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Abstract

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Keywords

immunoglobulin Y separation, ammonium sulfate, cation exchange chromatography, egg yolk

Disciplines

Agriculture | Animal Sciences | Poultry or Avian Science

Comments

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Preparation of Immunoglobulin Y from Egg Yolk Using Ammonium Sulfate Precipitation and Ion Exchange Chromatography

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ABSTRACT The objective of this study was to develop an economical, simple, and large-scale separation method for IgY from egg yolk. Egg yolk diluted with 9 volumes of cold water was centrifuged after adjusting the pH to 5.0. The supernatant was added with 0.01% charcoal or 0.01% carrageenan and centrifuged at $2,800 \times g$ for 30 min. The supernatant was filtered through a Whatman no. 1 filter paper and then the filtrate was concentrated to 20% original volume using ultrafiltration. The concentrated solution was further purified using either cation exchange chromatography or ammonium sulfate precipitation. For the cation exchange chromatography method, the concentrated sample was loaded onto a column equilibrated with 20 mM citrate-phosphate buffer at pH 4.8 and eluted with 200 mM citrate-phosphate buffer at pH

6.4. For the ammonium sulfate precipitation method, the concentrated sample was twice precipitated with 40% ammonium sulfate solution at pH 9.0. The yield and purity of IgY were determined by ELISA and electrophoresis. The yield of IgY from the cation exchange chromatography method was 30 to 40%, whereas that of the ammonium sulfate precipitation was 70 to 80%. The purity of IgY from the ammonium sulfate method was higher than that of the cation exchange chromatography. The cation exchange chromatography could handle only a small amount of samples, whereas the ammonium sulfate precipitation could handle a large volume of samples. This suggests that ammonium sulfate precipitation was a more efficient and useful purification method than cation exchange chromatography for the large-scale preparation of IgY from egg yolk.

Key words: immunoglobulin Y separation, ammonium sulfate, cation exchange chromatography, egg yolk

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INTRODUCTION

The production of antibodies from immunized chicken eggs is an excellent alternative to that from the serum of mammals (Svendsen et al., 1995). Chicken antibodies have many advantages to the traditional mammalian antibodies and have several important differences against mammalian IgG in regard to their functions. First, chicken IgY can react with many epitopes of mammalian antigens due to phylogenetic distance between birds and mammals, resulting in amplification of signals. Second, chicken antibodies do not react with rheumatoid factors, mammalian IgG, and bacterial Fc receptors; they do not induce false positive results in immunoassays because they do not activate mammalian complements (Larsson et al., 1991, 1992; Davalos-Pantoja et al., 2000; Stalberg and Larsson, 2001) and do not bind protein A and G, which are commonly used for the isolation of IgG, due to differences in the Fc regions (Akerstrom et al., 1985; Schwarzkopf and

Thiele, 1996). Therefore, IgY can be applied in many medical fields such as xenotransplantation (Fryer et al., 1999), diagnostics (Erhard et al., 2000), and antibiotic alternative therapies (Carlander et al., 1999). Furthermore, the amount of antibodies produced from an egg is equivalent to that from 200 to 300 mL of mammalian blood, and the costs for animal care per unit production of antibodies are much lower in chicken than in mammals (Larsson et al., 1993; Schade and Hlinak, 1996). However, the practical use of IgY in research and diagnostics is limited due to complex and time-consuming purification steps associated with the further purification of IgY (Akita and Nakai, 1992; Camenisch et al., 1999).

The first step of IgY separation from egg yolk usually involves the extraction of IgY from yolk. One of the major obstacles in isolating IgY from egg yolk is a high concentration of lipids and lipoproteins (Hansen et al., 1998; Verdoliva et al., 2000). Various strategies, such as the use of detergents such as SDS (Sriram and Ygeeswaran, 1999), carrageenan (Hatta and Kim, 1990), sodium alginate, or xanthan gum (Hatta et al., 1988); solvents such as acetone (Sriram and Ygeeswaran, 1999), chloroform (Ntakarutimana et al., 1992), and ethanol (Bade and Stegemann, 1984); precipitation of lipoproteins using polyethylene glycol (Polson et al., 1985; Akita and Nakai, 1993) or

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Table 1. Effect of pH on lipid content, turbidity, and protein and IgY content in the water-soluble fraction of egg yolk solution¹

Item	Lipid (%)	Turbidity	Protein (mg/mL)	IgY (mg/mL)
Control (pH 6.0)	1.00 ± 0.12 ^a	2.97 ± 0.15 ^a	0.41 ± 0.07 ^a	0.82 ± 0.25 ^a
pH adjustment to 5.0	0.08 ± 0.02 ^b	0.33 ± 0.01 ^b	0.51 ± 0.05 ^a	1.09 ± 0.34 ^a

^{a,b}Means within a column with no common superscript differ ($P < 0.05$).

¹n = 4.

dextran sulfate (Jensenius et al., 1981); aqueous 2-phase system with phosphate and triton X-100 (Stalberg and Larsson, 2001); simple freeze and thaw cycling (Svendson et al., 1995); and water dilution under acidic conditions (Akita and Nakai, 1992; Ruan et al., 2005), have been used to remove lipids and lipoproteins from egg yolk extract. Most of these methods, however, have drawbacks such as low IgY yield rates, complexity of procedures, or compatibility for human use.

