Preparation and Purification of Immunoglobulin Y from Egg Yolk of Immunized Hens

تحضير وتنقية الجلوبيولين المناعي Y من صفار البيض لدواجن ممينعة

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Abstract

Two purification methods of egg yolk immunoglobulin (IgY) based on precipitation using charcoal-PEG and dextran-PEG were developed. These methods were com- pared with chloroform extraction and polyethylene glycol (PEG) precipitation methods. The results showed that protein contents were high with chloroform method (12.8 mg/ml) followed by dextran-PEG (10.5 mg/ml), charcoal-PEG (8.13 mg/ml) and PEG method (4.4 mg/ml). The purity of resultant IgY was homogeneous with dextran-PEG method followed by charcoal-PEG method, less purity was in PEG method followed by chloroform extraction method.

Introduction

Most commonly used animals for the production of immune sera for diagnostic purposes are rabbits and guinea pigs, although horses, goats, and sheep are used to a lesser extent for the same purpose (1). However, chickens are an attractive alternative to mammals as antibody producers because large quantities of antibodies can be produced from the egg yolk making restraint from the blood sampling obsolete techniques to the benefit of the animals used for this purpose (2).

There are 3 classes of immunoglobulin, analogues to the mammalian immune - globulin classes have been shown to exist in chicken, IgA, IgM and IgY (IgG) (3). IgY is a systemic rather than a secretary antibody but IgY is also found in duodenal contents, tracheal washings and seminal plasma, it is called IgY rather than IgG to distinguish it from its mammalian counterpart (4). The overall structure of IgY is similar to mammalian IgG, with two light (L) and two heavy (H) chains, the molecular mass has been reported to be 167 250 Da, slightly larger than IgG (~160 kDa) (5). The H chain (Mw 65 105 Da), has one variable (V) region and four constant (C) regions, the light chain (Mw 18 660 Da) is composed of one variable and one constant domain (6). The concentration of Ig Y in the yolk is essentially constant through the oocyte maturation, and at maturity the yolk will contain about 10-20 mg/ml IgY (7). When hens immunize with an antigen, produce specific

antibodies against this antigen, these antibodies are transported in a large quantity to the egg yolk from the blood of laying hens (8).

IgY has been produced against a number of bacteria and viruses, and has been shown to bind to and inhibit the infection and disease symptoms, in-vitro and in-vivo, of gastrointestinal pathogens such as enteric colibacillosis, salmonellosis and human & bovine rotavirus (9, 10 , 11 and 12). Also, IgY has been applied successfully for scientific and diagnostic purposes (13, 14).

One of the major obstacles in isolating IgY from egg yolk is a high concentration of lipids and lipoproteins (15), this situation renders some purification of IgY necessary for scientific use regardless of the assay in which they are to be used (16). There are several procedures used for purifying IgY based on the strategy of separation of proteins from lipoproteins and the rest of the yolk lipids using extraction with organic solvents rather low yields of antibody (17). Other methods are based on dilution of the yolk followed by a freezing-thawing process after which the process consists of ion exchange chromatography (17). Moreover, by using of 3.5% (w/v) of a low molecular weight polyethylene glycol (PEG) (18) or natural gum (19).

The main aims of this study were: 1) test the use of charcoal and dextran in the purification of IgY against other methods including uses of chloroform extraction and uses of PEG 6000 precipitation. 2) evaluate the purity of these four methods and yield.

Material and Methods

Laying Hens

Four brown laying hens *Gallus domesticus* were obtained from a commercial farm. The hens were kept in an environmentally controlled room, and were subjected to regular light cycles. The hens were fed *ad libitum* with commercial diet.

Immunization of Hens

The procedure of chicken immunization was described by (20). For the first injection, 4 mg bovine serum albumin (BSA) was dissolved in phosphate buffer saline (PBS) [0.14 M NaCl, 0.0015 M KH2PO4, 0.0081 M Na2HPO4, and 0.0027 M KCl, pH 7.2], and emulsified with an equal volume of complete Freund's adjuvant to obtain the final concentration of 1 mg bovine serum albumin (BSA)/ml. Each hen was intramuscularly injected with 1 mg BSA at four different sites (0.25 mg per site) of breast muscles (two sites per left or right breast muscle). A booster injection was given intramuscularly 2 weeks after the first injection with the same dose emulsified with Freund's incomplete adjuvant.

