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Thesis  
For the Degree of Master of Science

# Sequential Separation of Value-Added Components from Egg Yolk

난황으로부터 부가가치물질들의  
연속분리공정에 관한 연구

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연속분리공정에 관한 연구

지도교수 안 동 욱

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## SUMMARY

Egg yolk contains a number of components that can play significant functional roles in foods and human. Although some of the egg yolk components are already isolated and commercialized, the separation methods are largely limited to a single component and the rest are discarded. Therefore, majority of vital natural resources can be wasted. At present, most of the developed methods for the separation of value-added components from egg yolk are for laboratory scale and cannot be used in commercial scales.

The objectives of this research are 1) to compare currently available separation methods for value-added elements from egg yolk, 2) to develop an efficient scale-up preparation method for each element for the practical use of egg components, and 3) to develop a sequential separation method for IgY, phosvitin, and phospholipids and neutral lipids from egg yolk. Because the separated components will be used for humans, we try to minimize or eliminate the use of harmful chemicals and organic solvents in the separation methods.

The main tools used for the separation of value-added components from egg yolk include dilution with water, pH adjustment, centrifugation, ultrafiltration, and salt precipitation. First, the phosvitin-containing granules were separated from the rest of the egg yolk components using 2 volumes of water (3x dilution) and centrifuged. The precipitant was collected and phosvitin was extracted using 10% NaCl (w/v) in 0.05N NaOH solution and homogenization. The homogenate was diluted with DW and the pH adjusted to 4.0, and then precipitant was removed by centrifugation. The resulting supernatant was desalted and concentrated using ultrafiltration, and the impurities were removed by heat treatment.

After centrifugation, the phosvitin-containing supernatant was freeze-dried.

The supernatant of the 3x-diluted egg yolk was used to separate IgY and lipids. The supernatant fraction was diluted with 3 volumes of water (4x dilution) and centrifuged. Phospholipids and neutral lipids were extracted from the solid fraction using ethanol and hexane, respectively. Phospholipids were collected after removing ethanol from the ethanol extract, and neutral lipids was recovered after removing hexane using a rotary evaporator. The liquid fraction was concentrated using ultrafiltration and then IgY was precipitated using 20% saturated  $(\text{NH}_4)_2\text{SO}_4$ +15% NaCl (w/v). The precipitant containing IgY was dissolved in water, desalted using ultrafiltration, and then freeze dried. The SDS PAGE and Western Blot analyses indicated that the phosvitin separated had over 90% purity and 91% yield, and IgY had over 90% purity and 80% yield.

The method developed protocol can not only be applied for individual component but also for sequential separation of multiple components, can be immediately applicable for scale-up processing, and the separated components can be used for humans as well as drug industry because no toxic compounds or organic solvents were used in the separation processes.

Keywords: Egg yolk, IgY, Phosvitin, phospholipids, neutral lipids,  
sequential separation, large scale production

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# **CHAPTER 1**

## **GENERAL INTRODUCTION**

Egg is considered as a complete food that contains all essential nutrients. However, total egg consumption in developed countries has declined steadily during past 50 years due mainly to high cholesterol and fat content in eggs. Medical communities have been categorized egg as a non-healthy, cholesterol-loaded high-fat food, and discouraged public from consuming it. The amount of cholesterol in a typical large egg (60 g) is 213 mg, which are over 75% of the daily recommended dietary amount for humans, and fat content in egg yolk is 33% of liquid egg yolk. Numerous efforts have been made to decrease cholesterol from eggs by dietary manipulation of hens, or cholesterol and fat content by chemical extraction from yolk and egg products only with limited success.

One of the major issues for the sustainable egg industry is the efficient movement of egg and egg products through market channels. Use of processed egg products, such as dried whole egg, yolk powder and liquid eggs in food processing can be a way to increase the consumption of egg but the increase through the conventional way is limited. Therefore, developing new value-added products and diversifying the utilization of egg in new food or non-food products is necessary. Egg yolk contains almost all the fat and cholesterol of egg, which negatively influence the consumption of egg. Increased utilization of egg yolk products, therefore, is the most critical factor increasing egg consumption. There had been sporadic attempts to separate few value-added components from egg yolk, but nobody used systemic approach to separate several useful components from egg yolk sequentially. Separation of value-added components from egg yolk is complicated and costly, but can increase the value of egg hundreds folds and expand the use of egg components to non-food products (e.g., pharmaceutical or antimicrobial agents).

Over the past several decades, many studies have been done on functional properties of bioactive compounds of egg but the materials they

used are from the intricate separation methods using organic solvents. And the use of organic solvents makes these materials to be classified as environmental waste to bring negative effect on the overall study.

Moreover, the separation efforts are largely limited to a single component and some of the separated components from egg yolk which are already commercialized are too expensive for the practical uses. The main problem for developing separation methods for a single component is that most of the residuals after separation of a target component is discarded, leading great loss of economical process and value for utilization of value added components from egg yolk. Therefore, development of a continuous separation method for multiple components from egg yolk with scale-up capability is important.

To develop an appropriate separation method for multiple value added components from egg yolk, each step should be focused on making simple, economic, and environment-friendly method for large scale production. Previous methods used organic solvents which caused low yield and are difficult to scale up. However, our approaches do not use harmful chemicals and solvents for protein separation and minimally use food-grade solvents for the extraction of lipids. The limited use of organic solvents minimizes the environmental wastes. Furthermore, this study does not require many chromatography methods such as affinity, ion-exchange, and gel filtration to increase the purity of separated value-added components because these columns are relatively expensive and inadequate for large scale separation.

To develop optimal continuous process for scale-up production, our separation method involves simple steps so as to bring high efficiency in isolating important elements from egg yolk with high purity and yield. General characteristics of egg yolk, value-added components from egg yolk, and the objective of this study will be reviewed in Chapter 2.

Separation methods on each egg yolk components are introduced in the subsequent Chapters: Separation method of immunoglobulin Y is covered in Chapter 3, Purification method for phosvitin without using solvents is described in Chapter 4, and separation of phospholipids and neutral lipids is in Chapter 5. Lastly, Chapter 6 discusses the overall sequential separation of value-added components from egg yolk.

## **CHAPTER 2**

### **LITERATURE REVIEW**

## **1. Overview of the egg yolk**

### **1.1. General characteristics and chemical composition**

The chicken eggs have been recognized as an excellent source of vital nutrients such as proteins, lipids, vitamins, and minerals for humans. Egg yolk is also a multifunctional ingredient widely used in many food products such as mayonnaise, salad dressings, and creams due to its emulsifying, gelling, and antioxidant properties. Each constituent of yolk possesses physical and chemical characteristics responsible for its own functional properties. These functional properties are greatly influenced not only by the environmental conditions such as pH and ionic strength but also by processing processes such as heating, freezing, and drying.

Egg yolk comprises about 30 to 36% of chicken egg. The dry matter of fresh egg yolk varies between 50 to 52% depending upon the age of laying hens and the duration of storage. Fresh egg yolk is composed of 51.1% water, 3.6% lipids, 16.0% proteins, 0.6% carbohydrates, and 1.7% minerals while dried yolk is composed of 62.5% lipids, 33.0% proteins, 1.2% carbohydrates, and 3.5% minerals (Powrie and Nakai, 1986). The main components of egg yolk are lipids and lipid to protein ratio is about 2:1 in dry matter. Lipids of yolk are highly associated with lipoprotein assemblies. They are composed of 62% tryglycerides, 33% phospholipids, and less than 5% cholesterol. Carotenoids represent less than 1% of yolk lipids, and are responsible for yolk color. Proteins exist as free and apoprotein forms. The interactions between lipids and proteins form lipoproteins such as low and high density lipoproteins, which constitute the major yolk components. Seventeen percent of liquid egg yolk is protein, which is composed of lipovitellins (36%), livetins (38%),



phosvitin (8%), and low-density lipoproteins (17%).

## **1.2. Structure of Egg yolk**

Egg yolk can be separated into plasma and granule fractions after 2x dilution with 0.3 M NaCl and centrifugation (McBee and Cotterill, 1979). The plasma makes up 77-81% of yolk dry matter and is composed of 85% LDL and 15% livetins (Burley and cook, 1961). It forms an aqueous phase where yolk particles are in suspension. It represents about 90% of yolk lipids and 50% of yolk proteins. Plasma contains approximately 73% lipids, 25% proteins, and 2% ash. Lipids of plasma are composed of 70% triglycerides, 25% phospholipids, and 5% cholesterol.

Granules represent 19-23% of yolk dry matter, accounting for about 50% of yolk proteins and 7% of yolk lipids (Anton and Gandemer, 1997). Lipids in granules are composed of 60% triglycerides, 35% phospholipids, and 5% cholesterol and are mainly distributed in HDL (70%) and LDL (30%) proteins. Granules are prone to make insoluble HDL-phosvitin complexes due to phosphocalcic bridges between phosphate groups of phosvitin and HDL at low ionic strength (Burley and Cook, 1961) because phosvitin contains large amount of highly phosphorylated serine residues (Causeret et al., 1991). These bridges make the granule structure very compact and poorly hydrated, which limits access by enzymes and protects against thermal denaturation and heat gelation. It is reported that the phosphocalcic bridges could be disrupted at high ionic strength (0.3M NaCl) (Cook and Martin, 1969). In this condition, the solubility of granules can be increased up to 80% as phosivitin is a water soluble protein and high density lipoproteins behave as soluble proteins (Anton and Gandemer, 1997). However, complete

disruption of granules occurs when the ionic strength becomes very high (1.71 M NaCl). On the other hand, acidification and alkalization are also able to disrupt granules and solubilize these constituents due to the electrostatic repulsion by positive and negative charges in those conditions.

## **2. Value-added components from egg yolk**

### **2.1. Egg yolk antibody (Immunoglobulin Y)**

The protein represents approximately 17.0% of egg yolk. One of the major protein fractions is livetin and is composed of  $\alpha$ -livetins (serum albumin),  $\beta$ -livetins ( $\alpha_2$ -glycoprotein), and  $\gamma$ -livetins ( $\gamma$ -globulin, IgY) (Sugino et al., 1997). All livetins are water soluble and the relative portion of the three livetins in egg yolk is 2:5:3 (Bernardi and Cook, 1960). Immunoglobulin Y is the predominant fraction of  $\gamma$ -livetins and is one of the most vital components of the livetin fraction (Kovacs-Nolan et al., 2005).

The  $\gamma$ -livetins in egg yolk are transported from the blood of chicken. Among the three immunoglobulins (IgM, IgA, and IgG) in the serum, only IgG can be transferred to yolk (Hatta et al., 1997). The  $\gamma$ -globulins or  $\gamma$ -livetins in egg yolk are referred to as immunoglobulin Y to distinguish them from mammalian IgG. Even though IgY is derived from chicken serum IgG, it has many different chemical and structural characteristics from mammalian IgG (Kovacs-Nolan and Mine, 2004). As IgG, IgY molecule has two light and heavy chains. However, the molecular weight of the heavy chains is greater than that of the mammalian IgG (180kDa for IgY and 150kDa for mammalian IgG).

Moreover, IgY heavy chain lacks in a hinge region, which results in a reduced flexibility of the Fab moiety. This structural difference can be a reason for the differences in the functional properties between IgY and IgG and antigen epitope recognition (Warr et al., 1995; Sun et al, 2001). IgY has a lower isoelectric point, does not associate with mammalian complement or rheumatoid factors, and binds less with human and bacterial Fc receptors (Kovacs-Nolan and Mine, 2004).

Recently, many research groups studied the use of IgY as an oral passive immunization against various bacteria and viruses (Kovacs-Nolan and Mine, 2004). Oral administration of IgY has proven effective to treat a variety of gastrointestinal pathogens and against dental plaque formation in humans (Smith et al., 2001), indicating that IgY can be used to cure various animal and human diseases, immunodiagnostic, and pathogen control.

## **2.2. Phosvitin**

Phosvitin is a phosphoglycoprotein that contains about 10% phosphorus and comprises approximately 7% of egg yolk proteins (Mecham and Olcott, 1949). It is one of the most highly phosphorylated proteins in nature. More than 50% of the amino acids in phosvitin are serine and out of which 90% are phosphorylated (Clark, 1985). Phosvitin is a mixture of two polypeptides including  $\alpha$ -phosvitin and  $\beta$ -phosvitin.  $\alpha$ -Phosvitin contains three or four subunits of 35 to 40 kDa and  $\beta$ -phosvitin contains four or five subunits of 45 kDa (Ito et al., 1962).

Phosvitin predominantly forms a random coil conformation at neutral pH while a  $\beta$ -sheet predominates at acidic pH (Taborski, 1974; Damodaran and Xu, 1996). Phosvitin is water soluble protein with

isoelectric point of 4.0 (Ternes, 1989), and is highly charged at neutral pH. Phosvitin is synthesized from a precursor, vitellogenin where high density lipoproteins are also derived (Taborsky, 1983). After cleavage of the precursor, the two polypeptides are liberated but they interact through phosphocalcic bridges to form the granular structure. Phosvitin is poorly modified by thermal treatment because of its unordered structure. Even heating at 110 °C for 20 minutes does not cause its aggregation or insolubility (Albright et al., 1984).

Phosvitin has a strong metal-chelating capability due to its polyanionic character. The unique primary structure of phosvitin enables this protein as one of the strongest metal chelating agents (Byrne et al., 1984). Whole egg contains approximately 2 mg of iron per 100 g, and 95% of egg yolk iron is bound to phosvitin (Albright et al, 1984). Phosvitin also can bind calcium and is possible to be involved in calcium and iron metabolism during embryonic development (Richards, 1997). Moreover, phosvitin structure is affected by pH because of modifications in electrostatic repulsion (Yasui et al., 1990). Even if the interaction between iron and phosvitin reduces electrostatic repulsion, iron binding does not affect the structure of phosvitin (Taborsky, 1980).

### **2.3. Phospholipids and Neutral lipids**

Lipids are the major component of egg yolk solids, and are composed of 65% triglycerides, 28-30% phospholipids, and 4-5% cholesterol. Phospholipids are amphiphilic molecules that contain one hydrophilic head group and one hydrophobic group. The major components of egg yolk phospholipids are phosphatidylcholine (PC), which is 76% of total phospholipids, and phosphatidylethanolamine (PE),

which make up approximately 22% of the phospholipids. Phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), cardiolipins (CL), lysoPC, and lysoPE are also components of yolk phospholipids. Egg yolk contains three-fold higher levels of phosphatidylcholine than natural soy phospholipids. Choline is a structural part of phosphatidylcholine and is known as a vital nutrient in brain development, liver function and cancer prevention (Gutierrez et al., 1997), and generally added to commercial infant formulas as an important nutrient. The consumption of phosphatidylcholine is reported to increase plasma and brain choline levels and boost neuronal acetylcholine synthesis. Also, it is indicated that consumption of yolk proteins are prone to alleviate the Alzheimer disease symptoms (Juneja, 1997).