Among the methods, Akita and Nakai (1993) suggested that the water dilution method under acidic conditions was the most efficient and economical procedure for large-scale production of IgY from egg yolk. Upon removal of lipids and lipoproteins from egg yolk using acidified water, IgY can be precipitated by ammonium sulfate (Akita and Nakai, 1993), sodium sulfate (Wooley and Landon, 1995), or caprylic acid-ammonium sulfate (McLaren et al., 1994; Ruan et al., 2005). However, the water dilution (10×) method involves an extreme volume increase, which makes it difficult to use NaCl precipitation in large scale. Ultrafiltration is one of the best methods of reducing the volume of egg yolk extract, and the efficacy of ultrafiltration is greatly influenced by the presence of lipids or lipoproteins in the solution. Therefore, complete removal of lipids or lipoproteins from the water extract of egg yolk is necessary. The changes of pH value in egg yolk solution influence the extent of interactions between polysaccharides and proteins, the precipitation of polysaccharide-lipoprotein complexes, and the recovery of immunoactivity in IgY (Gurov et al., 1983; Samant et al., 1993). Chang et al. (2000) reported that addition of 0.1% of λ -carrageenan was effective in removing lipoproteins from the water extract of egg yolk at pH 5.0.

After precipitation of IgY by NaCl, column chromatography (Jensenius et al., 1981) is frequently used as a final step for IgY purification. However, column chromatography is expensive and impractical for the large-scale production of antibodies. Therefore, appropriate strategies for the large-scale production of antibodies with high purity and yield are needed. The objective of this work was to develop an efficient and simple protocol for large-scale production of antibodies with high purity and yield rates. In this study, carrageenan or charcoal was added to the water-soluble fraction obtained by the water dilution method to remove lipoproteins, which tend to clog the membrane filter during ultrafiltration.

MATERIALS AND METHODS

Preparation of Egg Yolk Extract

Fresh eggs (less than 2 d old) were obtained from a local farm, and yolk was separated from white using yolk separators. Egg yolk (4°C) was diluted with 9 volumes of cold (4°C) distilled water and homogenized for 1 min using a Waring blender (Waring Laboratory, Torrington, CT) at high speed. The pH of egg yolk solution was adjusted to pH 5.0 with 1 N HCl. To determine the effect of NaCl on the extraction of IgY from egg yolk, various amounts of NaCl were added to the egg yolk before homogenization. To investigate the effect of storage temperature on the extraction of water-soluble proteins, homogenized egg yolk solutions were kept in room temperature (control), freezer, or refrigerator for 24 h before centrifugation at $2,800 \times g$ for 40 min at 4°C. The turbidity, protein content, and lipid content of the supernatant were measured.

After centrifugation, the supernatant collected was added with nothing added (control), 0.1% carrageenan, or 0.1% charcoal (final concentration) and centrifuged again at $2,800 \times g$ for 30 min at 4°C to remove residual lipoproteins in the supernatant. The supernatant was filtered through a Whatman no. 1 filter paper and then concentrated to one-fifth of the original volume using a Pellicon XL Biomax-50 ultrafiltration membrane filter (cut-off size: 100 kD) installed to a Labscale TFF System (Millipore, Billerica, MA). The concentrate was further purified for IgY either using a cation exchange chromatography or ammonium sulfate precipitation method. The

Table 2. Turbidity and yield of IgY from the water-soluble fraction of egg yolk solution after centrifugation¹

Item	Yield ² (%)	Turbidity (600 nm)	IgY (mg/mL)	Total IgY ³ (yield × IgY)
Control	77.03 ^a	0.065 ^a	1.11 ^a	86.73 ^a
Refrigerator	78.23 ^a	0.040 ^b	1.12 ^a	88.78 ^a
Freezing	72.03 ^b	0.014 ^c	1.24 ^a	90.05 ^a

^{a-c}Means within a column with no common superscript differ ($P < 0.05$).

¹n = 4.

²The percentage volume of supernatant obtained after centrifugation of egg yolk solution.

³IgY concentration × the volume of supernatant gained after centrifugation.

Table 3. Effect of NaCl on the turbidity, protein, and IgY content in the water-soluble fraction of egg yolk solution¹

NaCl (%)	Turbidity	Protein (mg/mL)	IgY (mg/mL)
0	0.07 ± 0.005 ^d	1.44 ± 0.04 ^a	2.02 ± 0.18 ^a
0.1	0.06 ± 0.01 ^d	0.52 ± 0.01 ^c	2.20 ± 0.36 ^a
0.2	0.09 ± 0.03 ^{cd}	0.55 ± 0.004 ^{bc}	2.04 ± 0.20 ^a
0.3	0.14 ± 0.01 ^c	0.57 ± 0.03 ^{bc}	2.70 ± 0.35 ^a
0.4	0.21 ± 0.01 ^b	0.59 ± 0.002 ^b	2.57 ± 0.11 ^a
0.5	0.51 ± 0.06 ^a	0.56 ± 0.002 ^c	2.70 ± 0.32 ^a

^{a-d}Means within a column with no common superscript differ ($P < 0.05$).

¹n = 4.

turbidity, protein content, and lipid content of the supernatant and filtrate were measured. The turbidity was determined by reading the absorbance of sample solutions using a spectrophotometer (Cary 50 Bio, Varian Inc., Palo Alto, CA) at 600 nm. Protein concentration was determined using the BioRad protein assay method (BioRad, Hercules, CA) based on the Bradford method. Bovine serum albumin (1 mg of protein/mL, Sigma-Aldrich, St. Louis, MO) was used as a reference protein. The absorbance at 595 nm after 30 min of reaction with Bradford solution was measured using a spectrophotometer. Lipid content was measured using Folch's method (Folch et al., 1957).

The concentrated sample solution by ultrafiltration was further purified using either cation exchange chromatography or the ammonium sulfate precipitation method. For cation exchange chromatography, an aliquot of sample was loaded onto a column (6 mL of bed volume), packed with preswollen carboxymethyl cellulose (Sigma-Aldrich), and equilibrated with 200 mM citrate-phosphate buffer, pH 5.0. The column was washed 2 times with 9 mL of 20 mM citrate-phosphate buffer, pH 5.0, and eluted with 200 mM citrate-phosphate buffer, pH 6.4. The elution profiles of samples were plotted, and fractions that make a peak were pooled and analyzed for antibody activity and purity using ELISA and SDS-PAGE, respectively.