Collection of Eggs and Separation of Yolk

The eggs were collected daily after 2 weeks of booster immunization and kept at 4˚C until suitable number were obtained. The yolk of ten eggs were separated according to (1) with minor modification, the egg yolks were separated from egg whites, washed with distilled water to remove as much albumen as possible and rolled on paper towels to remove adhering egg white. Intact yolks were broken by dropping through a funnel into a graduated cylinder and mixed thoroughly.

Purification of IgY from Egg Yolk
Lipid Removal

The water soluble protein was prepared from egg yolks by using three main protocols for lipid removal, each protocol was tested three times. These protocols included :

A- Used organic solvents (chloroform) according to (21). Briefly, 15 ml of yolk was brought to 25 ml with sodium phosphate buffer (100 mM, pH 7.6) and mixed vigorously. Subsequently, 20 ml of chloroform was added and the mixture was shaken until a semisolid phase was obtained. Then the mixture was centrifuged at 2000 rpm for 30 min, the supernatant was filtered through filter paper and decanted into another centrifuge tube for further purification of IgY.

B- Used polyethylene glycol in 3.5% according to (18). Briefly, an equal volume of buffer (0.01 M sodium phosphate, 0.1 M NaCl, pH 7.5) was added to yolk and stirred. Solid polyethylene glycol PEG 6000 (Sigma) was added to a concentration of 3.5%, stirred until it all dissolved, and the protein precipitate that formed was pelleted by centrifugation at 10,000 rpm for 15 minutes. The supernatant was filtered through filter paper and decanted into another centrifuge tube for further purification of IgY.

C- A novel and simple procedure modified from a combination of earlier protocols (18, 19). Briefly, egg yolk was diluted 1:2 with distilled water, homogenized for 30 seconds and filtered through filter paper. The mixture was mixed with two volumes of D.W. contain one of these materials charcoal (BDH) or dextran (BDH) , the final percentage of these material (0.01%).The resultant mixture was left for 30 min at room temperature, then centrifuged at 12000 rpm for 15 minutes. The supernatant was filtered through filter paper and decanted into another centrifuge tubes. To complete elimination of lipid, 3.5% of solid PEG 6000 (Sigma) was added to the supernatant and stirred until dissolved. The mixture was centrifuged at 12000 rpm for 15 min (to pellet the residual lipoprotein precipitate) . The supernatant was filtered through filter paper to remove any floating lipid debris and decanted into another centrifuge tube for further purification of IgY.

Precipitation of IgY

This step was conducted according to (18). Briefly, 12% w/v solid PEG was added to the supernatant and stirred thoroughly , and centrifugation at 10000 rpm for 15min, resulted in the precipitation of IgY. The pellet was redissolved to the original yolk volume in 0.01 M sodium phosphate buffer, 0.1 M NaCI, pH 7.5, and PEG was added to 12% w/v for a second precipitation. The supernatant was decanted and the pellet centrifuged twice more to remove any residual PEG trapped in the precipitate. This final IgY pellet was then dissolved in phosphate buffer (0.01 M, pH 7.5) to the original volume of yolk and stored at -20˚C.

Total Protein Estimation

Total protein concentration of product was determined according to (22) with bovine serum albumin (BSA) as standard in the range from 0 to 500 µg/ml.

Protein Electrophoresis

To determine the purity of IgY in the egg yolk final product, Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was used according to (23) . The resultant from the IgY precipitation steps was dissolved in sample buffer with 2% 2-mercaptoethanol and run on a 5% stacking gel and 10% separating gel. The gel was run at 20 mA for 1.5 h and stained with Coomassie Brilliant Blue.

Gel Precipitation

For gel precipitation (Ouchterlony double diffusion), agarose (0.75 % in phosphate buffer saline) was done according to (24). The mixture was autoclaved at 15 PSI for 15 minutes and then allowed to cool to 60˚ C. Ten milliliters of the agar was pipetted into petri dishes and allowed to solidify. A cluster of six wells (5 mm) surrounding a center well of 5 mm, separated by 3 mm from each other, was cut into the agar. Twenty microliters (μL) of the IgY sample was added to the outer wells, and 20 μL of antigen (BSA 2 mg/mL) was administered to the central well. The plates were incubated at room temperature in a humidified chamber for 24 h, and the precipitin reactions were determined.

Results

Purification of IgY

The IgY was purified by two steps including lipid removal and precipitation of IgY.

Lipid Removal

In this study two novel procedures were used to purify the IgY from egg yolks. Lipid removal from yolk was done with two combinations of salt precipitation including charcoal-PEG and other combination including dextran- PEG. These

methods were compared with 2 traditional methods include precipitation of lipid by using PEG 3.5% alone (18) and lipid extraction with chloroform (21).

