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Isolation and Estimation of Chicken Immunoglobulins (IgY) from Egg Yolk by Optimizing Polyethylene Glycol (PEG) Precipitation Method

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Abstract: Immunoglobulin Y (IgY) is found in high concentrations in the egg yolk of chicken. Birds concentrate immunoglobulins into their egg yolks to protect the offspring. The practical application of IgY in research, diagnostics and functional food is limited due to complex and time-consuming purification procedures. The objective of this study was to develop an economic, simple, safe, large-scale separation method for IgY from egg yolk. In the present study, we determined egg yolk IgY concentration in six lines of chicken by optimizing polyethylene glycol (PEG) precipitation method. Egg yolk samples were collected from studied lines of chicken. PBS (Phosphate Buffer Saline) was taken twice of the egg yolk volume and mixed with the yolk. Thereafter 3.5% PEG (Polyethylene glycol) of the total volume was added and centrifuged (4°C) for 30 min at 4500 rpm. The supernatant was poured through a folded filter paper into a new tube and 8.5 % PEG was added to the tube, and centrifuged again. The supernatant was discarded and PBS was added to make a volume of 10 ml. The solution was mixed with 12 % PEG and centrifuged again. The pellet was carefully dissolved in 2 mL PBS. Finally, the isolated IgY samples were stored at -20°C until further processing. SDS-PAGE was performed to check the quality of IgY. The purity of IgY was determined by electrophoresis. The result showed that the yield of isolated IgY was varied between 206.015 and 392.030 mg per egg in the experimental lines of chicken. In this optimized method, the yield of IgY was the highest 30.904 ± 7.621 mg/mL of egg yolk and the lowest was 16.753 ± 5.282 mg/mL. This optimized precipitation method was simple with a higher yield of IgY, more efficient and useful for the large-scale preparation of IgY from egg yolk.
Keywords: Immunoglobulin Y (IgY), Polyethylene Glycol (PEG), Phosphate Buffer Saline (PBS), SDS-PAGE, Egg yolk

INTRODUCTION:

Chicken egg yolk has been considered as an ideal source of immunoglobulin and IgY is understood to be the predominant antibody in egg yolk [1]. Egg yolk contains massive amount of immunoglobulin Y (IgY), the functional equivalent to mammalian IgG, which plays a central role in the protection of the newly hatched chick against infectious diseases [2]. In mammals, maternal Igs are transferred to the fetus and neonate through the placenta and breast milk, respectively. In birds, maternal Igs in blood are incorporated into egg yolks of maturing oocytes, and then they are transferred to the embryonic circulation through yolk sac membrane [3]. It has many significant advantages over mammalian IgG. Firstly, the production of IgY is non-invasive, which makes it suitable for large-scale production (~40 g IgY/y/hen). Secondly, due to the phylogenetic distance between chickens and mammals, IgY does not react with rheumatoid factors, bacterial fragment crystallisable (Fc) receptors, and mammalian IgG [4]. Also, IgY can recognize more epitopes of the highly conserved mammalian proteins than other mammalian IgGs are able to [4], IgY does not induce false positive results in immunoassays because they do not activate mammalian complements [5]. Immunoglobulin Y (IgY) have been applied successfully for scientific, diagnostic, prophylactic and therapeutic purposes [6], but the practical use of IgY in research and diagnostics is still also limited due to the complex and time-consuming purification steps [5]. Other reasons are lack of laboratory experience with the processing of egg yolk for the extraction of IgY and the methods which are used for the purification of mammalian immunoglobulins cannot be automatically applied to egg yolk and IgY [7]. IgY concentration in the egg yolk of chickens has been measured by many investigators, but the reported IgY concentrations have varied from 1to 25 mg/g yolk [8-11, 1]. It seems likely that the scattering of the yolk IgY concentration data is caused by multiple reasons including differences in strains of chickens [12] and daily fluctuation[11], but one of the main reasons is that the methods of preparing IgY yolk extract differed among the investigators [13]. The key of isolating IgY from egg yolk is to remove the water-insoluble components such as lipids and lipoproteins to get water-soluble protein fraction (WSF) [14]. Different researchers have proposed several methods