For the ammonium sulfate precipitation method, the concentrated sample by ultrafiltration was first precipitated by 40% ammonium sulfate at 4°C. Then, the pellet was resuspended in 0.01 M Tris-HCl (pH 8.0) to a volume equal to half of the supernatant. The sample was precipitated by 40% saturated ammonium sulfate again at 4°C, and the pellet was dissolved in PBS, pH 7.4, and dialyzed against 10 mM phosphate buffer, pH 7.0, for 24 to 48 h to remove NaCl. The schematic diagram for the isolation of IgY from egg yolk is shown in Figure 1. Antibody activity and purity were determined using ELISA and SDS-PAGE, respectively. All the sample preparation processes were replicated 4 times.

SDS-PAGE

Sodium dodecyl sulfate-PAGE was done under nonreducing conditions using Mini-PROTEAN II Cell (BioRad) following instruction of the manufacturer. The purity of

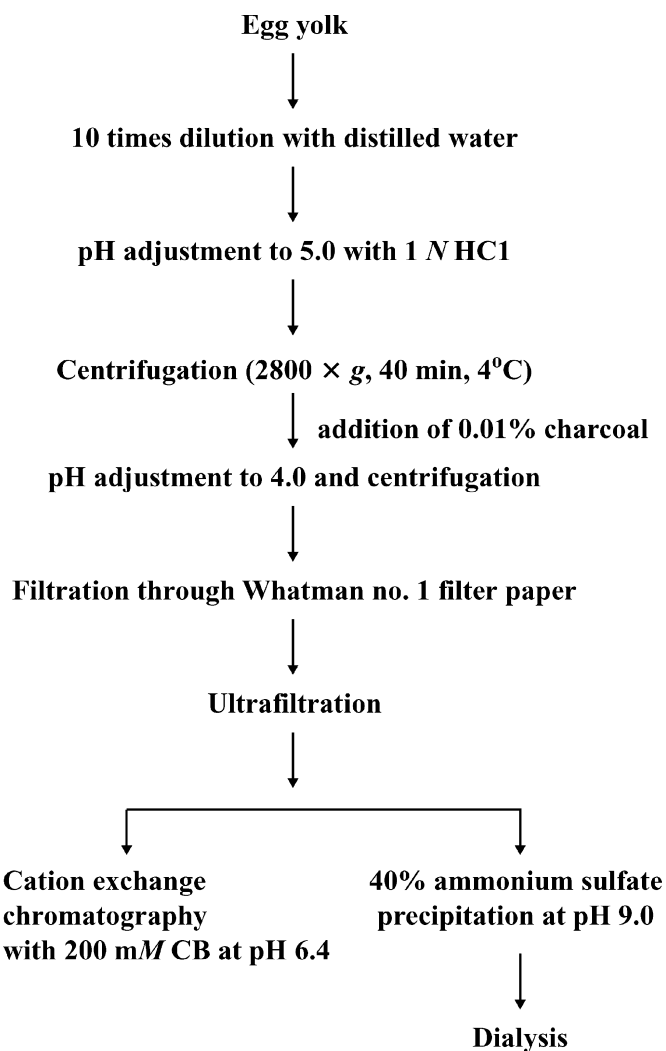


Figure 1. Schematic diagram for the isolation of IgY from egg yolk. CB = citrate-phosphate buffer.

various IgY preparations was estimated using 10% SDS-PAGE, and Coomassie brilliant blue R-250 (BioRad) was used to visualize the protein bands. Broad-range SDS-PAGE molecular weight standards of 44 to 200 kDa (BioRad) were used as markers. Quantification of proteins and determination of the molecular weight of protein bands were conducted with a Pharmacia Phast Imagine Gel Analyzer using the AlphaEaseFC software (Alpha Innotech Corp., San Leandro, CA).

ELISA

Immulon I micro titer 96-well plates (Dynatec Laboratories, McLean, VA) were used as the solid support. Polystyrene 96-well plates (Nalge Nunc Int., Rochester, NY) were coated with 100 μ L of IgY samples dissolved in a coating solution (0.05 M carbonate buffer, pH 9.6) and incubated overnight at 4°C. After washing the wells 3 times with PBS-Tween (10 mM phosphate, 0.15 M NaCl, pH 7.2, 0.05% Tween 20), 300 μ L/well of blocking solution [1% BSA solution in PBS (10 mM phosphate, 0.15 M NaCl, pH 7.2)] was added. After incubating for 1 h at

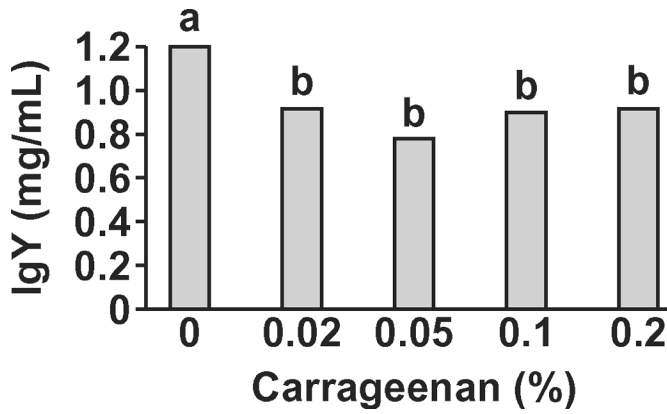


Figure 2. Effect of carrageenan on the yield of IgY from the water-soluble fraction of egg yolk solution. ^{a,b}Values with no common letter differ significantly ($P < 0.05$). $n = 4$.