Neutral lipids of egg present in yolk granules as well as in the plasma (Chang et al., 1977). The glycerol of the triglycerides is mainly esterified by palmitic acid in position 1, by oleic and linoleic acids in position 2, and by oleic, palmitic, and stearic acids in position 3 (Kuksis, 1992). Neutral lipids in egg yolk can be used as cooking oil or in animal feed.

### **3. Objectives**

There had been many attempts to isolate value-added components from egg yolk individually but no systemic approach to separate multiple components from egg yolk has been reported. Most of the developed methods for separating value-added components from egg yolk are mainly focused on simple component and rest of the yolk residues was discarded. The objectives of this research were: 1) to develop separation and purification methods for individual value-added components from egg

yolk, 2) to optimize the developed method for large-scale production in order to use them for food applications and drug industries, and 3) to develop simple, economical and continuous preparation method for separating immunoglobulin Y, phosvitin, phospholipids, neutral lipids, and other yolk proteins from egg yolk.

## **CHAPTER 3**

# **SEPARATION OF IMMUNOGLOBULIN Y FROM CHICKEN EGG YOLK USING SODIUM CHLORIDE AND AMMONIUM SULFATE PRECIPITATION**

# 1. Abstract

The objective of this study was to develop a simple, efficient and economical separation procedure for IgY using ammonium sulfate and sodium chloride combinations. Egg yolk was diluted with two volumes of cold distilled water, homogenized, and then centrifuged at  $3,400 \times g$  for 30 min to remove yolk granules. The supernatant was diluted again with three volumes of cold distilled water to precipitate phospholipids and lipoproteins, and then the precipitate was removed by centrifugation. The resulting supernatant was concentrated using ultrafiltration and then IgY was precipitated using 20% saturated ammonium sulfate + 15% sodium chloride (w/v). The IgY pellet was collected, dissolved with distilled water, desalted using ultrafiltration, and then freeze-dried. Separated IgY was confirmed using Western Blot. The purity determined using SDS-PAGE was  $> 95\%$ , and the yield was  $> 80\%$ . The method developed was simple, easy and has very high potential for the commercial production of IgY from egg yolk. The method can be applied to separate antibodies produced by specific antigens, which can be used to cure diseases, immunotherapy, and diagnostics for animals and human diseases.

Key words: Immunoglobulin Y, ammonium sulfate, sodium chloride, chicken egg yolk, separation



## 2. Introduction

The antibodies produced from immunized chicken has very high potentials to be used to cure various animal and human diseases, immunodiagnostic, and pathogen control in food products (Hatta et al., 1993; Reilly et al., 1997; Chalghoumi et al., 2009; Vega et al., 2011). Chicken antibodies have significant advantages over mammalian antibodies: in contrast to mammalian antibodies, chicken antibodies do not activate the human complement system nor react with rheumatoids factors largely due to different phylogenetic relationships and genetic backgrounds between the two species. Therefore, use of IgY can reduce interference in immunological assays and improve the performance in curing diseases (Larsson and Sjoquist, 1993; Otani et al., 1991). Also, chicken IgY has higher stability at high temperature and low pH conditions (Shimizu et al., 1992), and is more cost effective to produce than the mammalian antibodies.

The amount of IgY in egg yolk is approximately 5-7 mg/ml and around 25 eggs can be produced from one hen per month, which is equivalent to 375 ml of serum (Akita et al., 1992; Shimitzu et al., 1994). This productivity represents reduction in animal numbers and improved animal welfare because invasive blood collection from animals can be avoided. Also, direct use of egg yolk as an antibody source will be simple and cost less. Thus, chicken antibodies would have greater potentials than mammalian antibodies and egg yolk is an ideal source for antibodies (Fayer and Jenkins, 1992; Svendsen et al., 1995). At present, the application and efficiency of IgY have not been fully appreciated (Hatta et al., 1993). However, if immunoglobulin Y (IgY) from egg yolk is isolated and purified, its efficiency and application can be increased dramatically.

Several published antibody purification methods from egg yolk are available, but most of them are for laboratory scale and are complicated (Jensenius et al., 1981; Polson et al., 1985; Akita and Nakai, 1992; Dong et al., 2008). One of the main hindrances in separating IgY from egg yolk was high portion of insoluble lipids and lipoproteins in egg yolk, which have negative effects on further chromatography and filtration-based purification steps (Hansen et al., 1998). To improve extraction of IgY various dilution factors (Akita and Nakai, 1993) have been tested. To remove lipids in the IgY extract, xanthan gum (Hatta et al., 1988), carrageenan (Hatta and Kim, 1990), and chloroform (Ntakirutimana et al., 1992) have been used. Also, precipitation of IgY from egg yolk using polyethylene glycol (Polson et al., 1985), pectin (Chang et al., 2000), dextran sulfate (Jensenius et al., 1981) and ammonium sulfate (Akita and Nakai, 1993) had been tested. However, many of these approaches have limitations for commercial production of IgY due to low yield and complicated procedures.

The most popular strategy for separating IgY from egg yolk is extracting IgY from yolk using 10 volumes of acidic water and then precipitating IgY using ammonium sulfate or sodium sulfate (Akita and Nakai, 1992; 1993). However, extreme volume increase makes it difficult for handling. Ultrafiltration can help reduce the volume of IgY extract (Liu et al., 2010; Hernandez-Campos et al., 2010; Liu et al., 2011) but the efficacy of ultrafiltration can be significantly affected by the amount of lipids in the extract. To improve the purity of IgY separated, affinity chromatograph or ion-exchange chromatography are often employed (Ko and Ahn, 2007; Dong et al., 2008). However, the chromatographic methods are not recommended because the resins are expensive and have limitations for industrial scale preparations. Therefore, an appropriate separation procedure for IgY with high yield and purity, and scale-up

capability without using toxic compounds is necessary if the separated IgY is to be used for humans. The objective of this work was to develop a simple and easy purification method that can be used for commercial production of IgY from egg yolk.

### **3. Material and methods**

#### ***Materials***

Chicken eggs (less than 3 days old) were purchased from a local market. Ammonium sulfate and sodium chloride were purchased from Fisher Scientific (Thermo Fisher Scientific Inc., Waltham, MA, USA). IgY standard was purchased from MP biomedical (MP Biomedicals, LLC, Santa Ana, CA, USA) and anti-IgY HRP conjugated antibody was from US Biological (US Biological, Swampscott, MA, USA).

#### ***Separation of IgY***

Egg yolk was separated from egg white using egg separators. Egg yolk was diluted with 2 volumes of cold (4 °C) distilled water and homogenized for 1 min using a hand blender (Kitchen Aid) at high speed. After centrifugation at 3,400 x g for 30 min at 4 °C, the supernatant was collected and diluted again with 3 volumes of cold (4 °C) distilled water, kept in a cold room overnight to precipitate phospholipids and lipoproteins, and then centrifuged to remove the precipitant. The resulting supernatant was concentrated using ultrafiltration (membrane filter cut-off size: 50 kD,

GE healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Immunoglobulin Y in the concentrated solution was precipitated using different levels of ammonium sulfate, sodium chloride, or ammonium sulfate + sodium chloride combinations. The precipitant was collected after centrifugation, dissolved with distilled water, and then precipitated again with salt combinations for further purification if necessary. The precipitant was dissolved with distilled water and desalted using ultrafiltration. Protein concentration was determined using DC protein assay (BioRad, Hercules, CA) based on the Bradford assay. Bovine serum albumin (1.5 mg of protein/mL, Sigma-Aldrich, St. Louis, MO) was used as a reference protein. The absorbance was measured at 595 nm after 15-min reaction with Bradford solution using a microplate reader (Fisher Scientific, St. Louis, MO, USA). The purity of IgY was determined using SDS-PAGE and identified using Western Blot. All the sample preparations were replicated 3 times.

### ***Yield of IgY***

To determine the yield of IgY, precipitation of IgY using 20% saturated ammonium sulfate and 15% sodium chloride (w/v) combination was used because this protocol produced the best IgY separation. The precipitated IgY was dissolved with distilled water, desalted, and then freeze-dried (Labconco Corp., Kansas City, MO, USA). The yield was calculated using the reported value of igY in egg yolk (Stadelman and Cotterill, 2001). The whole separation process was replicated three times.

### ***SDS-PAGE***

The IgY separated using the optimum level of ammonium sulfate and sodium chloride combination was evaluated. SDS-PAGE was carried out under non-reducing conditions using Mini-PROTEIN Tetra cell (Bio-Rad Lab Inc.). Ten percent SDS gel and Coomassie Brilliant Blue R-250 (Sigma) staining was used. After destaining, gel pictures were taken using the Gel Doc (Bio-Rad Lab Inc.). The protein bands in the gel picture were analyzed using ImageJ software (NIH, Bethesda, MD, USA) and used to calculate the purity of IgY.

### ***Western Blot***

Western blot was conducted to identify separated IgY. Ten percent SDS-PAGE gel was run with a standard IgY, IgY samples separated, and a dual color molecular marker (Bio-Rad). Proteins in the SDS-PAGE gel were transferred to a nitrocellulose membrane (Bio-Rad) using a Mini TransBlot Cell (Bio-Rad) at 90 V for 2 h in cold room and then, the membrane was blocked with 5% skim milk (w/v) dissolved in PBST for 1 h in RT. After blocking, a membrane was put in primary antibody with HRP conjugated in PBST (Add company name and address). The anti-IgY antibody conjugated with HRP was diluted at 1:15,000 in PBST solution containing 5% skim milk and incubated overnight at 4 oC. The membrane was washed 3 times with PBST solution at 10 minutes intervals, exposed to ECL Prime (GE Healthcare) for 5 minutes, and checked with Chemidoc (Bio-rad Laboratory Inc.).

### ***Statistical Analyses***

The data were analyzed using SAS Institute software (Release 9.3,

SAS Institute Inc., Cary, NC) by the generalized linear model procedure. Differences in the mean values were compared by Tukey honestly significant difference test, and mean values and standard deviations were reported (Kuehl, 2000).

## **4. Results**

### ***Purification of Immunoglobulin Y from the crude extract of egg yolk***

One of the most frequently used methods to separate proteins is precipitating proteins using sodium chloride, ammonium sulfate and polyethylene glycol (PEG). Recently, Marcet et al. (2011) used multi-step PEG precipitation to separate IgY from egg yolk. They have precipitated IgY three times using different levels of PEG and then further purified using anion exchange chromatography. Pauly et al. (2011) also used a three-step PEG precipitation for IgY separation from egg yolk. PEG was used to remove lipids in the first step and then to precipitate IgY in the next two steps. However, more than two steps are needed to separate relatively pure form of IgY and it is difficult to remove PEG from IgY after precipitation. The molecular size of PEG is 6-8 kD but its size greatly increases because it can bind hundreds of water molecules, which significantly reduce the efficacy of ultrafiltration. Also, chromatography and dialysis used as the last step for IgY separation method are not appropriate for large scale purification. Their SDS-PAGE results showed high levels of impurities in precipitant. Therefore, using salt instead of PEG to precipitate IgY will be easier and better for scale-up process.

Our preliminary trials were focused on comparing the capabilities

of two salts, ammonium sulfate and sodium chloride, commonly used to precipitate IgY. Kim and Nakai (1998) reported that addition of sodium chloride makes IgY polymerized and facilitated the isolation of IgY from other proteins. Figure 1 showed the effect of sodium chloride at different concentrations: 15% sodium chloride produced the best IgY precipitation, but large amount of IgY were still remaining in the supernatant. Ko and Ahn (2007) suggested two times precipitation of IgY using 40% ammonium sulfate and revealed that ammonium sulfate produced better result than sodium chloride. The IgY remaining in supernatant (Figure 2) indicated that precipitation of IgY using ammonium sulfate was better than that with sodium chloride, but the precipitant contained impurities even at high concentration of ammonium sulfate. It was shown that 20% saturated ammonium sulfate produced the best precipitation although some IgY was still remaining in the supernatant. Therefore, ammonium sulfate and sodium chloride combinations were tested in the next study to improve separation procedure, increase the purity, and reduce the amount of salt uses.

Two salts combinations were tested to precipitate IgY effectively. The preliminary trials indicated that 20% saturated ammonium sulfate or 15% sodium chloride (w/v) was the best conditions to precipitate IgY when ammonium sulfate or sodium chloride was used alone. Thus, the combination studies used fixed level of ammonium sulfate (20% saturation) with varying levels of NaCl, and fixed level of sodium chloride (15%, w/v) with varying levels of ammonium sulfate. Figure 3 shows the comparison of IgY separation at different concentrations of ammonium sulfate with fixed level (15%) sodium chloride (w/v). The result showed that increasing ammonium sulfate higher than 15% saturation did not improve the precipitation of IgY. The DC protein assay produced the highest readings when 15% saturated ammonium sulfate and 15% sodium

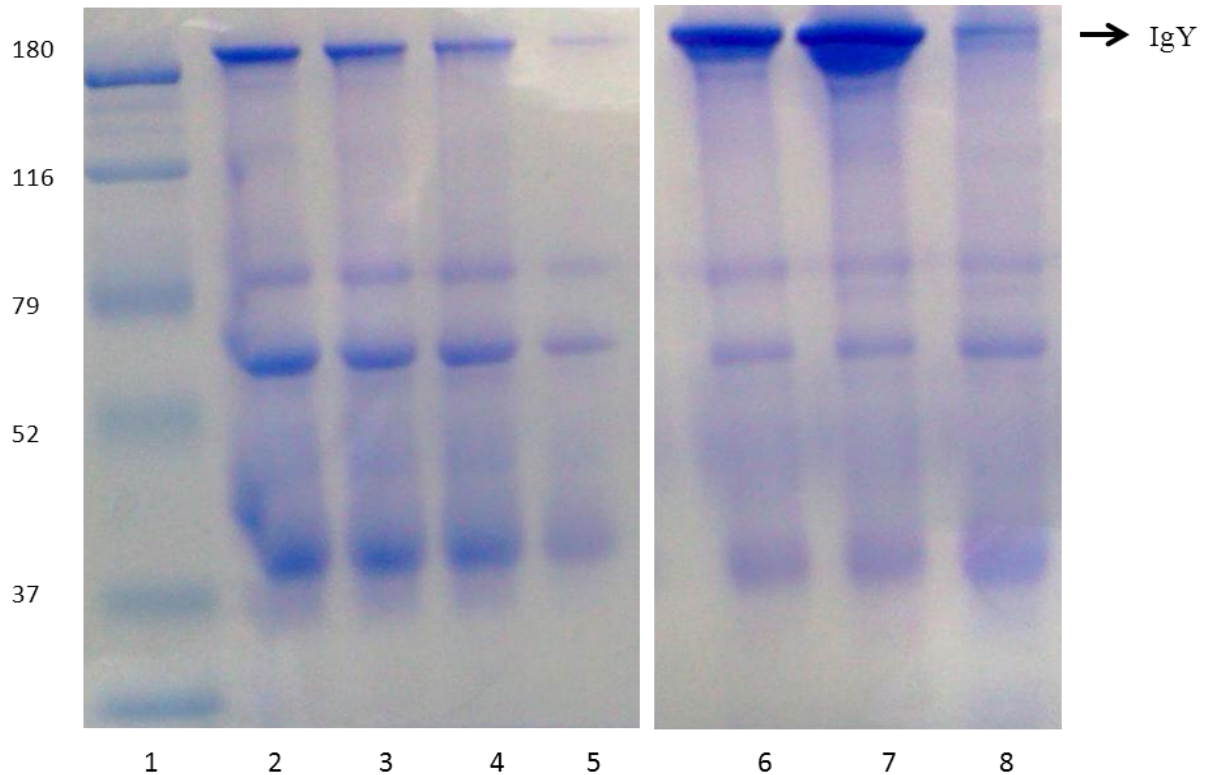
chloride (w/v) combination was used.