Table (1) and Figure (1) show the protein concentration of water soluble protein after lipid removal. Chloroform extraction method was gave the highest protein concentration (mg/ml) followed by Dextran-PEG, PEG then charcoal-PEG with mean ± SD, 21± 0.7, 18.1± 0.15, 16.8± 0.4 and 16.4± 0.3 respectively.

IgY Precipitation

The IgY was precipitated from water soluble protein resulted from different lipid removal methods. Table (1) and Figure (1) show the protein content of IgY precipitation by different methods. Chloroform extraction method was gave the highest protein concentration (mg/ml) followed by Dextran-PEG, charcoal-PEG then PEG method with mean ± SD, 12.8 ± 0.25, 10.5 ± 0.36, 8.13 ± 0.21 and 4.4 ± 0.36 respectively. The yield of protein obtained by various methods was high with chloroform method (61%) followed by dextran-PEG, charcoal-PEG then PEG method, 58%, 50% and 26% respectively.

Table (1): Protein concentrations of resultant solutions after lipid removal and IgY precipitation.

Methods used for purification of IgY	Protein concentration mean ± SD (mg/ml) After lipid removal	Protein concentration mean ± SD (mg/ml) After IgY precipitation with 12% PEG-6000	Protein yield %
Chloroform extraction method (Polson *et al*., 1990)	21 ± 0.7	12.8 ± 0.25	61
Charcoal – PEG 6000 precipitation method	16.4 ± 0.3	8.13 ± 0.21	50
Dextran – PEG 6000 precipitation method	18.1 ± 0.15	10.5 ± 0.36	58
PEG 6000 precipitation method (Polson *et al*., 1980)	16.8 ± 0.4	4.4 ± 0.36	26