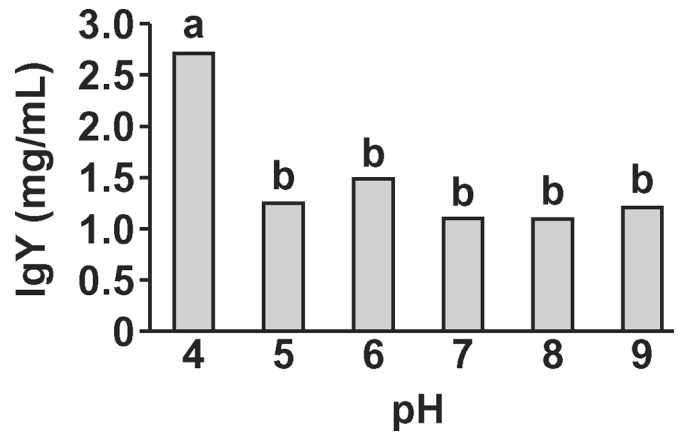


Figure 4. Immunoglobulin Y content in each pH condition in the addition of 0.01% charcoal to water-soluble egg yolk protein solution. ^{a,b}Values with no common letter differ significantly ($P < 0.05$). $n = 4$.

room temperature, the plate was washed with PBS-Tween. To each well of the plate, 100 μ L of primary anti-chicken IgG (1:10,000 solution diluted with 1% BSA-conjugated alkaline phosphatase) was added and then incubated for 1 h. After washing with PBS-Tween, 50 μ L of *p*-nitrophenyl phosphate solution was added to each well as a substrate for color development and incubated for 30 min. The enzyme reaction was stopped by adding 50 μ L of 3 N NaOH, and the color developed was read on an ELISA plate reader (THERMOMax, Molecular Devices Corp., Sunnyvale, CA) with a 405-nm filter. For each plate, 3 controls were prepared: a positive control with reagent-grade chicken IgG (Sigma-Aldrich), a nonspecific antigen BSA as another control, and a negative control without antigen. All the procedures for ELISA were conducted at room temperature (about 25°C).

Statistical Analysis

Data were analyzed using SAS Institute software (Release 6.11, SAS Institute Inc., Cary, NC) by the generalized linear model procedure. The Student-Newman-Keuls

multiple range test was used to compare differences among means. Mean values and SD of mean were reported. Significance was defined at $P < 0.05$.

RESULTS AND DISCUSSION

pH Adjustment on Delipidation and IgY Extraction

Lipid content of supernatant from egg yolk solution without pH adjustment (pH 6.0) was 1.0%, whereas that adjusted to pH 5.0 was 0.08%. Also, there was a significant difference in the turbidity of supernatant between pH-adjusted and pH-nonadjusted egg yolk solutions (Table 1). The low turbidity of supernatant from the pH-adjusted egg yolk solution was attributed to better dilapidation at pH 5.0 than at pH 6.0. Also, a greater amount of IgY was extracted from pH-adjusted yolk than nonadjusted yolk.

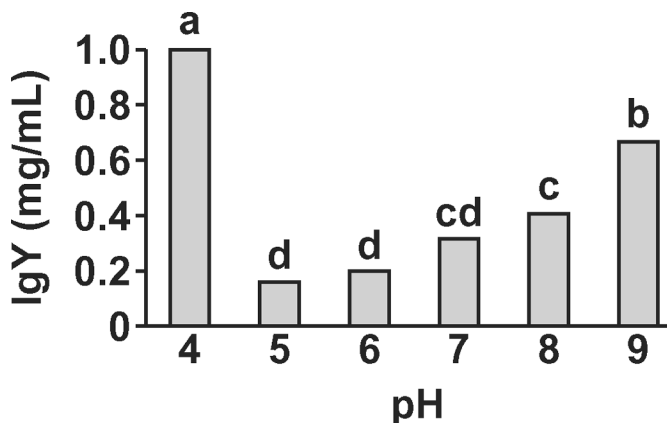


Figure 3. Changes of IgY content after adding 0.02% carrageenan to water-soluble fraction of egg yolk in different pH conditions. ^{a-d}Values with no common letter differ significantly ($P < 0.05$). $n = 4$.

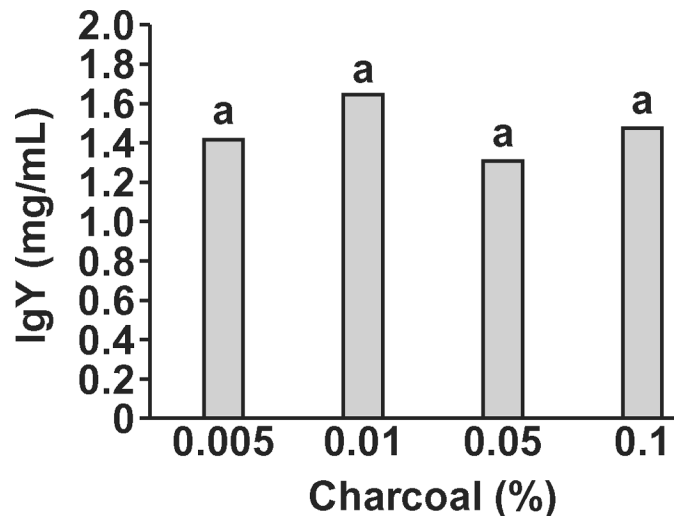


Figure 5. Comparison of IgY yields in charcoal amount added to water-soluble egg yolk protein solution.

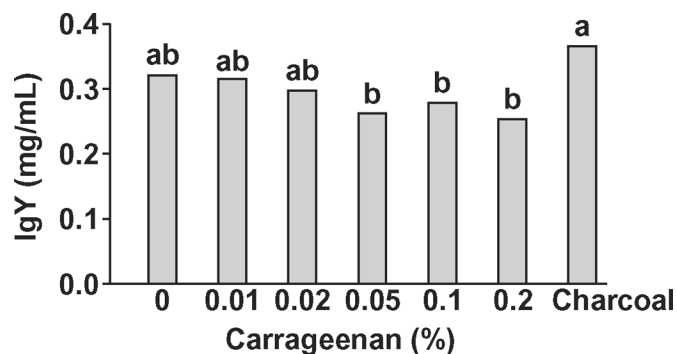


Figure 6. Comparison of IgY yields in the addition of different concentration of carrageenan and 0.01% charcoal to water-soluble egg yolk protein solution ^{a,b}Values with no common letter differ significantly ($P < 0.05$). $n = 4$.