Figure 4 showed no significant difference in the separation of IgY when 10 - 17.5% of sodium chloride was added to the concentrated supernatant with fixed level (20% saturated) of ammonium sulfate. The gel picture of supernatant indicated that 5% sodium chloride (w/v) was not enough to precipitate IgY and more than 17.5 % sodium chloride (w/v) did not increase the precipitation. The DC protein assay results showed that the best precipitation was obtained when 15.0 % sodium chloride (w/v) and 20% saturated ammonium sulfate combination was used. From Figures 3 and 4, the two best salt combinations were compared to confirm the final conditions to isolate IgY from the egg yolk extract. The SDS-PAGE and DC protein assay results showed that 15% sodium chloride (w/v) + 20% saturated ammonium sulfate precipitated IgY more effectively than 15% saturated ammonium sulfate + 15% sodium chloride (w/v) conditions. Therefore, 20% saturated ammonium sulfate and 15% sodium chloride combination was selected as the final conditions for separating IgY from the egg yolk extract. The purified IgY was confirmed by the Western blot (Figure 6).

### ***Yield and purity of purified Immunoglobulin Y***

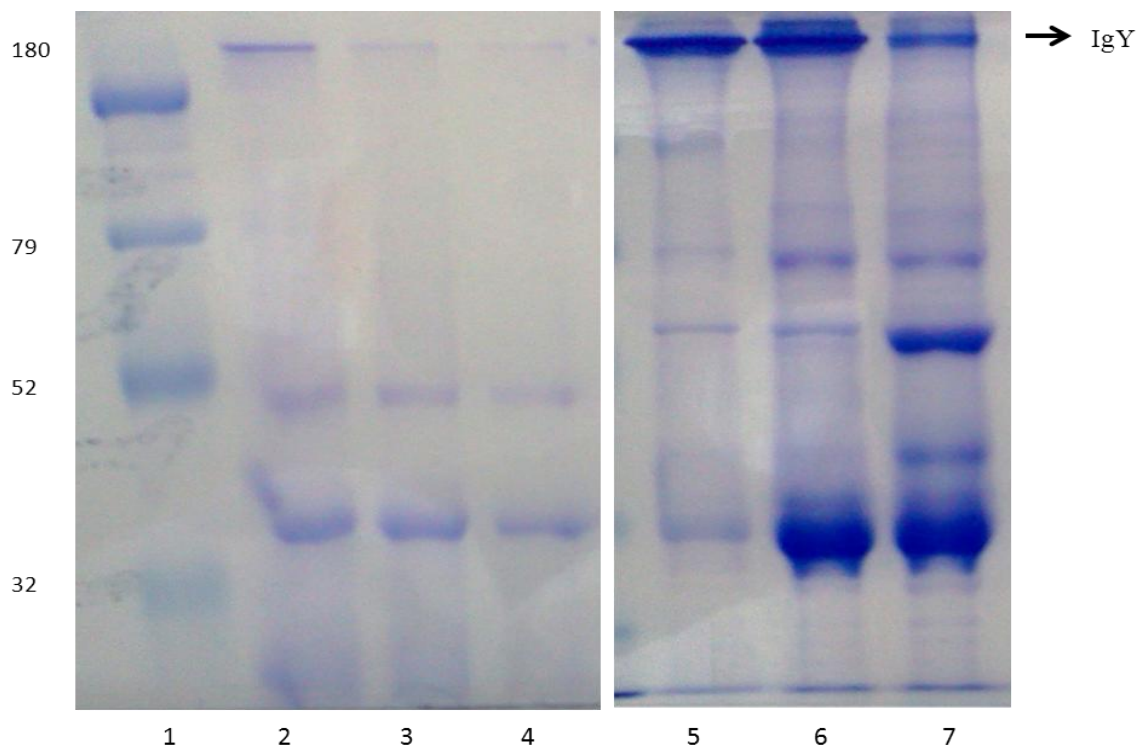
The sample fractions of egg yolk solutions obtained by each step involving dilution, centrifugation, ultrafiltration, and 20% saturated ammonium sulfate + 15% sodium chloride (w/v) precipitation are shown in Figure 5. The purity of purified IgY is as high as 95% and Table 1 showed that yield, which is more than 80%.





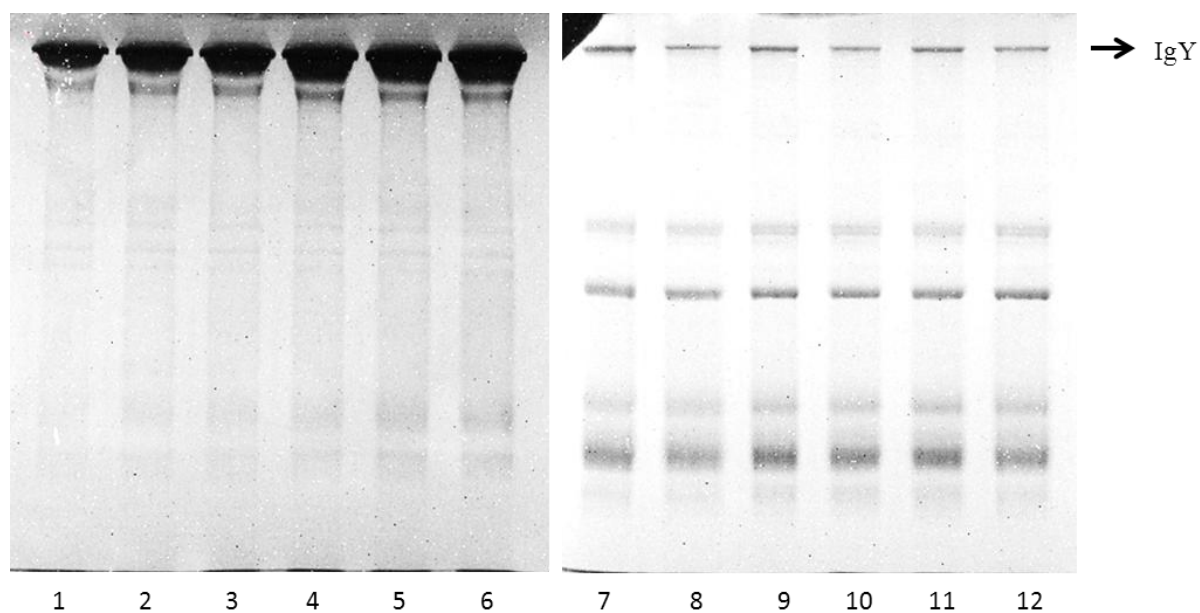
**Figure 1. Effect of sodium chloride (w/v) on the separation of immunoglobulin Y from egg yolk extract**

Lane 1: Marker; Lane 2: Egg yolk extract; Lanes 3 to 5: Supernatant obtained after 10, 15, and 20% sodium chloride (w/v) precipitation, Lanes 6 to 8: Precipitant obtained from 10, 15, and 20% sodium chloride (w/v) precipitation.



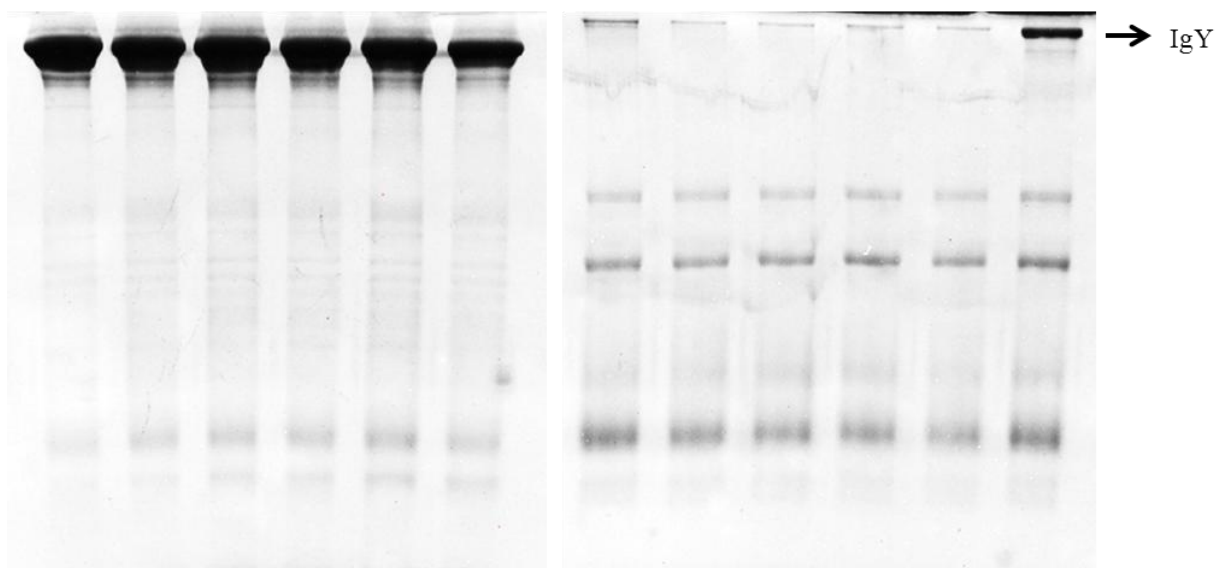
**Figure 2. Effect of ammonium sulfate on the separation of immunoglobulin Y from egg yolk**

Lane 1: Marker; Lanes 2 to 4: Supernatant obtained from 20, 30, 40% (saturated) ammonium sulfate precipitation; Lanes 5 to 7: Precipitant obtained from 20, 30, and 40% (saturated) ammonium sulfate precipitation.



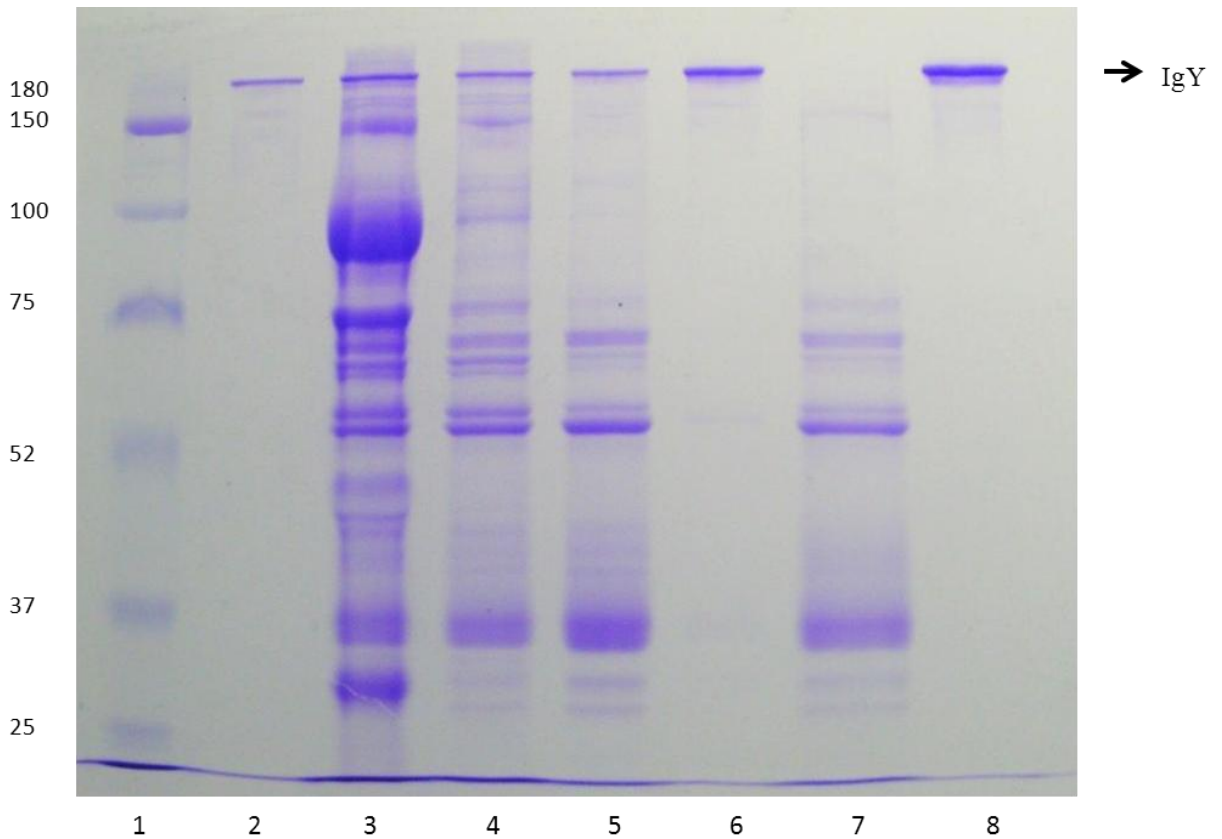
**Figure 3. Effect of ammonium sulfate and sodium chloride combinations on the separation of immunoglobulin Y from egg yolk extract**

Lanes 1 to 6: Precipitant obtained from 5.0, 10.0, 12.5, 15.0, 17.5, 20.0 saturated ammonium sulfate with fixed level (15%) of sodium chloride (w/v) precipitation; Lanes 7 to 12: Supernatant obtained from 5.0, 10.0, 12.5, 15.0, 17.5, 20.0 saturated ammonium sulfate with fixed level (15%) of sodium chloride (w/v) precipitation.