for efficient IgY separation, such as: the use of detergents such as SDS, carrageenan, sodium alginate, or xanthan gum; use of solvents such as acetone, chloroform and ethanol ; for the precipitation of lipoproteins using polyethylene glycol or dextran sulfate ; aqueous 2-phase system with phosphate and triton X-100; simple freeze and thaw cycling and water dilution under acidic conditions have been used to remove lipids and lipoproteins from egg yolk extract. Most of these methods have drawbacks due to low IgY yield rates, complexity of procedures or compatibility for human use [5]. Akita and Nakai [15] compared the water dilution method (WD) with three other methods, namely, polyethylene glycol (PEG), dextran sulphate (DS) and xanthan gum (Xan) in terms of yield, purity, ease of use, potential scaling up and immunoactivity of IgY. The WD method gave the highest yield, followed by DS, Xan and PEG methods in that order. A total of 9.8 mg IgY/ml egg yolk was routinely obtained from the WD method compared to 4.9 mg IgY/ml egg yolk with the popular PEG method with purities of 94% and 89%, respectively. Chang *et al*. [16] reported that addition of 0.1%of λ-carrageenan was effective in removing lipoproteins from the water extract of egg yolk at pH 5.0. They just focused on the interactions between polysaccharides and lipoproteins; they did not consider the purification of IgY. Tan *et al.* [17] reported a rapid way to isolate IgY using a combination solution followed by ammonium sulfate. But the yield and purity of IgY isolated in this way were not very satisfying. Pauly *et al*. [18] described a protocol of total IgY extraction from egg yolk by means of polyethylene glycol (PEG) precipitation procedure. The purity of the extracted IgY by this method is around 80%, the total IgY per egg varies from 40-80 mg, depending on the age of the laying hen. The objective of the present study was to optimize the protocol of polyethylene glycol (PEG) precipitation method for the extraction of IgY from egg yolk with higher yield rates. This optimized IgY extraction methodology can easily be performed in simply equipped laboratory. In this study, we also determined the yolk IgY concentration in different broiler sire and dam lines namely: Male Line White (MLW), Male Line Color (MLC), Male Line White 2 (MLW2), Female Line White (FLW), Female Line Color (FLC) and a two-way cross of MLW male and FLW female (MLW x FLW).

MATERIALS AND METHODS

Statement of the Experiment:

This study was conducted at Bangladesh Agricultural University Poultry Farm and Poultry Biotechnology and Genomics Laboratory, Department of Poultry Science, Bangladesh Agricultural University, Mymensingh in between February 2014 and November 2014.

Experimental Populations:

Hens from previously developed lines namely: Male Line White (MLW), Male Line Color (MLC), Male Line White 2 (MLW2), Female Line White (FLW), Female Line Color (FLC), and a 2-way cross of MLW male and FLW female (MLW x FLW) were used in this study. These lines of chicken were developed at BAU (Bangladesh Agricultural University) Poultry farm by index selection for rapid early growth and better egg production. The MLW, MLC and MLW2 were selected for early rapid growth for use as a male line for producing broiler chicks, whereas FLW and FLC selected for increased egg production and moderate early growth. All experimental lines were reared in an open sided house. Feeding, lighting and other management were similar in the studied lines.

Collection of Samples:

An average of 6-7 eggs per line was collected from a single hen in between 35-37 weeks of age. Freshly laid eggs were collected and immediately transferred to Poultry Biotechnology and Genomics Laboratory for further processing.

Isolation of IgY:

There are several methods of IgY isolation depending on the type of starting materials and laboratory facilities [19]. In this study, optimized protocol of Polyethylene Glycol (PEG) precipitation method was used for the isolation of IgY from egg yolk.

Yolk Separation

At first, the eggs were weighed and the eggshell was cracked carefully. The yolk was transferred to a modified "yolk spoon" in order to remove as much egg white as possible without piercing the vitelline membrane. Then the yolk was transferred to a filter paper and rolled to remove remaining egg white, and the vitelline membrane was punctured with a pipette tip. The yolk was carefully poured into 50 ml tube and the yolk volume was recorded.