Similar results were obtained by Chang et al. (2000), who reported that lowering the pH value of the water-soluble fraction of egg yolk from pH 6.0 to 5.0 resulted in a significant decrease in lipid content (from 6.0 to 7.5% to 1.6 to 7.5%). Akita and Nakai (1992) reported that the adjustment of pH to 5.0 and 10 times the dilution of yolk was crucial in removing lipids or lipoproteins from the water-soluble fraction of egg yolk. They reported that 93 to 96% of IgY was recovered after pH adjustment of water-soluble fraction to 5.0 to 5.2, and lowering the pH value of diluted egg yolk solution resulted in a clear water-soluble fraction. Sugano and Watanabe (1961) reported that the solubility of lipoproteins at low ionic strengths decreased as the pH of water-soluble fraction was adjusted to pH 4.3. The pH of water-soluble fraction was the most important factor for IgY recovery because pH influenced the interactions between polysaccharides and proteins and the precipitation of polysaccharide-lipopro-

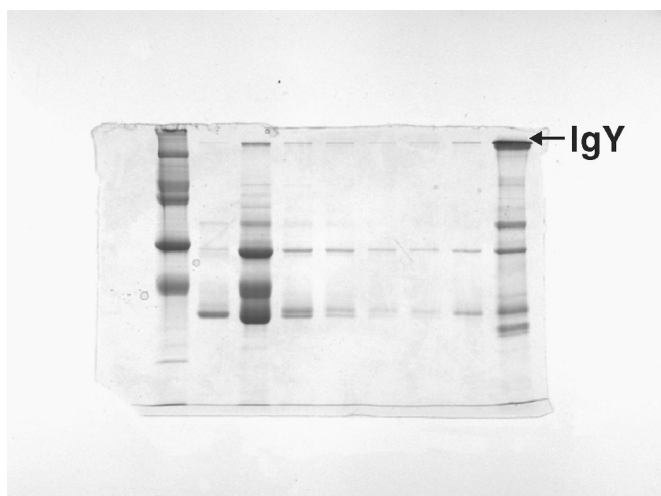


Figure 7. The SDS-PAGE patterns of egg yolk solutions obtained by cation ion exchange chromatography. Eight milliliters of concentrated water-soluble egg yolk solution by ultrafiltration was loaded on carboxymethyl cellulose chromatography, and the column was washed in 20 mM citrate phosphate buffer with pH 4.8. The IgY was eluted by 200 mM citrate phosphate buffer with pH 6.4. Lane 1 = marker; lane 2 = after loading; lanes 3 to 8 = washing; and lane 9 = elution.

tein complexes (Gurov et al., 1983; Akita and Nakai, 1992; Samant et al., 1993). The protein contents of the water-soluble fraction in both pH-adjusted and pH-nonadjusted treatments were substantially underestimated because Bradford's dye-binding assay resulted in significantly lower readings for IgY than other methods (Sdemak and Grossberg, 1977; Hansen et al., 1998). However, no significant difference in protein contents between pH-adjusted and pH-nonadjusted treatments was observed. Akita and Nakai (1992) reported that the water-soluble fraction of egg yolk solution was free of lipids at the pH 4.6 and 5.2 range, but lipid contents increased at pH below and above this range. The pH of diluted egg yolk was 5.8 to 6.3 and increased during storage. Because it is difficult to separate lipids or lipoproteins at this pH, pH adjustment to 5.0 before centrifugation is important to eliminate lipids.

Temperature Effect on Extraction of Water-Soluble Proteins

Egg yolk antibodies were separated by centrifuging diluted egg yolk after pH adjustment to 5.0. The volume of supernatant obtained from frozen (24 h) egg yolk solution was lower than that of the control and refrigerated solution, but the turbidity of supernatants was the lowest among the treatments (Table 2). The amounts of IgY in supernatant obtained after incubation at different temperature conditions was not significantly different. Also, there were no significant differences in total amount of IgY. Kim and Nakai (1996) and Yokoyama et al. (1993) reported that incubation of diluted egg yolk solution at freezing (-20°C) or refrigeration temperature (4°C) was helpful in removing lipoproteins from the water-soluble fraction. Larsson et al. (1993) reported that IgY was stable for 5 yr of storage at cold temperature (4°C) without changes in antibody activities. However, our result indicated that some IgY was precipitated during freezing and lost during storage at cold temperature, probably due to irreversible aggregation under these conditions.

Effect of NaCl in IgY Precipitation

As the concentration of NaCl in egg yolk solution increased, the turbidity and protein content in supernatant after centrifugation increased (Table 3). The solubility of proteins was significantly increased by the added NaCl, but IgY content was not increased. Gallaher and Vass (1970) and Kim and Nakai (1998) reported that addition of NaCl facilitated the separation of IgY from other proteins by polymerizing IgY molecules. Aggregation of IgY had no effect on Fab fragments of chicken antibody, but Fc fragments were precipitated by high NaCl concentration (Kubo and Benedict, 1969). Akita and Nakai (1992), however, reported that 0.16 M NaCl resulted in an inhibitory effect in separating egg yolk granules from plasma proteins in diluted egg yolk solution, and the adverse effect of NaCl increased as the concentration of NaCl increased. In addition, if NaCl is added during extraction of IgY, a

dialysis step to remove the NaCl is necessary. Therefore, addition of NaCl to the diluted egg yolk solution would not be beneficial.