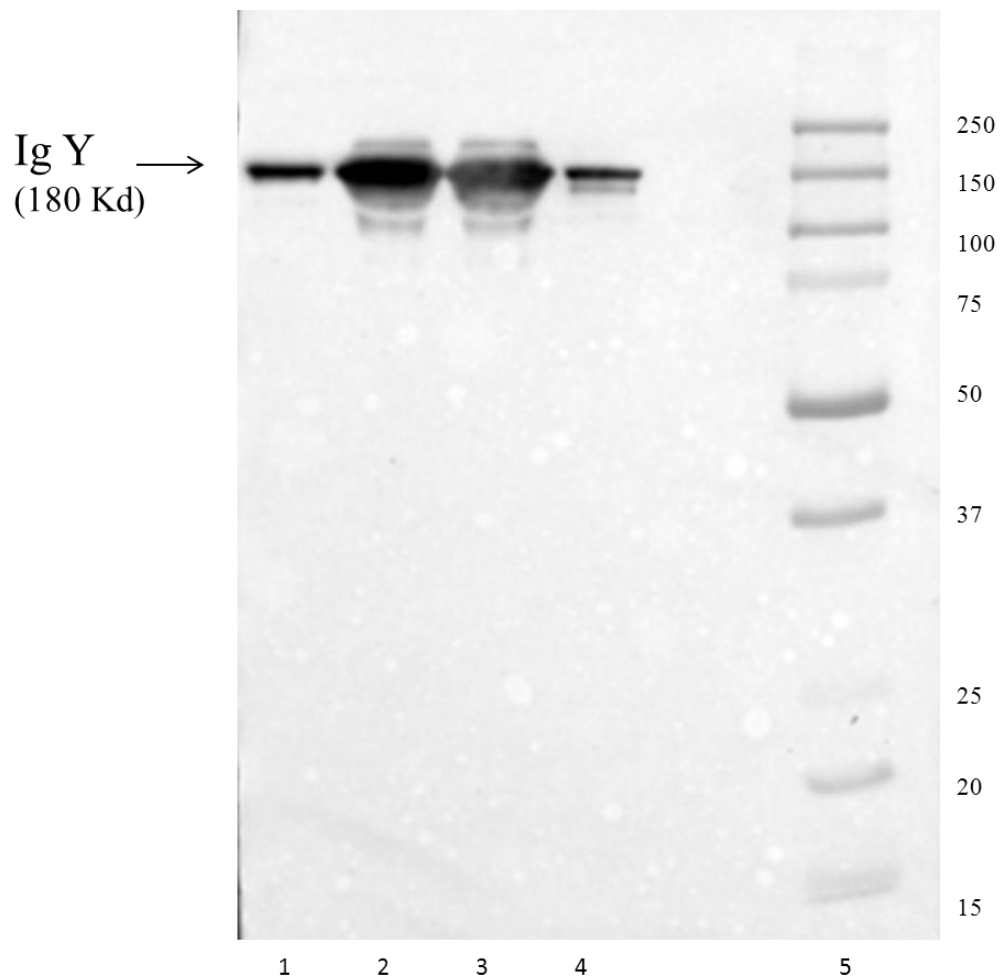
**Figure 4. Effect of ammonium sulfate and sodium chloride combinations on the separation of immunoglobulin Y from egg yolk extract**

Lanes 1 to 6: Precipitant obtained from 5.0, 10.0, 12.5, 15.0, 17.5, 20.0 % sodium chloride (w/v) with fixed level (20% saturated) of ammonium sulfate precipitation; Lanes 7 to 12: Supernatant obtained from 5.0, 10.0, 12.5, 15.0, 17.5, 20.0 % sodium chloride (w/v) with fixed level (20% saturated) of ammonium sulfate precipitation.



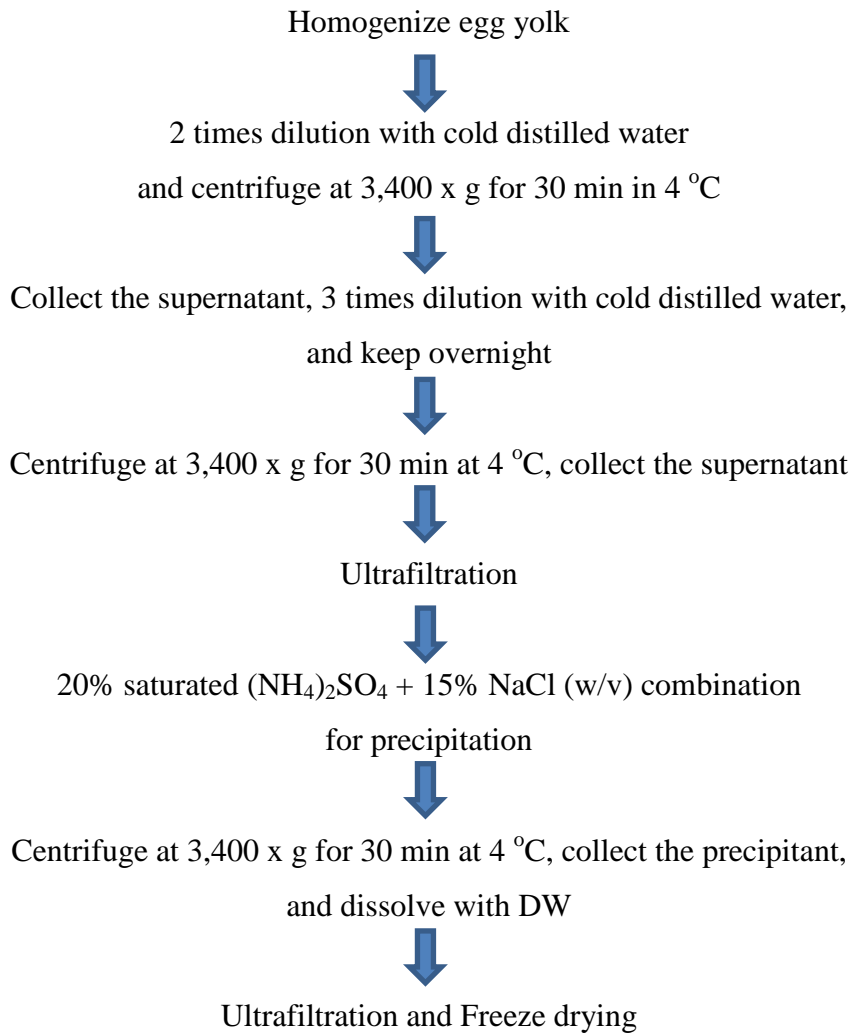
**Figure 5. The SDS-PAGE patterns of sample obtained at different step in the separation process**

Lane 1: Marker; Lane 2: Immunoglobulin Y standard; Lane 3: Diluted egg yolk; Lane 4: Supernatant prepared by centrifuge after adding distilled water; Lane 5: After ultrafiltration; Lane 6: Dissolved precipitant obtained from 20% saturated ammonium sulfate and 15% sodium chloride (w/v) combinations; Lane 7: Supernatant obtained from 20% saturated ammonium sulfate and 15% sodium chloride (w/v) combinations; Lane 8: Supernatant after desalting.



**Figure 6. Western blot of immunoglobulin Y**

Lane 1: 10 times diluted purified immunoglobulin Y; Lane 2 to 3: Purified immunoglobulin Y without dilution; Lane 4: immunoglobulin Y Standard (400 ng/ml); Lane 5: Molecular markers.



**Figure 7. Schematic diagram for the separation of immunoglobulin Y from egg yolk**

**Table 1. Yield of immunoglobulin Y using the protocol developed**

<b>Sample</b>	<b>Weight (g)</b>	<b>Yield (%)</b>
Egg yolk Immunoglobulin Y <sup>1</sup>	1.00	100
Final Immunoglobulin Y <sup>2</sup>	0.81± 0.01	80.94± 0.63

<sup>1</sup>The theoretical amount of immunoglobulin Y in egg yolk (Immunoglobulin Y is 0.6% of total egg yolk). The original amount of egg yolk was 165 g per replication.

<sup>2</sup>Immunoglobulin Y produced with 20% saturated ammonium sulfate and 15% sodium chloride combination; n=3



## 5. Discussion

Recent studies suggested modified ammonium sulfate precipitation to separate IgY from egg yolk. Wen et al. (2012) adapted two former studies (Polson et al., 1980; Ko and Ahn, 2007): they applied a two-step PEG precipitation of IgY to remove lipid portion, and then 50% and 33% saturated ammonium sulfate precipitation. The IgY yield was 76.5 mg per yolk, and the purity was 98.2%. Tan et al. (2012) also suggested a mix of  $\kappa$ -carrageenan, pectin and  $\text{CaCl}_2$  to remove lipids, and then used 30% and 20% ammonium sulfate in sequence to precipitate IgY. This study produced 60 mg IgY per egg yolk and the purity was around 80%. Although, the methods produced IgY with high yield and purity, they are not recommendable for the large scale preparation because the number of chemicals used and the steps involved are too many for scale-up processes. Also, use of gums and PEG makes it difficult to remove them at the final stage of purification. Ko and Ahn (2007) demonstrated that addition of 0.01% charcoal and 40% saturated ammonium sulfate precipitation at pH 9.0 produced 69 mg IgY per egg yolk with high purity. Compared with previous studies, precipitation of IgY using ammonium sulfate and sodium chloride combination does not require pH adjustment and use the original crude egg yolk supernatant.

To extract water-soluble proteins from egg yolk, former protocols used 10 times dilution with pH adjustment to 5.0. However, our protocol uses only 3x dilution of egg yolk without pH adjustment at the beginning step. Centrifugation of the 3x diluted egg yolk separated yolk granules and other fractions containing water soluble proteins, phospholipids and lipids in the supernatant fraction. This procedure enables the use of precipitated yolk granules for phosvitin separation. Further dilution of supernatant

fraction with 3 volumes of water coagulated a fraction containing lipids, lipoproteins and phospholipids upon storage in a cold room overnight. After centrifugation, the fraction containing water-soluble proteins can be concentrated using ultrafiltration and used to separate IgY. The precipitant can be used to separate phospholipids and neutral lipids. The two-step water dilution makes it easy for the separation of multiple value-added components from egg yolk. Also, this protocol involves with fewer steps than other reported methods and can be easily applied for scale-up process.

Immunoglobulin Y from egg yolk could be separated using dilution with cold distilled water, low speed centrifugation, salt combination, and ultrafiltration. The best combination for isolating immunoglobulin Y from the concentrated extract was 20% saturated ammonium sulfate + 15% sodium chloride (w/v) combination. The protocol is simple and applicable for a large scale production. Furthermore, the purified IgY can be used for food and drug industry because only distilled water and two salts are used to separate IgY, and the salts added could be removed easily by ultrafiltration at the last stage of the separation process. The yield of the IgY was over 80% and the purity was higher than 95%.

## **CHAPTER 4**

# **SEPARATION OF PHOSVITIN FROM CHICKEN EGG YOLK WITHOUT USING SOLVENT**

## 1. Abstract

Phosvitin is one of the major yolk proteins with extraordinary molecular structure and functions. More than half of the amino acids in phosvitin are phosphorylated, and thus phosvitin can be an excellent substrate for functional phosphopeptides production. The objective of this study was to develop a simple separation protocol to separate phosvitin from egg yolk without using organic solvents. Egg yolk was diluted with 2 vol. of cold distilled water, homogenized and centrifuged. The precipitant was collected and homogenized with 4 vol. of 10% NaCl (w/v), 0.05 N NaOH solution to extract phosvitin. The homogenate was diluted with 1 volume of DW, the pH adjusted to 4.0, and then the precipitant was removed by centrifugation. The supernatant containing phosvitin was collected, and salts removed and concentrated using ultrafiltration. The supernatant was heat-treated at 70 °C for 30 min to remove impurities and improve purity by centrifugation, and then collected supernatant is freeze-dried. The phosvitin separated was verified using Western blotting, and the purity checked using SDS-PAGE. The purity of the phosvitin separated was > 90% and the yield was > 85%. This study proved that phosvitin can be separated from egg yolk without using solvent. Also, the method developed is very simple and has a high potential for scale-up process.

**Key words:** Phosvitin, separation, sodium hydroxide, sodium chloride, pH adjustment, egg yolk

## 2. Introduction

Phosvitin is one of the most highly phosphorylated proteins in nature, and represents approximately 7-11% of yolk proteins and 7% is mostly used to estimate phosvitin content in egg yolk (Mecham and Olcott, 1949; Clark, 1985; Anton, 1998). About 60% of the amino acids in phosvitin is serine residues and almost all of them are phosphorylated. Phosvitin accounts for 60% of total egg yolk phosphoproteins and 90% of the total egg yolk phosphorous (Mecham and Olcott, 1949; Taborsky and Mok, 1967). Phosvitin comprises a mixture of two polypeptides called  $\alpha$ -phosvitin and  $\beta$ -phosvitin, and their molecular weight ranges from 18.5 to 60 kDa (Abe et al., 1982; Connelly and Taborsky, 1961). Both  $\alpha$ - and  $\beta$ -phosvitin have multi-subunits and  $\beta$ -phosvitin with molecular weight of 45 kDa is the predominant one. Phosvitin has excellent physiological functions and can be used in foods as a metal-chelating, emulsifying, antioxidant or antibacterial agent (Grizzuti and Perlmann, 1973; Khan et al., 1998; Ishikawa et al., 2004; Khan et al., 2000).

Mecham and Olcott (1949) are the first ones who purified phosvitin using  $\text{MgSO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$ , ethyl ether, and NaCl. Over the past 60 years, various separation methods had been developed using a three-step process: lipid removal using organic solvents such as methanol, ether, chloroform, hexane and ethanol from yolk, extraction of phosvitin from the lipid-free fraction using NaCl and then precipitation of extracted phosvitin using  $\text{MgSO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$ , and NaCl (Joubert and Cook, 1958; Sundararajan et al., 1960; McBee and Cotterill, 1979; Tsutsui and Obara, 1982; Losso and Nakai, 1994; Castellani et al., 2003; Belhomme et al., 2007; Ko et al., 2011). Causeret et al. (1991) reported that granular structure and solubilized phosvitin from granules can be greatly affected

by pH variations or changes in ionic strength.

