the ovotransferrin was freeze-dried. Most of the citric acid added to adjust pH at the iron-removal step was removed as a ferric citrate form. However, the citrate remaining in solution after the iron removal step can affect the iron-binding capacity of ovotransferrin. So, citrate-free solution was prepared by dialyzing the solution against 20 mM phosphate buffer, pH 6.5, and the effect of residual citrate on the iron-binding capacity of ovotransferrin was determined.

**Iron-Binding Capacity**

The quantity of ovotransferrin was determined using iron-binding capacity, which was measured by the modified method of Williams (1974). The calibration curve was obtained using commercial ovotransferrin (from chicken egg white, substantially iron-free, Sigma, St Louis, MO). To support its iron-binding capability, NaHCO3 and NaCl solution (50 mM and 0.15 M final concentration, respectively) were added to the apo-ovotransferrin solution. After pH adjustment to 8.0, 0.4 mL of 10 mM Fe-nitriloacetate in 50 mM Tris-HCl at pH 8.0 was added to 20 mL of prepared iron-free ovotransferrin, and then the solution was kept at room temperature for 1 h to develop color. Finally, iron-binding capacity of the solution was

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein (mg/mL)</th>
<th>Ovotransferrin (mg/mL)</th>
<th>Residual iron (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without dialysis</td>
<td>8.59 ± 0.14</td>
<td>8.00 ± 0.57</td>
<td>0.52 ± 0.08</td>
</tr>
<tr>
<td>Dialysis</td>
<td>8.57 ± 0.17</td>
<td>8.42 ± 0.67</td>
<td>0.53 ± 0.06</td>
</tr>
</tbody>
</table>

1Protein concentrations measured by the Lowry method.
2The amount of iron remaining in the solution after iron removal was measured.
Figure 6. Amounts of residual iron in ovotransferrin solution after the first and the second treatment with AG1-X2 resin. The ovotransferrin concentration of the solution used in this study was around 6.5 mg/mL. Bars with different letters are significantly different ($P < 0.05, n=4$).

determined by measuring absorbance at 468 nm. To eliminate trace amounts of metal contaminants, water used in all the procedures was treated with Chelex (Chelex-100 sodium form, Sigma) according to the method reported by Willard et al. (1969). To determine the effect of pH on the iron-binding capacity of ovotransferrin, the pH of apo-ovotransferrin solution was adjusted to pH 4, 5, 6, 7, 8, or 9, and then the iron-binding capacity of each solution was measured by the same method as described above.

Ferrozine Test

After iron removal from the holo-ovotransferrin precipitation, the amount of residual iron in ovotransferrin was measured using the Ferrozine method (Carter, 1971). Disposable polyethylene vessels, which were essentially free of metal contamination, were used for the analysis. Aqueous solutions were prepared with deionized distilled water. Fresh ascorbic acid [1 mL, 0.1% (wt/vol) in 0.2 N HCl] was added to 1 mL of iron-free ovotransferrin solution, and then the mixture was stirred and allowed to stand at room temperature for 5 min. Trichloroacetic acid (11.3% solution, 1 mL) was added to the mixture and centrifuged at 2,500 $\times$ g for 10 min. Each standard iron solution under certain concentrations was prepared by serial dilution from 100 ppm Fe(NH$_4$)$_2$SO$_4$·6H$_2$O solution containing 50 ppm concentrated sulfuric acid with distilled water. Ferrozine color reagent (0.8 mL), which was prepared with 75 mg of ferroine (Sigma), 75 mg of neocuproin (Sigma), and 1 drop of 10 N HCl in 25 mL of distilled water, was added to 2 mL of sample and a standard iron solution, and then the mixture was stirred gently. Finally, the optical density of the sample was measured at 562 nm after standing for 5 min.