Carrageenan and Charcoal Effect on IgY Isolation

Ultrafiltration is an important tool for the large-scale preparation of IgY from egg yolk by concentrating the supernatant of egg yolk solution that contains IgY. However, lipids or lipoproteins in the water-soluble fraction (supernatant) should be minimized to prevent clogging of the ultrafiltration membrane and improve filtration efficiency. Charcoal and carrageenan decreased the amount of lipids or lipoproteins in the water-soluble fraction of egg yolk. The optimal condition for the highest recovery of IgY from the the water-soluble fraction of egg yolk was removing lipoproteins using 0.02% of λ -carrageenan at pH 4.0 (Figures 2 and 3) or 0.01% charcoal at pH 4.0 (Figures 4 and 5). Chang et al. (2000) reported that 0.1% λ -carrageenan at pH 5.0 was the optimal condition for the removal of lipoproteins from 6-fold diluted egg yolk solution. However, our result indicated that the recovery of IgY at pH 5.0 was lower than that at pH 4.0. This should be caused by the differences in dilution of egg yolk. Even though some researchers suggest that addition of λ -carrageenan results in effective delipidation of egg yolk solution (Hatta and Kim, 1990; Kim and Nakai, 1996), some IgY should be precipitated by electrostatic forces that occur from interactions between proteins and polysaccharides (Imeson et al., 1978). Addition of 0.01% charcoal at pH 4.0 produced the highest IgY yield among the carrageenan and charcoal treatments (Figure 6).

Cation Exchange Chromatography

The purity of IgY solution obtained by cation exchange chromatography was not high and contained many egg yolk proteins other than IgY (Figure 7). MacCannel and Nakai (1989) used DEAE-Sephacel anion exchange chromatography to separate IgY from the water-soluble fraction of egg yolk and found that the purity of IgY was 50 to 60%. The antibody preparation recovered by cation exchange chromatography had 60 to 69% of purity (Fichtali et al., 1992, 1993). Therefore, we assumed that there are some limitations such as low purity and efficiency when ion exchange chromatography is used to purify IgY.

Ammonium Sulfate Precipitation

The precipitation of IgY using 40% ammonium sulfate was influenced by pH of the solution, and the optimal conditions for the highest yield of IgY was at pH 9.0 (Figure 8). The purity of IgY obtained after the second precipitation of the first precipitant using 40% ammonium sulfate at pH 9.0 was also significantly improved (Figure 9). This suggested that 2-time precipitation of IgY using 40% ammonium sulfate at pH 9.0 produced IgY with much higher purity than cation exchange chromato-

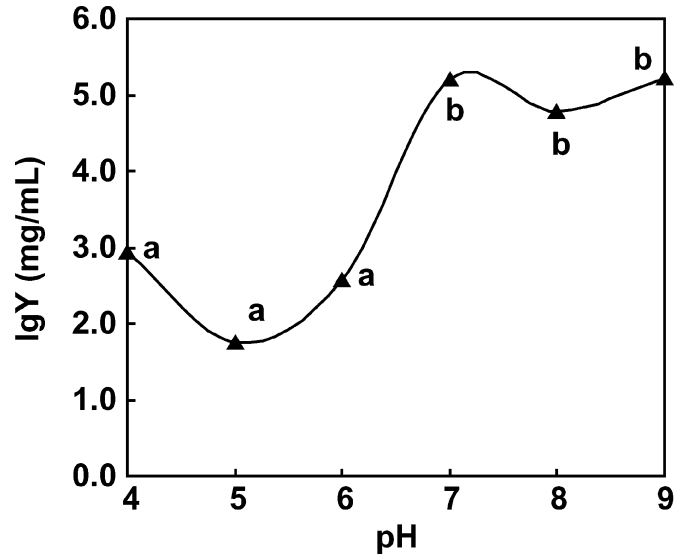


Figure 8. Changes of IgY yields on each pH condition in precipitation of 40% ammonium sulfate with concentrated water-soluble egg yolk solution by ultrafiltration. ^{a,b}Values with no common letter differ significantly ($P < 0.05$). $n = 4$.

graphy, and the efficiency of IgY purification was greater than the cation exchange chromatography methods. Akita and Nakai (1992) reported that use of 60% ammonium sulfate produced a high IgY recovery and removed contaminating proteins, especially 38 and 53 kDa proteins.

Recovery of IgY

The recovery rates of IgY at each purification step are shown in Table 4. Ammonium sulfate (40%) and 0.01% charcoal protocol were used for the recovery study. Addition of charcoal lowered the turbidity of supernatant by

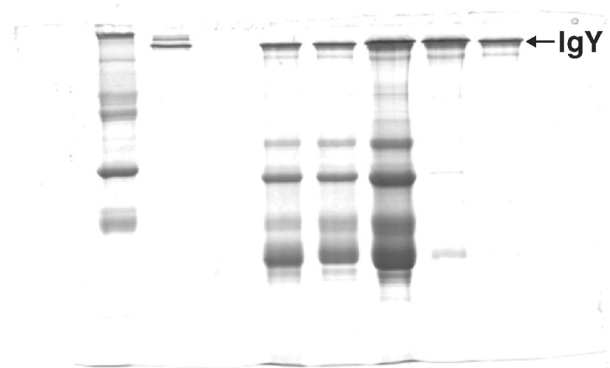


Figure 9. The SDS-PAGE patterns of sample fractions obtained from each step, including centrifugation, ultrafiltration, and 40% ammonium sulfate precipitation. Lane 1 = marker; lane 2 = chicken IgG; lane 3 = after centrifugation of water-soluble fraction; lane 4 = addition of 0.01% charcoal following difiltration; lane 5 = after ultrafiltration; lane 6 = after first precipitation by 40% ammonium sulfate at pH 9.0; and lane 7 = second precipitation with ammonium sulfate.