For further purification of extracted phosvitin, anion exchange chromatography has been applied in particular because phosvitin is negatively charged due to large number of phosphate groups in phosphoserine residues.

Among the anion exchange chromatography, DEAE-cellulose resin was the most commonly used (Connelly and Taborsky, 1961; Castellani et al., 2003; Zhang et al., 2011; Lei and Wu, 2012), but this method consumes large amounts of acid and alkali for regeneration of resins, which are relatively expensive. Recently, Zhang et al. (2012) purified phosvitin using immobilized metal affinity nanoparticles (IMANs) with calcium, instead of ion exchange chromatography, but the method was not applicable for commercial scale-up process.

Most of the current methods for separating phosvitin use non-food-grade solvents to remove lipids in granules from egg yolk, and thus the phosvitin cannot be accepted for human use. Also, the solvents caused denaturation and modification of phosvitin structure, which led low phosvitin recovery and loss of functions (Castellani et al., 2003). Recently, several separation methods for phosvitin without using toxic solvents, which include polyethylene glycol, heat treatment, and carbonate-bicarbonate buffer, have been published (Zhang et al., 2011; Liu et al., 2012; Lei and Wu, 2012). However, all these methods used anion exchange chromatography or dialysis for further purification, which has limitation to apply for industry scale production. The objective of this work was to develop a simple and easy method for separating phosvitin from egg yolk without using organic solvents.

### **3. Material and methods**

#### ***Chemicals***

Sodium chloride and NaOH were purchased from Fisher scientific (Thermo Fisher Scientific Inc., Waltham, MA, USA). Phosvitin standard was purchased from Sigm-Aldrich Inc. (St. Louis, MO, USA), and phosvitin primary antibody (mouse monoclonal IgG<sub>2a</sub>) and secondary antibody (goat anti-mouse IgG-horseradish peroxidase) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and ECL prime Western Blotting Detection Reagents from GE Healthcare Life Sciences (Pittsburgh, PA, USA).

#### ***Protocol for phosvitin purification***

Fresh chicken eggs were purchased from a local market. Egg yolk was separated from egg white and 2 volumes of cold distilled water (4 °C) were added to the egg yolk, homogenized for 1 min using a kitchen hand blender (Kitchen Aid), and then centrifuged at 3,400 x g for 30 min. The precipitant containing egg yolk granules was homogenized with 4 volumes of 10% sodium chloride (w/v) in 0.05 N sodium hydroxide solution, diluted with an equal volume of distilled water, the pH of the homogenate adjusted to pH 4.0 using 6 N HCl, and then centrifuged at 3,400 x g for 30 min at 4 °C to remove precipitant containing phospholipids and lipids. The supernatant was collected. The precipitant was re-extracted using 4 volumes of 10% NaCl (w/v) in 0.05 N sodium hydroxide solutions, treated the same way as the first extraction, and then the supernatant was collected and pooled with the first supernatant. The pooled supernatant

was desalted and concentrated using ultrafiltration (membrane filter cut-off size: 10 kD, GE healthcare Bio-Sciences Corp., Piscataway, NJ, USA), and heated at 70 °C for 30 min, and then centrifuged at 3,400 x g for 30 min. The resulting supernatant was freeze-dried (Labconco Corp., Kansas City, MO, USA). The yield was calculated using the reported value of phosvitin in egg yolk (Stadelman and Cotterill, 1977). The schematic diagram for the purification of phosvitin from egg yolk is shown in Figure 6. The purity of phosvitin was determined using SDS-PAGE and identified by Western Blot (Figure 5).

### ***SDS-PAGE***

Freeze dried phosvitin was dissolved in distilled water at 1 mg protein/mL. Sample (10 µl) was mixed with 40 µl of Laemmli sample buffer solution under reducing conditions, heated at 95 °C in a block heater for 5 min, and then 10 µl of sample was loaded on the Mini-PROTEIN Tetra cell (Bio-Rad Laboratory Inc.). The purity of phosvitin preparations was evaluated using 10% SDS-PAGE gel and a low-range SDS-PAGE molecular weight standard (Bio-Rad Laboratory Inc.) was used as a marker. The proteins in the gel were stained using 0.1 M aluminum nitrate for proteins containing phosphorus from the modified Coomassie Blue method (Hegenauer et al., 1977). After destaining, gel pictures were taken using the Gel Doc (Bio-Rad Lab Inc.). The protein bands in the gel picture were analyzed using ImageJ software (NIH, Bethesda, MD, USA) and used to calculate the purity of phosvitin.



## ***Western Blot***

10% SDS-PAGE gel was run with a standard phosvitin, phosvitin samples separated, and a dual color molecular marker (Bio-Rad). Proteins in the SDS-PAGE gel were transferred to a nitrocellulose membrane (Bio-Rad) using a Mini TransBlot Cell (Bio-Rad) at 90 V for 2 h in cold room, and then the membrane was blocked with the blocking solution containing 5% skim milk (w/v) dissolved in PBST for 1 h at room temperature. After blocking, a membrane was placed in the phosvitin primary antibody solution (mouse monoclonal IgG<sub>2a</sub>, Santa Cruz Biotechnology Inc.), which was diluted to 1:2,000 ratio with 5% skim milk (w/v) dissolved in PBST and incubated overnight at 4 °C. The membrane was washed 3 times with PBST solution at 10 minutes intervals at RT and then incubated with a secondary antibody (goat anti-mouse IgG-horseradish peroxidase, Santa Cruz Biotechnology Inc.) for 1 h at room temperature. The secondary antibody was diluted at 1:5,000 with 5% skim milk (w/v) dissolved in PBST solution. After completion of the secondary antibody incubation, the membrane was washed 3 times with PBST solution at 10 minutes intervals, exposed to ECL Prime (GE Healthcare) for 5 minutes, and detected with Chemidoc (Bio-Rad).

## ***Yield of Phosvitin***

To determine the yield of Phosvitin, 10% NaCl in 0.05N NaOH solution and heating at 70 °C for 30 min was used because this protocol produced the best phosvitin separation. After extraction, phosvitin was desalted and concentrated by ultrafiltration and then freeze-dried (Labconco Corp., Kansas City, MO, USA). The yield was calculated using

the reported value in egg yolk (Stadelman and Cotterill, 2001). This was replicated three times.

### ***Statistical Analyses***

The data were analyzed using SAS Institute software (Release 9.3, SAS Institute Inc., Cary, NC) by the generalized linear model procedure. Differences in the mean values were compared by Tukey honestly significant difference test, and mean values and standard deviations were reported (Kuehl, 2000).

## **4. Results**

### ***Separation of phosvitin from egg yolk granules***

There are three major proteins in yolk granules, which include low-density lipoprotein, lipovitellin recognized as a high-density lipoprotein (HDL), and phosvitin. Phosvitin is present inside the micelle structure of egg yolk granules and interacts with lipovitellin as a complex through a phosphocalcic bridge (Radomsky and Cook, 1964). Lipovitellin is composed of  $\alpha$ - and  $\beta$ -lipovitellin and these are prone to soluble at alkaline pH (Burley and Cook, 1961; Anton and Gandemer, 1997). If the granule portion from egg yolk could be completely disrupted and solubilized to release phosvitin, it would be easier to make simplified phosvitin purification process. To disrupt the granule structure and extract phosvitin, 10% NaCl (w/v) was used after removing phospholipids from the egg yolk granules (Losso and Nakai, 1994; Ko and Ahn, 2011). The

basic concept of the current study was to break the micelle structure of yolk granules and modify lipovitellin through high ionic strength and high pH. In this study, extraction of phosvitin from yolk granules using 10% NaCl in various concentration of NaOH (ranging from 0.05N to 0.1N), 10% NaCl with heating, and 10% NaCl, NaOH and heating combination was tested. As shown in Figure 1, a mild alkaline condition with 10% NaCl (w/v) was better than higher concentrations of sodium hydroxide in terms of phosvitin yield and purity in the extract. Heat treatment at 80 °C for 30 min with 10% NaCl (w/v) has the greatest purity but yield was low. LDL and HDL started to denature at 70 °C and formed gels at 75 °C while phosvitin is less sensitive to thermo-coagulation than LDL (Yang and Cotterill, 1989). The result also showed that combination of high ionic strength (10% NaCl) + alkaline condition (0.05-0.1N NaOH) + heating was too harsh to extract phosvitin from egg yolk granules. Use of 10% NaCl (w/v) + heating denatured phosvitin and the results was not reproducible. Thus, heating was eliminated from the extraction parameter and used it as a tool to remove impurities after the initial extraction of phosvitin from egg yolk with 10% NaCl in 0.05N NaOH solution.

The pH of 10% NaCl (w/v) in 0.05 N NaOH solution for extracting phosvitin from granules of egg yolk considerably affected the recovery rate of phosvitin. Phosvitin and lipoproteins have different isoelectric points, and thus pH adjustment can help separating these two proteins. Sugano (1957) indicated that both  $\alpha$ - and  $\beta$ -lipovitellin would precipitate at pH < 7.5-7.8 and pH < 6.5-7.0, respectively. Castellani et al. (2003) adjusted the pH of phosvitin extract from 7.0 to 5.0 or lower (pH 3.6 and 2.5) to precipitate lipovitellin. However, there was no significant difference in the solubility of phosvitin because it was not influenced much by the pH adjustment. Causeret et al. (1991) reported that the viscosity and solubility of granules was greatly diminished from pH 3.0 to

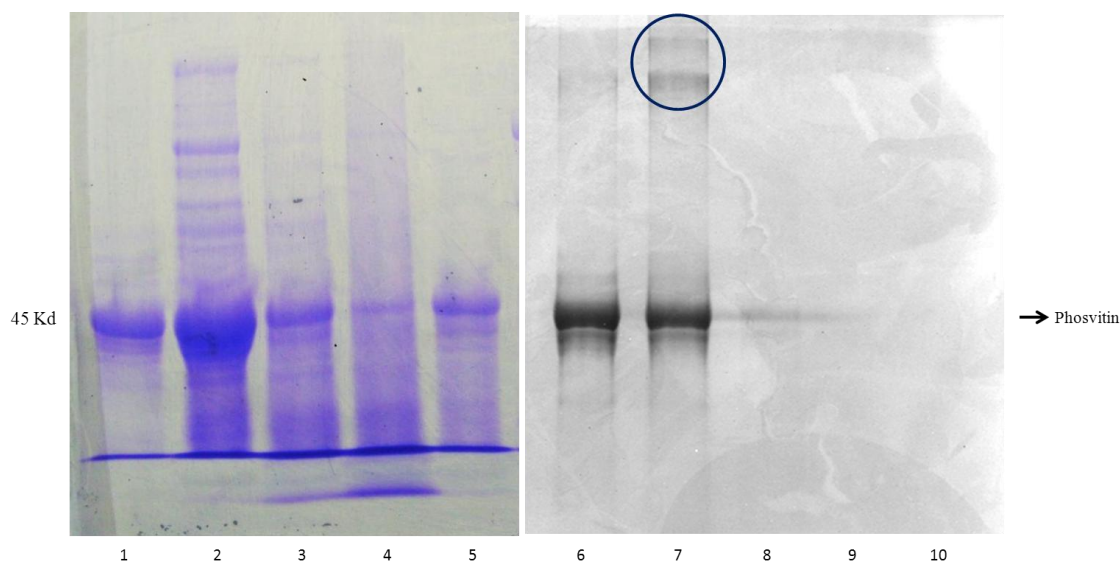
6.4. This step was used to determine the best pH conditions for removing impurities. We narrowed down the pH range from 3.0 to 4.5 and tested the effect of pH adjustment. As shown in Figure 2, the yield of phosvitin was the highest when the pH of the solution was adjusted to pH 4.0. When the pH of the extract solution was adjusted to pH < 4.0 phosvitin recovery started to decrease, while the pH was > 4.0, the impurity in the extract increased. Therefore, adjusting the pH of the extract to 4.0 and centrifugation will be the best conditions for the the maximal recovery of phosvitin from the extract.

On the other hand, cleaning up impurities with additional heating was also tested in case pH adjustment could not remove impurities completely. Different temperatures from 70 to 90 °C at 5 C interval and heating time from 30 to 60 min at 10 min interval were tested to determine the optimal heating temperature and time conditions to eliminate impurities from the phosvitin extract using 10% NaCl (w/v) in 0.05 N and 0.075 N NaOH solutions. As shown in Figure 3, some impurities were still found above the phosvitin band in the extract using 10% NaCl (w/v) in 0.075 N NaOH solution. However, extract from 10% NaCl (w/v) in 0.05 N NaOH solution had no bands in the area. Liu et al. (2012) used 0.5M NaCl to dissolve granule portion of egg yolk and heated at 80 °C for 30 min to extract phosvitin, but the purity of phosvitin in SDS-PAGE gel was not clear and the majority of phosvitin band was shown at around 35 kDa, which is smaller than the natural phosvitin at around 45 kDa. Therefore, phosvitin extraction using 10% NaCl (w/v) in 0.05 N NaOH solutions would be the best conditions for the highest phosvitin extraction from egg yolk granules and additional heating at 70 °C for 30 min significantly improved its purity. Figure 4 verified the effect of this additional heat treatment. Therefore, 10% NaCl in 0.05N NaOH solution with additional heating at 70 °C for 30 min was selected as the final conditions for

separating phosvitin from the granule portion of egg yolk. The purified phosvitin was confirmed by the Western blot (Figure 5).