Yield of Ovotransferrin

To determine the yield of ovotransferrin, egg white was extracted twice using 43% ethanol. The holo-ovotransferrin from the first ethanol extraction was precipitated with 59% ethanol (final concentration), but the holo-ovotransferrin from the second extraction was precipitated with 64% ethanol, because 64% ethanol was the best for precipitating the second extract. The precipitate was dissolved with distilled water and then the iron bound to

<p>| Table 3. The yields of each supernatant obtained by adding 43% ethanol to egg white solution |
|---------------------------------|------------------|------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Extraction</th>
<th>Concentration (mg/mL)</th>
<th>Volume (mL)</th>
<th>Volume $\times$ concentration (mg)</th>
<th>Yield$^1$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First$^2$</td>
<td>11.99 $\pm$ 0.23</td>
<td>43.95 $\pm$ 0.13</td>
<td>527.30 $\pm$ 8.64</td>
<td>75.33 $\pm$ 1.23</td>
</tr>
<tr>
<td>Second$^3$</td>
<td>7.94 $\pm$ 0.37</td>
<td>22.32 $\pm$ 0.36</td>
<td>170.56 $\pm$ 1.80</td>
<td>24.37 $\pm$ 0.26</td>
</tr>
</tbody>
</table>

$^1$The yields were calculated based on the theoretical value (700 mg) of ovotransferrin in 100 mL of 2x-diluted egg white solution.

$^2$Ethanol was added to 100 mL of 2x-diluted egg white to make 43% ethanol (final) and then the holo-ovotransferrin was precipitated by 59% ethanol (final). The holo-ovotransferrin in the precipitate was dissolved with distilled water (precipitate weight: water volume = 2:1).

$^3$In second extraction, the holoform of ovotransferrin in supernatant was precipitated by 64% ethanol; $n=3$.  

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3.In second extraction, the holoform of ovotransferrin in supernatant was precipitated by 64% ethanol; $n=3$.  

ovaltine was removed using AG1-X2 resin. After iron removal, the concentration of apo-ovaltine from each supernatant was determined by measuring iron-binding capacity. Also, after combining the first and second supernatants, the holo-ovaltine was precipitated with 59% ethanol to determine the total yield of apo-ovaltine. In calculating the yield of apo-ovaltine, 7 mg/mL of ovaltine in 2×-diluted egg white solution was used, because ovaltine comprises 12 to 13% of egg white proteins (Stadelman and Cotterill, 1986) and 11 to 11.5% of egg white is protein (Ahn et al., 1997).

**SDS-PAGE**

The most suitable amount of ethanol required for extraction and precipitation of holo-ovaltine was evaluated using SDS-PAGE. The SDS-PAGE was conducted under nonreducing conditions using Mini-Protein II cell (Bio-Rad). Ten percent SDS gel and Coomassie Brilliant Blue R-250 (Bio-Rad) staining were used. Broad-range SDS-PAGE molecular weight standards of 44 to 200 kDa (Bio-Rad) were used as a 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 Innotech Corp., San Leandro, CA).

**Statistical Analysis**

All analyses were replicated 3 times, and data were analyzed using the JMP software (version 5.1.1, SAS Institute, Cary, NC). Differences in the mean values were compared by Tukey’s honestly significant difference procedure, and mean values and standard deviations were reported (Kuehl, 2000).

**RESULTS AND DISCUSSION**

**Effects of FeCl3 Addition in Ethanol Treatment**

The yield of ovaltine was calculated by multiplying the concentration and volume of ovaltine and was significantly different depending upon the amount of FeCl3 added. Addition of 2× and 3× the iron required for saturating ovaltine produced higher absorbance at 468 nm than those of lower amounts of iron. There was no significant difference in absorbance values at 468 nm between adding 2× and 3× amounts of iron required to saturate ovaltine, indicating that all the apo-ovaltine in egg white solution was saturated by 2× iron treatment (Figure 2).

The theoretical amount of iron required for saturating all the apo-ovaltine in 100 mL of 2×-diluted egg white is around 1 mg, because an ovaltine molecule can bind 2 iron molecules (Azari and Feeney, 1958). Therefore, addition of 0.25, 0.5, 1.0, 2.0, or 3.0 mL of 20 mM FeCl3·6H2O to 100 mL of diluted egg white solution corresponds to 1/4, 1/2, 1, 2, or 3× the iron required to saturate ovaltine in the solution with iron, theoretically. Fraenkel-Conrat (1950) reported that a particular spatial configuration of native ovaltine is required for the formation of colored iron-bound ovaltine, and most chemical reagents and denaturing conditions lowered the affinity of ovaltine for iron. As suggested by Azari and Feeney (1958), the formation of holo-ovaltine prevented denaturation of ovaltine in ethanol. The result indicated that addition of FeCl3 was a critical step for the purification of ovaltine using ethanol. Also, this study confirmed the suggestion of Vacher et al. (1995), who reported that apo-ovaltine is denatured easily by ethanol.