Separation of IgY

i. PBS (Phosphate Buffer Saline) taken twice of the egg yolk volume was mixed with the yolk, thereafter 3.5% PEG6000 (in gram, pulverized) of the total volume was added and vortexed, followed by 10 min rolling on hand. This rolling process separates the suspension in two phases. One phase consists of "yolk solids and fatty substances" [20] and a watery phase containing IgY and other proteins.

ii. The tubes were centrifuged in a pre-cooled centrifuge (4°C) for 30 min at 4500 rpm (HARRIER 18/80, UK). The supernatant was poured through a folded filter paper and transferred to a new tube.

iii. Then 8.5 % PEG 6000 in gram (calculated according to the new volume) was added to the tube, vortexed and rolled for 10 min. The tubes were centrifuged again in a pre-cooled centrifuge (4°C) for 30 min at 4500 rpm.

iv. The supernatant was discarded and the resulting pellet was carefully dissolved in 1 mL PBS by means of a vortexer. PBS was added to a final volume of 10 mL. The solution was mixed with 12 % PEG 6000 (w/v, 1.2) and treated as in step iii (vortex and rolling mixer).

v. Again the tubes were centrifuged at 4°C for 30 min at 4500rpm and the supernatant was discarded. The pellet was carefully dissolved in 2 mL PBS.

vi. Finally, the isolated IgY samples were stored at -20°C until further processing.

SDS-PAGE of IgY:

To determine the purity of IgY in the egg yolk final product, Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was performed as described by Laemmeli [21]. SDS gel electrophoresis of IgY was done according to manufactures recommendations (Bio Craft, Ever Seiko Corporation, Tokyo, Japan). It was performed in a slab type vertical gel system. 10µL of each sample was placed in each well on one of the slab gels. Migration was performed at 20 mA per gel (or 40 mA totals) and it was taken approximately 1 hour to move down the slab. After migration, gels were stained with Coomassie Brilliant Blue R-250.

It was used to visualize the protein bands. When protein bands were visualized, then the gels were carefully washed in distilled water and placed on the scanner to capture the image.

Determination of IgY Concentration in Egg Yolk:

The IgY content (mg/mL) of the samples is measured photometrically at 280 nm (1:40 dilution with phosphate buffer saline) and calculated according to the Lamber-Beer law with and extinction coefficient of 1.33 for IgY.

Statistical Analysis:

Collected data were analyzed by using a linear mixed model implemented in R (R Core Team, 2014) [22]. Effects of line, egg weight, yolk volume, yolk weight and batch of IgY separation were treated as fixed effects. The covariates and their interactions that had significant effects at the nominal 5% level were included in the final model for comparisons of IgY levels among the lines of chickens. Line differences of IgY levels in egg yolk were determined by Tukey HSD post hoc test.

RESULTS AND DISCUSSION:

Isolation of Immunoglublin Y (IgY): The eggs used for IgY separation were collected from six lines (MLW, MLC MLW2, FLW and FLC and MLW × FLW) of chickens in between 35 to 37 weeks of their age. In this study, the egg yolk IgY was separated by Polyethylene Glycol (PEG) precipitation method. It is an excellent method to precipitate a specific protein from a complex mixture of proteins [18]. This method involves two important steps. The first one is the removal of lipids and the second is the precipitation of total IgY from the supernatant of step one. Using 3.5% PEG in the egg yolk volume helps to separate the "yolk solids and fatty substances" and it also removes the fatty substances from the egg yolk. In this method, for the precipitation of IgY 12% PEG was used in the last step and the IgY-extract can be stored at -20°C for more than a year. The purity of the isolated IgY was examined by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A representative SDS-PAGE of individual IgY preparation from different eggs under reducing conditions is shown in Figure 1. It is evident that both heavy and light chain of IgY fragments is present in the isolated total IgY without the existence of other protein impurities. The presence of heavy chain in the gel electrophoresis pattern of antibody is an indication of appropriate extraction method [23]. IgY can also be isolated from eggs using a water dilution method [15], ammonium sulfate [24], dextran sulfate [25], polyethylene glycol (PEG) and ion exchange chromatography [26]. The purity of IgY preparation can be increased by a combination of methods; for example, PEG precipitation can be combined with affinity chromatography [18]. The choice of a suitable IgY purification method is influenced by scale of purification (laboratory or industrial), cost effectiveness, technology (laboratory equipment), and impact on the environment (waste management) [18]. The technique described in this experiment with polyethylene glycol is widely accepted as the standard technique to isolate IgY [27]. Typically IgY of 89% purity is obtained in this method compared with 70–80% with dextran sulfate or ion exchange chromatography or 60–70% with ammonium sulphate [27]. The polyethylene glycol protocol does not require dialysis steps and therefore can be completed within 3 h [28]. Pauly *et al.* [18] have reported that the IgY-sample that was obtained by PEG-precipitation, worked very well in a lot of different immunological assays. Therefore, the purity of IgY samples obtained by PEG-precipitation in this study was acceptable and may be worked well in different immunological assays. The final protocol is shown in figure 2.

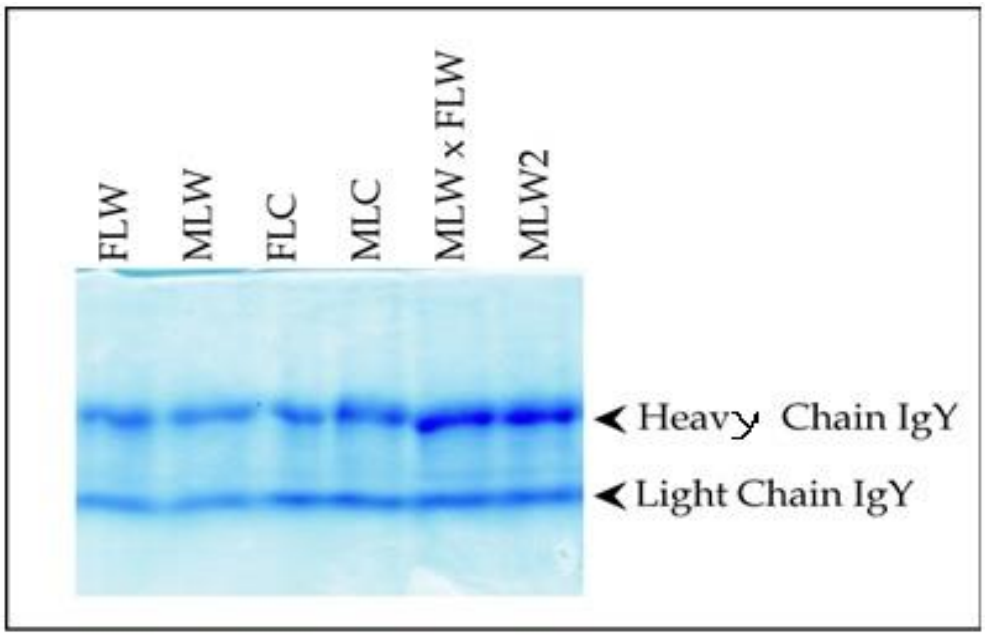

Fig-1: SDS-PAGE patterns of IgY purified from egg yolk of different lines of chicken under reducing conditions. FLW: Female Line White, MLW: Male Line White, FLC: Female Line Color, MLC: Male Line Color, MLW2: Male Line White 2, MLW x FLW: Two way cross of MLW male and FLW female.