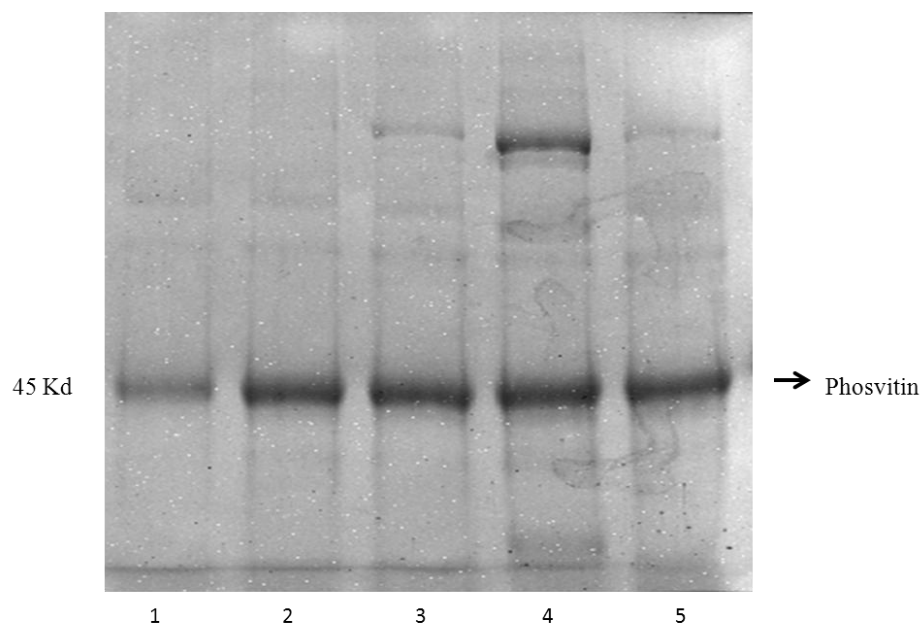
### ***Yield and purity of purified Phosvitin***

The purity and yield of purified phosvitin from final optimized method using 10% NaCl in 0.05N NaOH with additional heating at 70 °C for 30 min is shown in figure 4 and table 1 respectively. The results indicated that the purity is over 95% and the yield is approximately 91%.



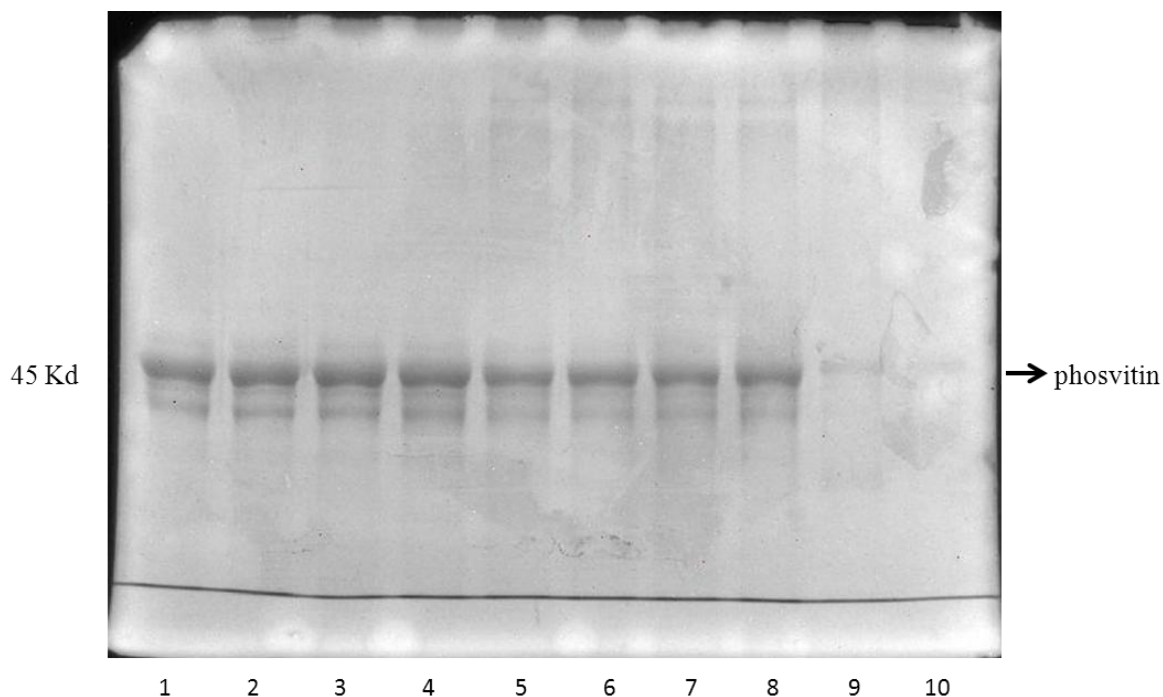
**Figure 1. Effect of 10% sodium chloride (w/v) in alkaline solution, heat treatment, and their combinations on the separation of phosvitin from egg yolk**

Lane 1: Phosvitin Standard; Lane 2: 10% NaCl in 0.05 N NaOH solution; Lane 3: 10% NaCl in 0.075 N NaOH solution; Lane 4: 10% NaCl in 0.1 N NaOH solution; Lane 5: 10% NaCl with heat treatment at 70 °C for 30min; Lane 6: 0.05 N NaOH solution; Lane 7: 0.075 N NaOH solution; Lane 8: heat treatment; Lane 9: 0.05 N NaOH solution with heat treatment; Lane 10: 0.075 N NaOH with heat treatment.



**Figure 2. Effect of pH on the recovery of phosvitin extracted using 10% NaCl (w/v) in 0.05N NaOH solution from yolk granules**

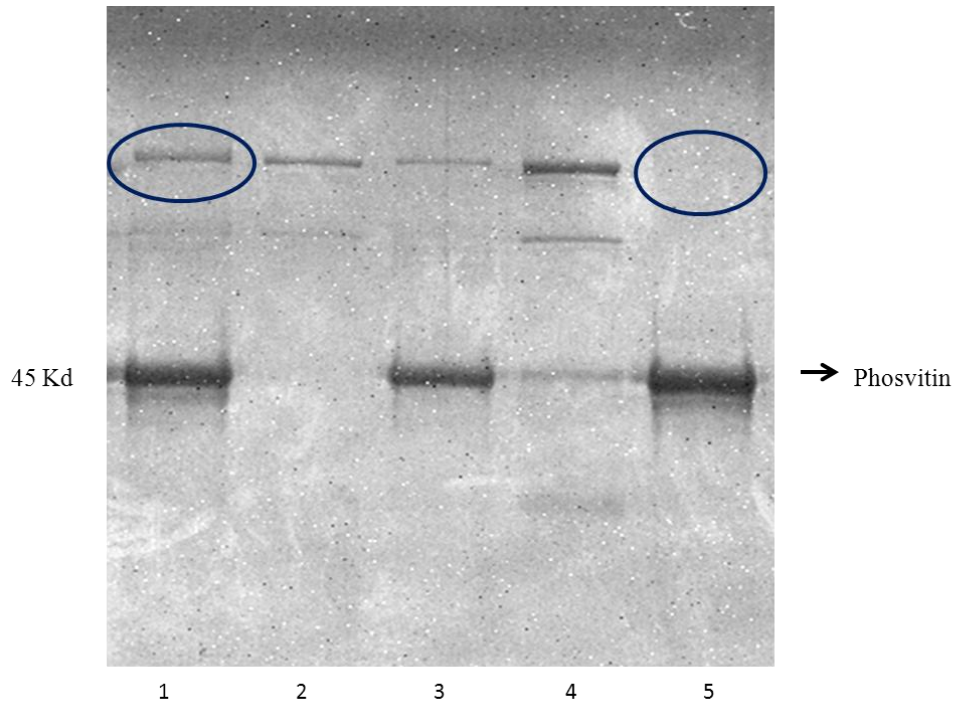
Lane 1: pH 3.0; Lane 2: pH 3.5; Lane 3: pH 4.0; Lane 4: pH 4.5; Lane 5: Phosvitin Standard.



**Figure 3. Effect of additional heat treatment at 70 °C on the purity of phosvitin extracted from egg yolk granules using 10% NaCl (w/v) in 0.05 N and 0.075 N NaOH solution**

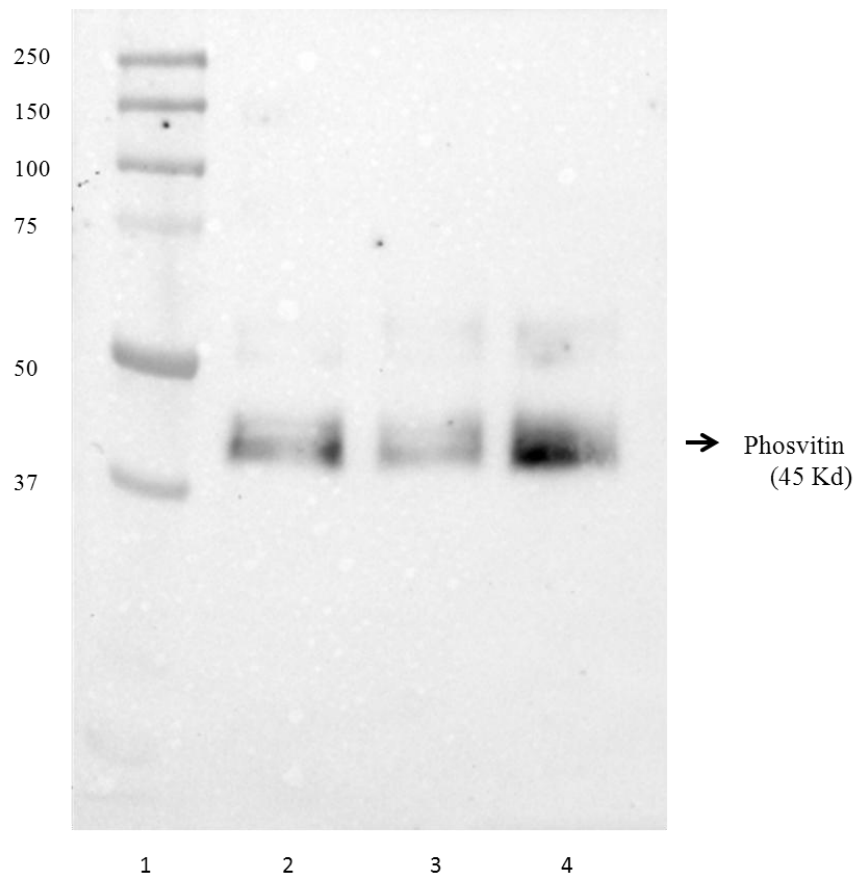
Lanes 1 to 4: Supernatant obtained from 0.05 N NaOH solution after heating for 30 min, 40 min, 50 min, and 60 min; Lanes 5 to 8: Supernatant obtained from 0.075 N NaOH solution after heating for 30 min, 40 min, 50 min, and 60 min; Lane 9: Precipitant obtained from 0.05 N NaOH solution after heating; Lane 10: Precipitant obtained from 0.05 N NaOH solution after heating.





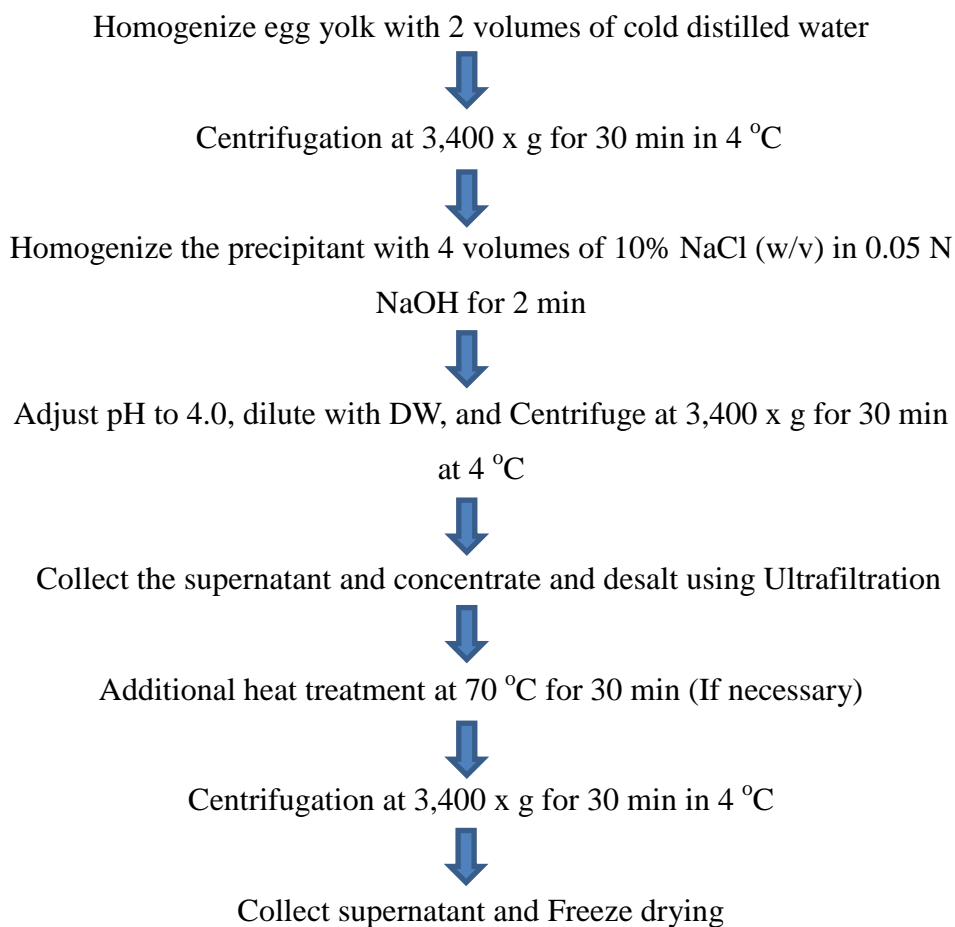
**Figure 4. Effect of additional heating at 70 °C for 30 min on the removal of impurities from the extract prepared using 10% NaCl (w/v) in 0.05N NaOH solution**

Lanes 1 and 3: Supernatant after centrifugation; Lanes 2 and 4: Precipitant after centrifugation; Lane 5: supernatant obtained from additional heating and centrifugation.



**Figure 5. Western Blot of Phosvitin**

Lane 1: Marker; Lane 2: Standard phosvitin (1mg/ml); Lanes 2 and 3: Purified phosvitin.



**Figure 6. Schematic diagram for the separation of phosvitin from egg yolk**

**Table 1. Yield of Phosvitin from egg yolk using the developed protocol**

<b>Sample</b>	<b>Weight (g)</b>	<b>Yield (%)</b>
Egg yolk Phosvitin <sup>1</sup>	2.07	100
Final Phosvitin <sup>2</sup>	2.04± 0.05	91.1± 0.04

<sup>1</sup>The theoretical amount of Phosvitin in egg yolk (Phosvitin is 8% of egg yolk protein). The original amount of egg yolk was approximately 165 g per replication.