Addition of ethanol to diluted egg white solution without adding FeCl3 denatured apo-ovaltine, which co-precipitated with the other proteins present in egg white. Also, addition of 2× iron required for ovaltine saturation was the most appropriate for preventing loss of ovaltine during ethanol extraction.

**pH Effect on Ovatransferrin Separation**

The pH of egg white solution had a significant effect on the recovery of the apo-ovaltine during ethanol extraction (Table 1). Among the different pH conditions, pH 9.0 produced the highest recovery rate for ovaltine, which was around 96%. Even though iron saturation stabilized ovaltine by converting the apo-form to the holo-form, high pH (pH 9.0) stabilized holo-ovaltine. Also, the effect of pH conditions on the iron-binding capacity of apo-ovaltine indicated that iron binds well with ovaltine at pH >6.0, but the iron-binding capacity of ovaltine rapidly decreased at <pH 6.0 (Figure 3). Griffiths and Humpherys (1977) reported that the N-lobe of ovaltine displayed lower iron-binding stability and more accelerated release of Fe3+ than the C-lobe at acidic pH. Also, pH influenced the efficiency of citrate-mediated release of Fe3+ from ovaltine.  

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (mg/mL)</th>
<th>Volume (mL)</th>
<th>Volume × concentration (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg white1</td>
<td>7.00</td>
<td>100</td>
<td>700</td>
<td>100</td>
</tr>
<tr>
<td>Final solution2</td>
<td>6.36 ± 0.37</td>
<td>108 ± 2.10</td>
<td>693.21 ± 45.53</td>
<td>99.03 ± 6.50</td>
</tr>
</tbody>
</table>

1 The theoretical concentration of ovaltine in 2×-diluted egg white solution used was 7 mg/mL.
2 Apo-ovaltine solution produced after iron removal; n = 3.
transferrin in the absence of NaHCO₃. Warner and Weber (1951) reported that even though ovotransferrin can be separated from egg white at any pH using ethanol, the intensity of salmon-pink color of iron-bound ovotransferrin solution decreased at pH 5.5. Williams (1975) reported that iron was bound preferentially to the N-terminal site at pH 8.0, whereas iron binding occurs preferentially at the C-terminal site in acid pH conditions (Williams et al., 1978).

**Extraction and Precipitation of Iron-Bound Ovotransferrin by Ethanol**

When 33% of ethanol was added to egg white solution, about 1/2 of the total ovalbumin remained in the supernatant. However, only a small amount of ovotransferrin precipitated at 43% ethanol solution, and most of the ovalbumin precipitated at 43% ethanol (Figure 4). The ovotransferrin separated at 50% ethanol had higher purity than 43% ethanol, but a larger amount of ovotransferrin was precipitated. This suggested that 43% of ethanol was the most suitable condition to produce ovotransferrin possessing high purity and yield from 2×-diluted egg white solution.

Ovotransferrin in supernatant started to precipitate as the amount of ethanol increased (Figure 5). When 2.5 mL of ethanol was added to 5 mL of supernatant (corresponding to 62% ethanol), the amount of residual ovotransferrin in the supernatant was the smallest. When ethanol added was less than 2.5 mL, some proteins possessing less than 45 kDa of molecular weight appeared in the supernatant. For the production of ovotransferrin both with high purity and yield, therefore, adding 2 mL of ethanol to 5 mL of supernatant obtained from the 43% ethanol extraction (59% ethanol, final) is the most appropriate condition for precipitating iron-bound ovotransferrin.

**Iron Release from Holo-Form Ovotransferrin**

When 0.6 or 0.9 g of AG1-X₂ resin was added to a 100-mL iron-bound ovotransferrin solution, residual iron content was 1.8 and 1.6 ppm, respectively (Figure 6). The concentrations of residual iron decreased to <0.5 ppm after second addition of 0.6 or 0.9 g of AG1-X₂ resin to the same volume of solution. Therefore, the most appropriate amount of AG1-X₂ resin for releasing iron from 100 mL of holo-ovotransferrin was around 0.6 g. Because the iron separated from or bound to holo-ovotransferrin could not be removed completely by the first addition of 0.6 g of AG1-X₂ resin/100 mL of ovotransferrin solution was needed.