Table 4. Yields of IgY at each processing step¹

Purification step	Yield (%)
Water-soluble fraction	100
Charcoal and diafiltration	103.47 ± 0.03
Ultrafiltration	80.02 ± 13.72
Ammonium sulfate (first)	74.82 ± 12.57
Ammonium sulfate (second)	69.07 ± 7.58

¹n = 4.

removing lipoprotein or lipids that existed in the supernatant of egg yolk solution. This step caused no loss of IgY but speeded up the flow of ultrafiltration. About 20% of IgY was lost at the ultrafiltration step, which removes proteins smaller than 50 kDa from the supernatant. Kim and Nakai (1998) also observed 15 to 20% loss in IgY by ultrafiltration. However, the percentage loss of IgY can be reduced if larger volumes of supernatant are used for ultrafiltration. Kim and Nakai (1996) suggested that direct application of the water-soluble fraction to ultrafiltration resulted in loss of IgY due to clogging of mucous lipoproteins on the membrane.

The first precipitation of the water-soluble fraction with 40% ammonium sulfate showed 75% IgY yield (Table 4). The second precipitation of IgY using 40% ammonium sulfate, however, resulted in 69% yield of IgY (Table 4 and Figure 9). Even though 6% of IgY was lost at the second precipitation step, the purity of IgY increased significantly. Akita and Nakai (1992) used 60% ammonium sulfate precipitation for purification of IgY, which resulted in 89% of IgY yield with 30% purity. Svendsen et al. (1995) used 25 to 45% of ammonium sulfate precipitation, which resulted in 58% yield of IgY with high purity. Most of the studies for purification or separation of specific proteins used combination methods adopting ion exchange or gel filtration chromatography for further purification after precipitation with ammonium sulfate at the first step. However, the protocol developed in this study depends largely on ammonium sulfate precipitation at pH 9.0 after charcoal addition to the water-soluble fraction to remove extra lipoproteins. In conclusion, the ammonium sulfate precipitation method produced IgY with higher purity and yield than the HPLC (Stec et al., 2004) or ion exchange chromatography method and was suitable for a large-scale IgY preparation from egg yolk, particularly with minimal steps.

REFERENCES

- Akerstrom, B., T. Bodin, K. Reis, and L. Bjorck. 1985. Protein: A powerful tool for binding and detection of monoclonal and polyclonal antibodies. *J. Immunol.* 135:2589–2592.
- Akita, E. M., and S. Nakai. 1992. Immunoglobulins from egg yolk: Isolation and purification. *Food Sci.* 57:629–634.
- Akita, E. M., and S. Nakai. 1993. Comparison of four purification methods for the production of immunoglobulins from eggs laid by hens immunized with an enterotoxigenic *E. coli* strain. *J. Immunol. Methods* 160:207–214.
- Bade, H., and H. Stegemann. 1984. Rapid method of extraction of antibodies from hen egg yolk. *J. Immunol. Methods* 72:421–426.
- Camenisch, G., M. Tini, D. Chilow, I. Kvietikova, V. Srinivas, J. Caro, P. Spielmann, R. H. Wenger, and M. Gassmann. 1999. General applicability of chicken egg yolk antibodies: The performance of IgY immunoglobulins raised against the hypoxia-inducible factor 1 α . *FASEB* 13:81–88.
- Carlander, D., J. Stalberg, and A. Larsson. 1999. Chicken antibodies: A clinical chemistry perspective. *Ups. J. Med. Sci.* 104:179–189.
- Chang, H. M., T. C. Lu, C. C. Chen, Y. Y. Tu, and J. Y. Hwang. 2000. Isolation of immunoglobulin from egg yolk by anionic polysaccharides. *J. Agric. Food Chem.* 48:995–999.
- Davalos-Pantoja, L., J. L. Orteta-Vinuesa, D. Bastas-Gonzalez, R. Hidalgo-Alvarez. 2000. A comparative study between the adsorption of IgY and IgG on latex particles. *J. Biomater. Sci. Polym. Ed.* 11:657–673.
- Erhard, M. H., P. Schmidt, P. Zinsmeister, A. Hoffman, U. Munster, B. Kaspers, K. H. Wiesmuller, and W. G. Bessler. 2000. Adjuvant effects of various lipopeptides and interferon- γ on the humoral immune response of chickens. *Poult. Sci.* 79:1264–1270.
- Fichtali, J., E. A. Charter, K. V. Lo, and S. Nakai. 1992. Separation of egg yolk immunoglobulin using an automated liquid chromatography system. *Biotechnol. Bioeng.* 40:1388–1394.
- Fichtali, J., E. A. Charter, K. V. Lo, and S. Nakai. 1993. Purification of antibodies from industrially separated egg yolk. *J. Food Sci.* 58:1282–1285, 1290.
- Folch, J., M. Less, and G. M. Slaone-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226:497–509.
- Fryer, J., J. Firca, J. Leventhal, B. Boondie, A. Malcolm, D. Lyan-cic, R. Gandhi, A. Shah, W. Pao, M. Abecassis, D. Kaufman, F. Stuart, and B. Anderson. 1999. IgY antiporcine endothelial cell antibodies effectively block human antiporcine xenoantibody binding. *Xenotransplantation* 6:98–109.
- Gallaher, J. S., and E. W. Voss Jr. 1970. Immune precipitation of purified chicken antibody at low pH. *Immunochemistry* 7:771–785.
- Gurov, A. N., M. A. Mukhin, N. A. Larichev, N. V. Lozinskaya, and V. S. Tolstoguzoc. 1983. Emulsifying properties of proteins and polysaccharides. I. Methods of determination of emulsifying capacity and emulsion stability. *Colloids Surf.* 6:35–42.
- Hansen, P., J. A. Scoble, B. Hanson, and N. J. Hoogenraad. 1998. Isolation and purification of immunoglobulins from chicken eggs using thiophilic interaction chromatography. *J. Immunol. Methods* 215:1–7.
- Hatta, H., and M. Kim. 1990. A novel isolation method for hen egg yolk antibody, "IgY". *Agric. Biol. Chem.* 54:2531–2535.
- Hatta, H., J. S. Sim, and S. Nakai. 1988. Separation of phospholipids from egg yolk and recovery of water-soluble proteins. *J. Food Sci.* 53:425–427, 431.
- Imeson, A. P., R. R. Watson, J. R. Mitchell, and D. A. Ledward. 1978. Protein recovery from blood plasma by precipitation with polyuronates. *J. Food Technol.* 13:329–338.
- Jensenius, J. C., I. Andersen, J. Hau, M. Crone, and C. Koch. 1981. Eggs: Conveniently packed antibodies. Methods for purification of yolk IgG. *J. Immunol. Methods* 46:63–68.
- Kim, H., and S. Nakai. 1996. Immunoglobulin separation from egg yolk: A serial filtration system. *J. Food Sci.* 61:510–514.
- Kim, H., and S. Nakai. 1998. Simple separation of immunoglobulin from egg yolk by ultrafiltration. *J. Food Sci.* 63:485–490.
- Kubo, R. T., and A. A. Benedict. 1969. Comparison of various avian and mammalian IgG immunoglobulins for sal-induced aggregation. *J. Immunol.* 103:1022–1028.
- Larsson, A., R. Balow, T. L. Lindahl, and P. Forsberg. 1993. Chicken antibodies: Taking advantage of evolution —A review. *Poult. Sci.* 72:1807–1812.
- Larsson, A., A. Karlsson-Parra, and J. Sjoquist. 1991. Use of chicken antibodies in enzyme immunoassays to avoid interference by rheumatoid factors. *Clin. Chem.* 37:411–414.