<sup>2</sup>Phosvitin produced with 10% NaCl in 0.05N NaOH and heat treatment at 70 °C for 30 min; n=3

## Discussion

Lei and Wu (2012) recently studied granule solubilization by changing the pH of egg yolk solution from pH 5 to 12 and then loaded the phosvitin solution to an anion exchange column for further purification. They reported that egg granules could be completely dissolved in 0.05 M carbonate-bicarbonate buffer at pH 9.6. However, using carbonate-bicarbonate buffer (0.05 M, pH 9.6) could not extract phosvitin from egg yolk granules, and most of the phosvitin was still remaining in the granules after 2 extractions. Although, further purification method using anion exchange chromatography improved the purity of phosvitin, the recovery rate of phosvitin was 35.4%, which is much lower than currently available method (Ko et al., 2011) as well as the method we have developed in this study. Our results suggested that combination of high ionic strength (10% NaCl) and alkali conditions (0.05N NaOH) would be an excellent tool to isolate phosvitin from egg yolk granules, and further heat treatment of the extracted phosvitin significantly improved the purity of phosvitin. The method is simple, effective and scalable, and the yield and purity of the phosvitin separated are similar to or better than any methods reported. The purified phosvitin can be used in food products and pharmaceutical industry because the method does not involve with any solvents or harmful ingredients because it uses only salt, pH adjustment, centrifugation, heating and ultrafiltration.

## **CHAPTER 5**

# **SEPARATION OF PHOSPHOLIPIDS AND NEUTRAL LIPIDS FROM CHICKEN EGG YOLK USING FOOD GRADE SOLVENTS**

## 1. Abstract

Lipids are the major component of egg yolk, which takes approximately 32-36% of yolk. The composition of yolk lipids is about 65% triglyceride, 28-30% phospholipids, and 4-5% cholesterol. The objective of this study was to develop a separation method for neutral and phospholipids from the residues of IgY and phosvitin separation using food grade solvents. Egg yolk solid, collected after separating yolk granules and then IgY extraction, was treated with four volumes of 100% ethanol and homogenized to extract phospholipids. The homogenate was centrifuged at 3,400 x g for 30 min and the supernatant containing phospholipids was collected. The precipitant was treated with four volumes of hexane, homogenized, and centrifuged to extract neutral lipids. After removal of ethanol and hexane from the respective fraction using rotary evaporator, lipid classes were identified using thin layer chromatography. The majority of phospholipids were present in ethanol fraction and neutral lipids in hexane fraction, and their purities were > 85%. However, their purity can be improved by adding an extra purification steps. This method is considered to be non-toxic and can be incorporated to the sequential separation of value-added components from egg yolk.

Key words: Egg yolk, phospholipids, neutral lipids, ethanol, hexane, thin layer chromatography (TLC)

## 2. Introduction

Dried egg yolk is consisted of 61% lipids, 30% protein, 4% water, and 3% ash. The lipids from egg yolk can be divided into three parts: neutral lipids (65%), phospholipids (32%), and cholesterol (3%) (Stadelman and Corrterill, 1977). Neutral lipid is one of the major components of egg yolk lipids and its nutritional value is equivalent to that of plant oils or even better. Phospholipids are currently used in food and non-food products and are well known as an outstanding emulsifier (Juneja et al., 1994). Most of the phospholipids available to food and non-food applications are separated from plant oils. However, the amount of phospholipids in plant oils is only about 10% of egg yolk lipids, and thus egg yolk is much better source for phospholipids than plant oils. Egg yolk phospholipids are composed of 77% phosphatidylcholine (PC), 18% phosphatidylethanolamine (PE), and 3% sphingomyelin (SM) (Stadelman and Corrterill, 1977).

Phosphatidylcholine (PC) is a major component of biological membranes and is regarded as an important factor in the functions of nerve cells. Because PC contains choline, which is a vital nutrient for human, PC isolated from egg yolk can be used as a dietary supplement especially for infants whose nerve-system is developing quickly (Ueland, 2011). Also, choline plays a decisive role in serving as a precursor of phospholipids and acetylcholine and element for neurodevelopment as well as nutrient for liver function and cancer prevention (Miller, 2002). Phosphatidylethanolamine (PE) is found in all living cells and nervous tissues such as the white matter of brain, nerves, neural tissue, and in spinal cord. PE also creates a more viscous lipid membrane than PC due to its polar head group (Li et al., 2011). Sphingolipids are a vital component



of central nerve system myelin sheaths, and affect the viability of brain cells and signal transduction in T-cell activation (Podbeisliska et al., 2012; Dong et al., 2012).

The components of egg yolk are distributed in the yolk granules and plasma portions in a distinct way. Neutral lipids of yolk granules are present inside the myelin structure and those of plasma are present inside the low density lipoprotein spheres surrounded by proteins and phospholipids of LDL (Chang et al., 1977). Therefore, breaking the outer structure of granules and LDL spheres using solvents for solubilizing phospholipids should be performed first in order to extract neutral lipids from egg yolk.

At present, a number of isolation and purification methods for phospholipids from egg yolk in industrial scale have been developed, but the neutral lipids portion remaining in the residues after phospholipids extraction are not fully used. Neutral lipids can be easily removed from the residue of phospholipids extraction, which leaves protein as the major remaining main component of yolk. The yolk protein, recovered after removing all lipids from egg yolk, contains well balanced essential amino acids. So, they can be used for many food products as a protein supplement or raw materials for functional peptides production. With the continuous separation method for lipids and yolk proteins with food-industry-compatible protocol, the efficient and economic uses of egg will be increased. The objectives of this research were to develop optimal sequential separation method for phospholipids, neutral lipids, and yolk proteins with environmental friendly method. Developing this method using water, ethanol, and hexane will increase the value and utilization of egg yolk as well as separate many essential yolk components, which have high potential to be used for human.

### **3. Material and methods**

#### ***Materials***

Chicken eggs were purchased from a local market. Acetone, hexane, diethyl ether, and methanol were purchased from Fisher scientific (Thermo Fisher Scientific Inc., Waltham, MA, USA). Standard of PC, PE, SM, TG, Cholesterol and 2'7'-dichlorofluorescein was purchased from Sigm-Aldrich Inc. (St. Louis, MO, USA), silica gel-G plate was purchased from Merck (Merck, Whitehouse Station, NJ, USA)

#### ***Separation of phospholipids and neutral lipids from egg yolk***

Egg yolk was separated from egg white and 2 volumes of cold distilled water (4 °C) were added to the egg yolk, homogenized for 1 min using a kitchen hand blender (Kitchen Aid), and then centrifuged at 3,400 x g for 30 min. The supernatant was diluted with 3 volumes of cold distilled water again and kept in cold room for overnight. After centrifugation, precipitant is collected for extraction of phospholipids and neutral lipids from water-insoluble fraction of egg yolk. Because this method is aimed for human uses, three food-grade solvents including ethanol, acetone, and hexane were selected and tested with various volumes. Solvents were added to the water-insoluble fraction of yolk, homogenized using a kitchen blender (KitchenAid, St. Joseph, MI, USA) for 2 min, and then centrifuged at 3,400 x g for 30 min. The supernatant was collected and the solvent used was removed using a rotary evaporator under vacuum. Separated phospholipids and neutral lipids are identified to check the purity by a thin layer chromatography.

## ***Thin Layer Chromatography***

Dried solvent extract were dissolved in Chloroform to make the lipid content approximately 100mg/ml. Separated samples and each standard about 20 to 50 µl are applied onto pre-coated silica gel-G plate (20x20 cm, Merck, Whitehouse Station, NJ, USA) which had been pre-activated under the heat at 120 °C at 2 hr. The plates were developed in chloroform: methanol: water (98:38:6) solution mixture to 10 cm from the origin approximately 40 min inside of the chamber. The plate was air dried and then developed in hexane and diethyl ether (120:30) solution for another 20 min. The plates were air-dried and then sprayed with 0.1% (w/v) 2',7'-dichlorofluorescein in ethanol. The spots corresponding to PC, PE, TG, SM, and cholesterol were identified under UV light. The schematic diagram for the extraction of phospholipids and neutral lipids is shown in Figure 3.

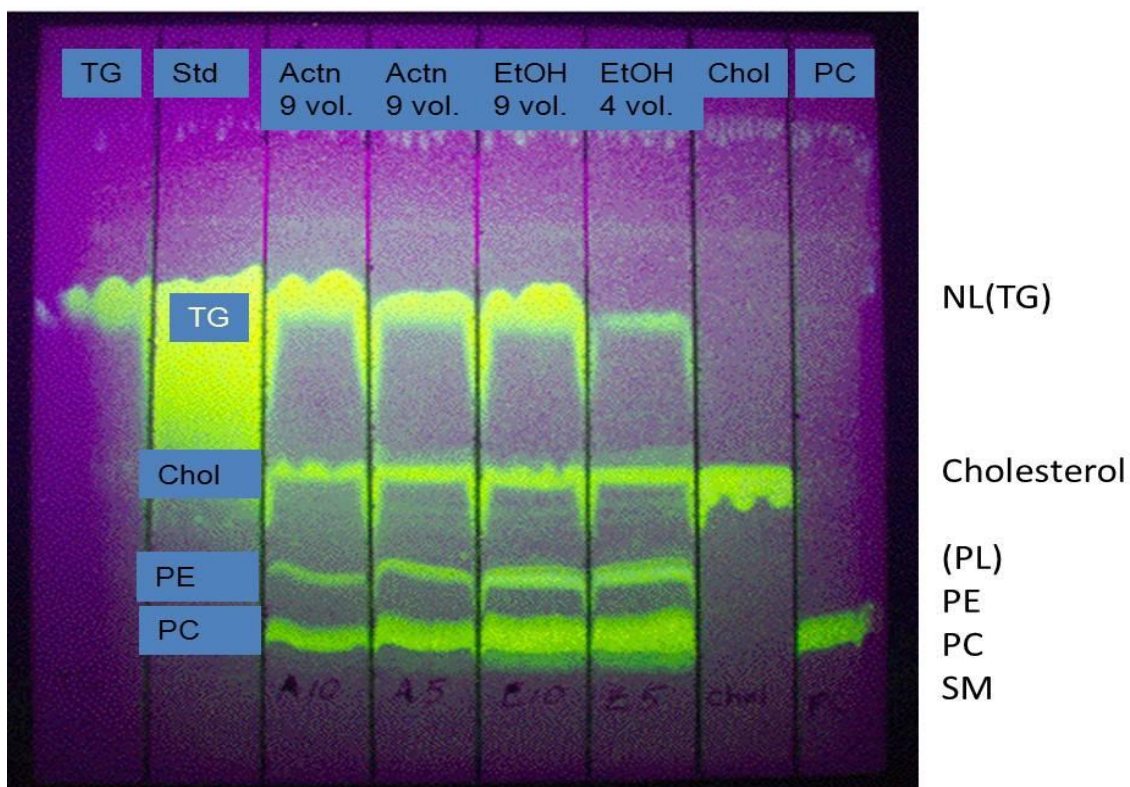
## **4. Results and discussion**

To extract phospholipids and neutral lipids from water-insoluble fraction of egg yolk, food grade solvents were tested for phospholipids extraction first. The result showed that acetone and ethanol have an excellent capability to extract phospholipids. However, hexane was not a good solvent for phospholipids extraction (Figure 1). The majority of the lipids separated using ethanol or acetone was phospholipids but some neutral lipids and cholesterol were also extracted as the volume of those solvents was increased from acetone in particular. It was also indicated that ethanol was better than acetone and 4 volumes of ethanol or acetone

was better than 9 volumes, representing high purity phospholipids and very little amount of neutral lipids in the TLC chromatogram.

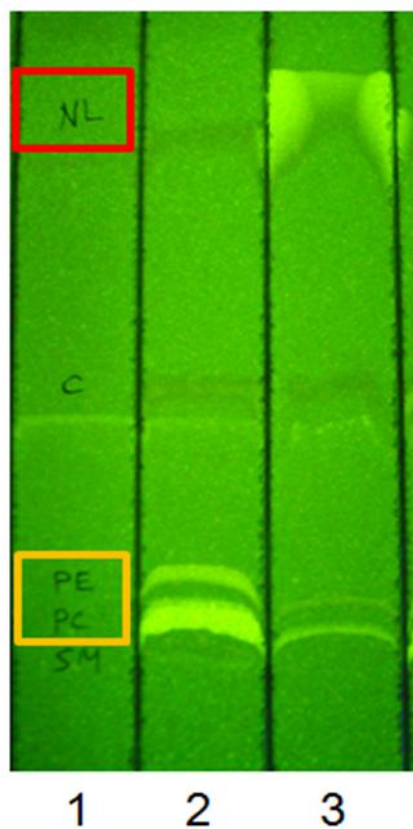
Result also showed that using 4 volumes of ethanol was better than 4 volumes of acetone resulting in little neutral lipids in ethanol extract even without further purification steps. Phospholipids content in the ethanol extract was very high portion but a cholesterol band is also seen. The remaining precipitant after ethanol extraction and centrifugation contained mostly neutral lipids, and was treated with 4 volumes of hexane. It is largely because hexane is the most broadly used solvent to extract lipids from plant seeds, have a strong power to extract neutral lipids. Figure 2 showed that the hexane extract was largely composed of neutral lipids but it also showed some phospholipids bands including PC and PE. Although almost all phospholipids was extracted using ethanol and neutral lipids using hexane, and their purities were relatively high (>85%), there are still potentials to increase the purity with additional purification steps.

After the second solvent extraction with hexane, the remaining precipitant was mainly composed of proteins. The final product after lipids and phospholipids removal and drying contained more than 90% proteins with small amounts of lipids and carbohydrates. Therefore, the final products can be used as a protein supplementing agent for many processed food products or substrate for functional peptides production. This method can be incorporated as a part of continuous separation process for multiple value-added components from egg yolk.