Citrate was added to the holo-ovotransferrin solution, because the release of Fe³⁺ from ferric transferrin requires presence of a simple anion to open a domain in either the C- or N-lobe of ovotransferrin (Baldwin and de Sousa, 1981; Cheuk et al., 1987; Bailey et al., 1997). However, it
is known that citrate binds iron (Masson and Heremans, 1968), and thus any residual citrate may result in underestimation of ovotransferrin by the iron-binding capacity method. Due to such a concern, the effect of residual citrate on the amount of ovotransferrin in the final solution was determined. There was no significant difference in the concentrations of ovotransferrin and other proteins in the ovotransferrin solution before and after citrate removal by dialysis (Table 2). As in previous reports, citrate played an important role in iron release from holo-ovotransferrin. In this study, citric acid was added to adjust the pH of the holo-ovotransferrin solution to pH 4.7. The added citrate formed ferric citrate with iron released from ovotransferrin. The ferric citrate could be easily removed by AG1-X2 resin. Although some residual citrate can remain in the final apo-ovotransferrin solution, the amount of residual citrate did not affect iron-binding capacity of ovotransferrin. Therefore, it can be concluded that the dialysis step to remove residual citrate was not necessary.

**Recovery and Purity of Ovotransferrin**

Iron-bound ovotransferrin (holo-form) in supernatant was stable at alkaline pH (Figure 7). When the pH of the solution was changed to 4.7 by citric acid for iron removal, however, most of the holo-form of ovotransferrin was changed to apo-form. The apo-ovotransferrin formed in acidic conditions was denatured easily by ethanol in the solution. Therefore, it was very difficult to measure the concentration of ovotransferrin in the supernatant containing ethanol, and the yield was measured only at the final stage in which iron-bound ovotransferrin was precipitated with ethanol. The ethanol precipitate was dissolved with distilled water and used to determine the yield of ovotransferrin by measuring iron-binding capacity.

The amount of the apo-ovotransferrin obtained from the first extraction with 43% ethanol was around 75% of total amount, whereas the second extraction recovered an additional 24% of the total amount (Table 3). Also, the total yield of ovotransferrin using the protocol in Figure 7 was around 99% (Table 4), indicating that almost all ovotransferrin in egg white was recovered through the ethanol extraction method. Also, the purity of the separated ovotransferrin was >80% (Figure 7). Ovalbumin comprising about 50% of total egg white proteins displayed the broadest band on SDS-PAGE (lane 3). Almost all ovotransferrin was precipitated by 59% of ethanol (lane 6), but some proteins still remained in the supernatant (lane 5). The purity of apo-ovotransferrin solution could be increased by reextracting the final holo-ovotransferrin preparation using ethanol before iron removal.

In conclusion, most of the ovotransferrin in the natural egg white exists in apo-form. Thus, the conversion of apo-ovotransferrin to iron-bound form was the most critical step to minimize the loss or denaturation of ovotransferrin by ethanol. The amount of iron required to saturate all the ovotransferrin in egg white was about 2x the theoretical amount to bind all apo-form of ovotransferrin. The holo-ovotransferrin in the presence of excess iron was more stable to ethanol treatment than that at low iron concentrations. At pH 9.0, the iron-binding capacity of holo-ovotransferrin was significantly higher than that at pH 7.0 or 8.0, and high pH conditions stabilized ovotransferrin during ethanol extraction and precipitation steps. Holo-ovotransferrin could be easily separated from egg white using 43% ethanol extraction and 59% ethanol precipitation. The AG1-X2 ion exchange resin was excellent in removing iron from holo-ovotransferrin, and the citrate added to adjust pH played a critical role in iron release from holo-ovotransferrin. The preparation method was simple and economical, and the ovotransferrin produced had high purity (>80% purity), and yield was excellent (99%). Therefore, the protocol developed is appropriate for a large-scale production of ovotransferrin. The produced ovotransferrin is applicable for food products, because only ethanol was used to separate ovotransferrin from egg white.

**REFERENCES**