- Larsson, A., P. Wejaker, P. Forsberg, and T. Lindahl. 1992. Chicken antibodies: A tool to avoid interference by complement activation in ELISA. *J. Immunol. Methods* 156:79–83.
- MacCannel, A. A., and S. Nakai. 1989. Isolation of egg yolk immunoglobulins into subpopulations using DEAE-ion exchange chromatography. *Can. Inst. Food Sci. Technol. J.* 23:42–46.
- McLaren, R. D., C. G. Prosser, C. J. Grieve, and M. Borissenko. 1994. The use of caprylic acid for the extraction of the immunoglobulin fraction from egg yolk of chicken immunized with ovine α -lactalbumin. *J. Immunol. Methods* 177:175–184.
- Ntakarutimana, V., P. Demedts, M. Van Sande, and S. Scharpe. 1992. A simple and economical strategy for downstream processing of specific antibodies to human transferrin from egg yolk. *J. Immunol. Methods* 153:133–140.
- Polson, A., T. Coetzer, J. Kruger, E. Maltzahn, and K. J. van der von Merwe. 1985. Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. *Immunol. Invest.* 14:323–327.
- Ruan, G. P., L. Ma, X. W. He, M. J. Meng, Y. Zhu, M. Q. Zhou, Z. M. Hu, and X. N. Wang. 2005. Efficient production, purification, and application of egg yolk antibodies against human HLA-A*0201 heavy chain and light chain (β 2m). *Protein Expr. Purif.* 44:45–51.
- Samant, S. K., R. S. Singhal, P. R. Kulkarni, and D. V. Rege. 1993. Protein-polysaccharide interaction: A new approach in food formulations. *Int. J. Food Sci. Technol.* 28:547–562.
- Schade, R., and A. Hlinak. 1996. Egg yolk antibodies, state of the art and future prospects. *ALTEX* 13:5–9.
- Schwarzkopf, C., and B. Thiele. 1996. Effectivity of different methods for the extraction and purification of IgY. *ALTEX* 13:35–39.
- Sdemak, J. J., and S. E. Grossberg. 1977. A rapid, sensitive and versatile assay for protein using Coomassie brilliant blue G250. *Anal. Biochem.* 79:544–552.
- Sriram, V., and G. Ygeeswaran. 1999. Improved recovery of immunoglobulin fraction from egg yolk of chicken immunized with AsialoGM1. *Russ. J. Immunol.* 4:131–140.
- Stalberg, J., and A. Larsson. 2001. Extraction of IgY from egg yolk using a novel aqueous two-phase system and comparison with other extraction methods. *Ups. J. Med. Sci.* 106:99–110.
- Stec, J., L. Bicka, and J. Kuzmak. 2004. Isolation and purification of polyclonal IgG antibodies from bovine serum by high performance liquid chromatography. *Bull. Vet. Inst. Pulawy* 48:321–327.
- Sugano, H., and I. Watanabe. 1961. Isolation and some properties of native lipoproteins from egg yolk. *J. Biochem. (Tokyo)* 50:473–480.
- Svensen, L., A. Croeley, L. H. Ostergaard, G. Stodulski, and J. Hau. 1995. Development and comparison of purification strategies for chicken antibodies from egg yolk. *Lab. Anim. Sci.* 45:89–93.
- Verdoliva, A., G. Basile, and G. Fassina. 2000. Affinity purification of immunoglobulins from chicken egg yolk using a new synthetic ligand. *J. Chromatogr. B Biomed. Sci. Appl.* 749:233–242.
- Wooley, J. A., and J. Landon. 1995. Comparison of antibody production to human interleukin-6 (IL-6) by sheep and chickens. *J. Immunol. Methods* 178:253–265.
- Yokoyama, H., R. C. Peralta, S. Sendo, Y. Ikemori, and Y. Kodama. 1993. Detection of passage and absorption of chicken egg yolk immunoglobulins in the gastrointestinal tract of pigs by use of enzyme-linked immunosorbent assay and fluorescent antibody testing. *Am. J. Vet. Res.* 54:867–872.