**Figure 1. Effect of solvents and solvent volumes on the extraction on phospholipids and neutral lipid from egg yolk**

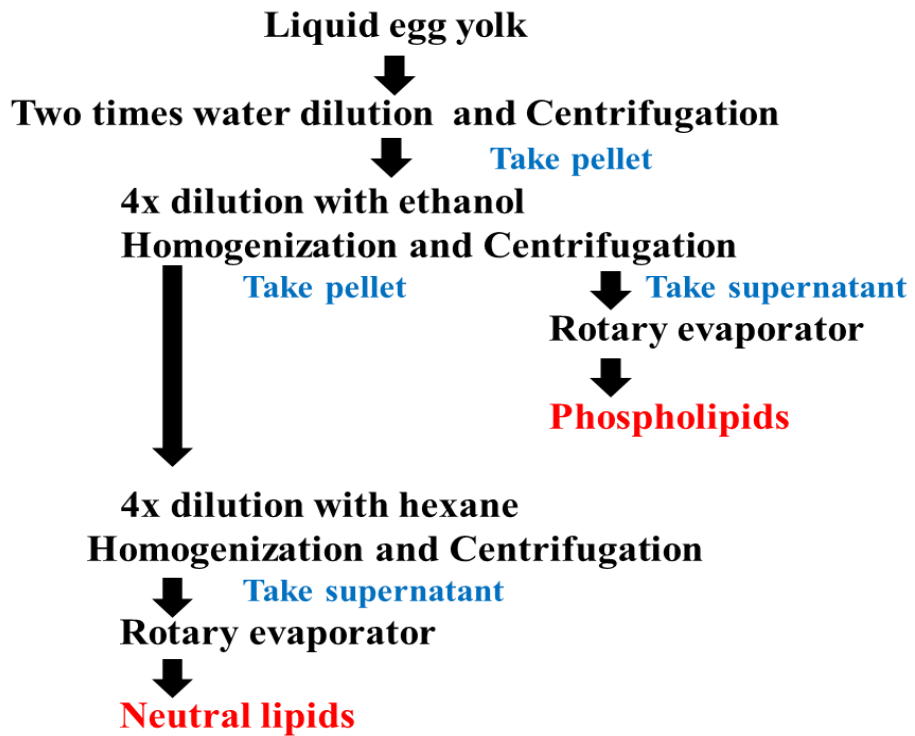
NL(TG): Neutral lipid; TG: Triglyceride; PL: Phospholipid; PE: Phosphatidyl Ethanolamine; PC: Phosphatidyl Choline; SM: Sphingomyelin.



**Figure 2. Effect of ethanol and hexane on the extraction of phospholipids and neutral lipid**

Lane 1: Standard indication; Lane 2: Ethanol extraction; Lane 3: Hexane extraction.

NL: Neutral lipid; C: Cholesterol; PE: Phosphatidyl ethanolamine; PC: Phosphatidyl choline; SM : Sphingomyelin.



**Figure 3. Schematic diagram for the separation of phospholipids and neutral lipid from egg yolk**

## **CHAPTER 6**

### **GENERAL DISCUSSION**



## General Discussion

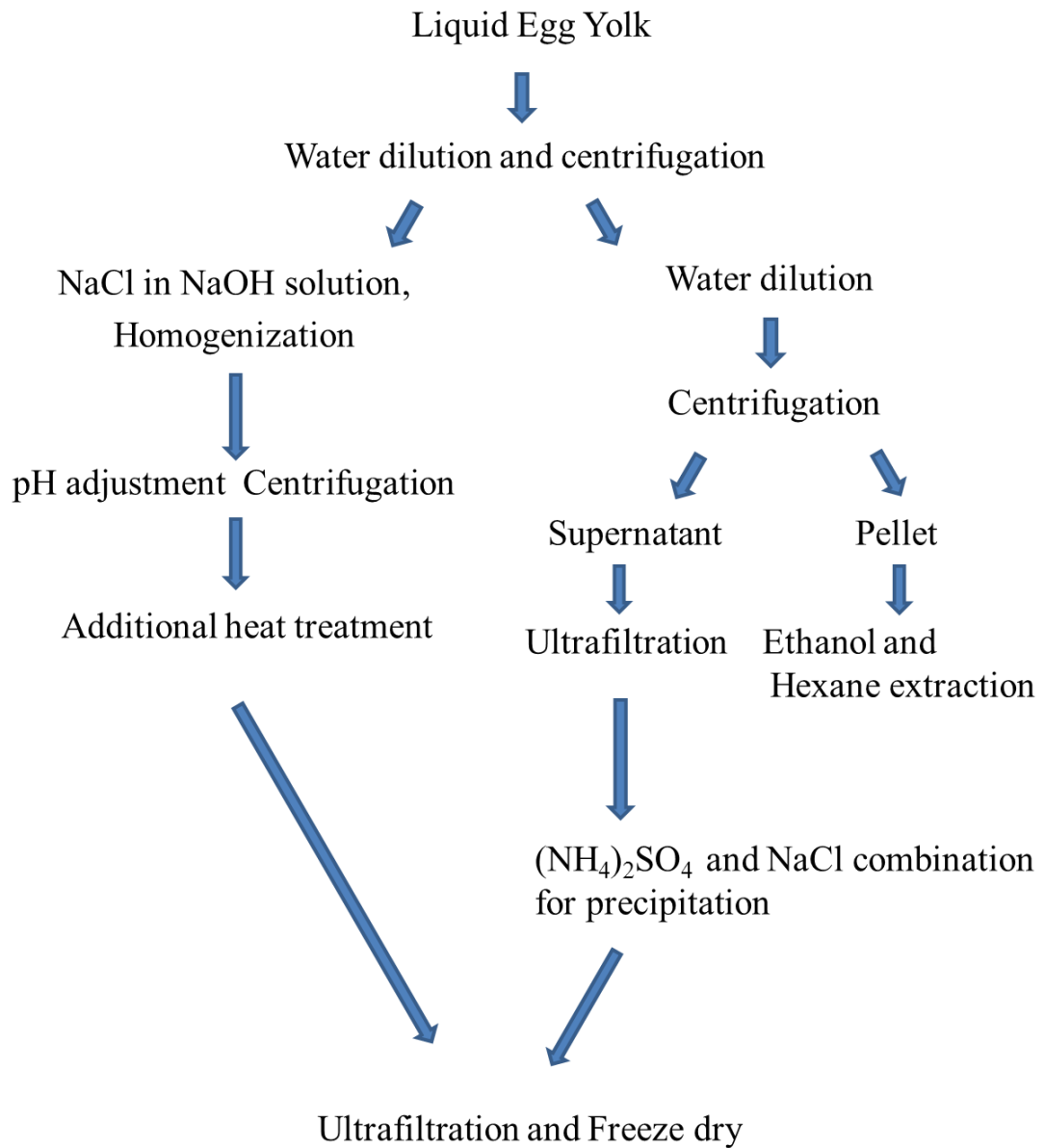
This study was conducted to develop optimal conditions for sequential separation of value-added components from egg yolk and implement the methods for large scale production of the value-added components in food and drug industries. From the liquid egg yolk, multiple elements such as immunoglobulin Y (IgY), phosvitin, phospholipids, and neutral lipids were extracted and separated sequentially using water, salt in alkaline solution, ethanol, and hexane. The final residue recovered was yolk proteins.

The developed protocols for individual component can be combined and make an integral scheme for sequential separation of all the components studied. The proposed sequential separation protocol for the value-added components from egg yolk is as follow:

Egg yolk separated from egg white was diluted with 2 volumes of cold water and then centrifuged at 3,400 x g for 30 min. Pellet, which is the granule portion of egg yolk was used to separate phosvitin and the supernatant (S1) used for IgY, lipids, and phospholipids separation. Yolk granules were treated with 4 volumes of 10% NaCl in 0.05N NaOH solution, homogenized, pH adjusted to 4.0, and then centrifuged. The supernatant was collected, and desalted and concentrated using ultrafiltration. The resulting supernatant mainly containing phosvitin was heat-treated at 70 °C for 30 min, and centrifuged. And then the supernatant was freeze-dried. The supernatant collected at the first step (S1) was diluted with 3 volumes of cold water again and centrifuged. The resulting supernatant was concentrated using ultrafiltration (cut-off size of 50 kDa) and IgY was precipitated using 20% ammonium sulfate + 15% sodium chloride combination. After centrifugation, the pellet containing IgY was

dissolved with water, salts removed by ultrafiltration, and then freeze dried. Lastly, the water-insoluble fraction (ppt) of the second dilution of egg yolk was added with 4 volumes of ethanol, homogenized, and centrifuged. The supernatant was collected after centrifugation, and phospholipids was recovered after removing ethanol using a rotary evaporator under vacuum. The precipitant of the ethanol extraction was added with 4 volumes of hexane, homogenized, and centrifuged. Neutral lipids was collected from the supernatant after removing hexane. The final solids after hexane extraction was dried and used as yolk protein fraction. The protocol successfully separated multiple value-added components from egg yolk in sequence and the purity and yield of the components were >85%.

The overall process is simple, efficient to separate value-added components, cost effective, and continuous separation system for multiple components from egg yolk. Also, the products can be used for various food and non-food purposes because no toxic compounds or harmful solvents were used for the process. With this sequential separation method of egg yolk components, natural resources can be maximally utilized with minimal wastes and costs. In addition, the protocol can be easily scaled-up for commercial production of many components, which will increase the use of egg and contribute to the sustainability of egg industry.



**Figure 1. Schematic diagram for the overall sequential separation method of value-added components from egg yolk**

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## SUMMARY IN KOREAN

난황에 있는 많은 요소들은 식품과 인간에게 있어서 중요한 기능성 역할을 하고 있다. 난황으로부터 몇몇의 요소들이 분리되고 상품화 되어왔지만, 분리방법들은 한 가지 요소만 분리하고 나머지는 버리기 때문에 상당히 제한적인 단점을 가지고 있다. 이는 중요한 자연 원천의 손실 및 경제적인 손실을 초래한다. 또한 많은 분리방법들이 실험실 스케일 수준에서만 개발되었고, 대량생산하여 산업화하기에는 부적합하다.

본 연구의 목적은 현재 사용되고 있는 난황의 유효물질 분리방법들을 비교하고, 면역글로불린 Y, 포스비틴, 인지질 및 중성지질들을 연속하여 분리정제하는 방법을 개발하는 것이다. 그리고 각각의 난황에서 분리된 유효물질들이 실용화 되어 쓰일 수 있도록 효율적인 대량생산화 할 수 있는 방법을 개발하는데 그 목적을 두었다. 또한, 모든 분리된 요소들은 사람을 대상으로 사용될 것이므로 본 연구에서는 유독한 시약 및 용매를 최소화 하거나 사용하지 않는 방법을 개발하고자 하였다.

본 연구에서 쓰인 주요 방법으로는 물을 이용한 회석, pH 조절, 원심분리, 한외여과거르기, 그리고 염침전법 등이 있다. 첫

단계에서 난황 볼륨의 2배의 물을 이용한 희석 및 원심분리를 통해 포스비틴을 함유한 과립이 난황으로부터 분리되었다. 포스비틴은 10%의 염화나트륨을 0.05N 농도의 수산화나트륨 용매에 녹인 용액을 이용하여 추출되었고, pH를 조절하여 불순물을 제거하였다. 원심분리 후, 포스비틴을 함유하고 있는 상층액은 한외여과거르기를 통하여 염 제거 및 농축이 되었다. 그 상층액을 다시 열처리를 하여 나머지 불순물을 제거한 후 원심분리하고 상층액을 모아 동결건조 시켰다. 2배의 물을 이용한 희석을 거친 상층액은 면역글로불린 Y와 지질들을 분리하기 위해 이용되었다. 상층액을 다시 그 볼륨의 3배만큼의 물을 이용하여 희석하였고, 이 단계에서 지질단백질을 함유하고 있는 고체부분이 침전이 되었다. 인지질 및 중성지방은 이 고체부분으로부터 분리가 되었다. 인지질과 중성지방은 에탄올과 헥산을 처리하여 각각 추출하였고, 회전증발기를 이용하여 처리한 에탄올 및 헥산을 제거하여 각 물질들을 분리하였다. 두 번째 물 희석을 거친 액상 부분은 한외여과거르기를 통하여 농축이 되었고 20%의 포화 황산암모늄과 15%의 염화나트륨 혼합염을 이용하여 면역글로불린 Y를 침전시켰다. 침전된 면역글로불린 Y를 다시 물에 녹여 한외여과거르기를 통하여 염을 제거하고 동결건조시켰다. 전기영동 및 웨스턴블롯 분석을 통해 분리된 포스비틴의 순도가 90% 이상, 수율이 98% 이상이고, 면역글로불린 Y의 순도가 90% 이상, 수율이 80% 이상임을 확인하였다.



본 연구로부터 개발된 방법은 대량생산에 바로 적용될 수 있도록 고안되었다. 그리고 분리된 난황으로부터의 유효물질들은 그 분리 공정에 있어서 유기 용매나 유독한 화학물질들을 사용하지 않았으므로, 사람을 대상으로 하여 식품 및 제약산업에 이용 가능할 것으로 기대된다.

## 감사의 글

실험실에서 생활한 지 어느 새 2년이 다 되어가고 졸업할 시기가 왔습니다. 그 동안 저의 주변에서 많은 도움을 주신 분들께 부족하나마 이 글을 통해 감사의 마음을 전하고 싶습니다.

먼저 많이 부족한 저를 지금까지 이끌어주시고 많은 가르침으로 지도해주신 안동욱 교수님께 진심으로 감사 드립니다. 교수님께서 신뢰해 주시고 지지해 주셔서 대학원 생활 동안 열심히 연구에 임하여 좋은 결과를 낼 수 있었습니다. 또한 바쁘신 와중에도 저의 부족한 논문심사를 해 주시고 세심한 조언을 해 주신 이무하 교수님과 남기창 교수님께도 깊은 감사의 말씀 전해드립니다.

지금까지 함께한 단백질화학공학 연구실 식구이자 늘 성실함으로 모범을 보여준 산둥에게 감사의 말씀 전하고 싶습니다. 미국 실험실에 있을 때 많은 도움을 주시고 격려해 주시며 아낌없이 조언을 해 주신 이은주 교수님, 실험실에 오셔서 도와주시고 늘 힘차게 응원해주신 김일석 교수님, 대학원 생활을 보람차게 보낼 수 있도록 따뜻하게 조언해주신 조철훈 교수님과 이경행 교수님, 그리고 석사 첫 학기에 많은 가르침과 깊은 신앙심으로 지지해주신 고경욱 박사님께 감사 드립니다. 더불어, 대학원 생활이 힘들고 지칠 때 옆에서 많은 응원과 격려의 말씀을 아끼지 않았던 생물물리학 및 나노생물학 연구실과 여성 건강 : 생식 및

중앙 생물학 연구실 식구들에게도 모두 감사 드립니다. 마지막으로 지금까지 큰 딸 뒷바라지 해 주신 사랑하는 부모님과 동생에게 감사의 말씀 전합니다.

이곳에서의 소중한 경험을 바탕으로 한 걸음 더 나아가 앞으로 귀하게 쓰임 받을 수 있는 더욱 발전한 이현용이 될 수 있도록 노력하겠습니다. 감사합니다.