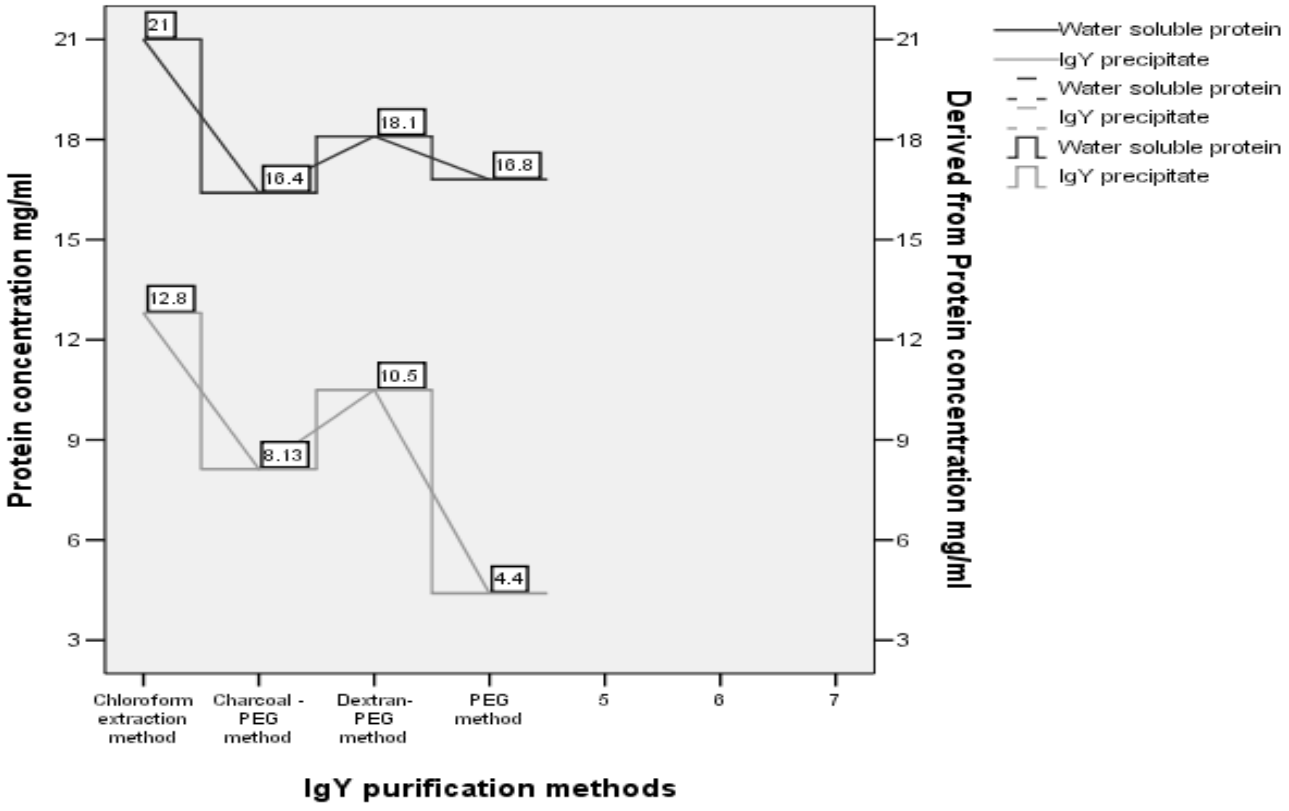

Figure (1): Comparison between protein concentrations of resultant solutions after lipid removal and IgY precipitation. The upper line represent protein concentration of water soluble protein after lipid removal with different methods, however the lower line represent protein concentration of IgY precipitate (the last purification step).

From left to right Chloroform extraction method, Charcoal – PEG 6000 method, Dextran – PEG 6000 method and PEG 6000 precipitation method respectively.

Purity of IgY

Purity of IgY (the final step of purification) was detected with using SDS-PAGE under reducing conditions. Figure (2) demonstrate that IgY purified with dextran-PEG contained two distinctive protein bands with a few contaminant bands, IgY purified with charcoal-PEG 3 distinctive protein bands with 3 minor bands. However, IgY extracted with chloroform method contained 4 major protein bands and 5 minor bands, also IgY purified by PEG method contained 4 major protein bands and 3 minor bands.

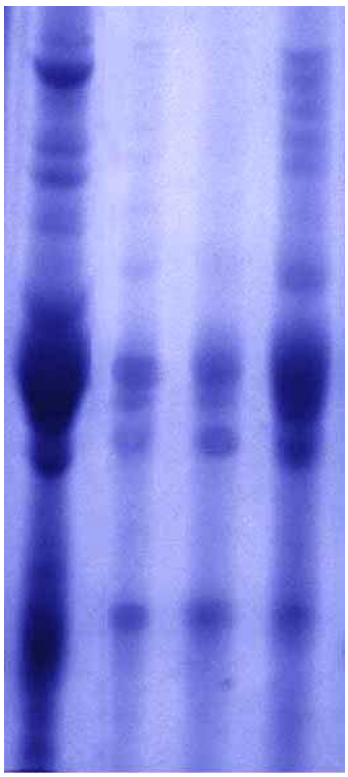

Figure (2): SDS-PAGE of IgY precipitate of various purification methods under reducing conditions, from left to right chloroform extraction methods, charcoal-PEG precipitation method, dextran-PEG precipitation method and PEG 6000 precipitation method.

Discussion

The main components of yolk are lipids (about 65% of the dry matter) and the lipid to protein ratio is about 2:1, lipids of yolk exclusively associated with lipoprotein assemblies (25), the major problem in isolation of IgY is removal of lipids (26). Therefore, the first step of isolation of IgY is to separate the water soluble protein from lipids and lipoproteins.

From a yield point of view the content of protein obtained by various methods was high with chloroform extracted method followed by dextran-PEG, charcoal-PEG and at the last PEG method. In recent study the reported concentration of IgY extracted with chloroform was 12.8 mg/ml, this result is slightly higher than that reported by (16). SDS-PAGE analysis of IgY purified with chloroform extracted method appears to confirm previous observation by (16) whom reported that the IgY extracted with chloroform is contaminated with 20% unwanted non-sense proteins.

IgY purified with PEG method resulted in a significantly low total protein content compared with other purification methods, this result is in accordance with (16, 26, 27 and 28). Result of SDS-PAGE analysis of IgY purified with PEG-6000 procedure are in agreement with (1 and 29).

A high purity of the IgY preparation is desirable for many immunoassays and for production of labeled second antibodies (30). Results of SDS-PAGE analysis of IgY purified with dextarn-PEG and charcoal-PEG showed minor contaminant proteins in

comparison with IgY purified with other methods of purification. However, the purity of IgY purified with dextran-PEG appeared more homogeneous in comparison with that purified by charcoal-PEG method.

In conclusion results of this study indicated that the purification of IgY from egg yolk by using dextran-PEG and charcoal-PEG have good purity in comparison with other two methods. Moreover, the number of protein bands obtained in the final product after using dextran-PEG were significantly decreased.

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