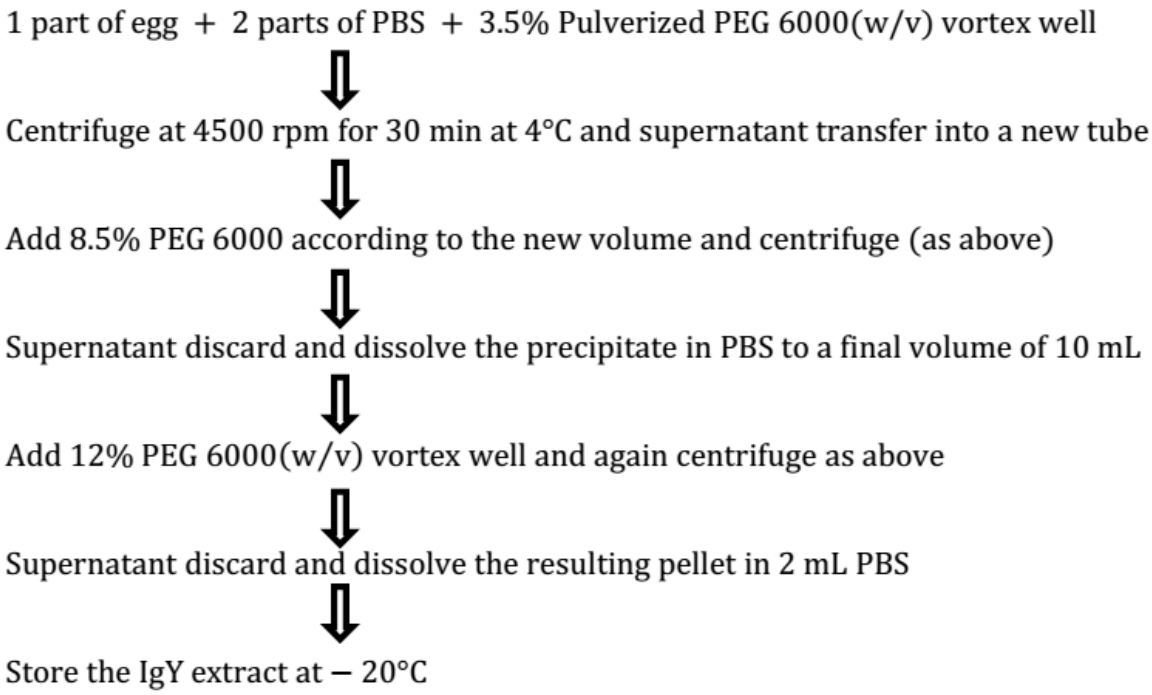

Fig-2: Schematic diagram of the separation of IgY from egg yolk by means of optimization of polyethylene glycol precipitation method.

Concentration of Egg Yolk IgY of Different Lines of Chickens:

We sought to compare the concentration of yolk IgY among six lines of chickens: MLW, MLC, MLW2, FLC, FLW and MLW x FLW. In this study, to obtain the concentration of yolk IgY, egg and yolk parameters of collected eggs were determined (Table 1). The weights of egg, yolk, yolk-egg ratio, yolk volume and yolk % were significantly different among the studied lines of chicken. The egg weight of MLW (65.97 ± 4.53) was the highest and the lowest in FLC (47.31 ± 2.84) among the lines. The egg weight was almost similar between MLW, MLW2 and MLW × FLW ($P>0.05$). On the other hand, the egg weight of FLW and MLC were similar ($P>0.05$) with FLC as determined by Tukey HSD. In contrary, the yolk weight

and yolk volume were significantly different among the lines. The yolk weight was the highest in MLW2 (17.40 ± 3.11) and the lowest in FLW (10.72 ± 1.15). Similar trend was also observed in yolk volume. Since the egg and yolk weights were significantly different among the lines and the trend was dissimilar, the ratio of yolk weight to egg weight was also calculated. The highest yolk to egg weight ratio was found in MLW2 (0.29 ± 0.02) and the lowest in FLW (0.21 ± 0.03), whereas MLW and MLC showed intermediate values. Since the egg weight, yolk weight and volume were significantly different among lines (Table 1), egg yolk IgY content was expressed in three different ways such as: milligrams of IgY per gram of egg (mg/g egg), milligrams of IgY per millilitre of yolk (mg/mL yolk) and milligrams of IgY per egg (mg/egg) to know the concentration of IgY among the lines (Table 2). The MLC showed the highest (6.822 ± 2.309) yolk IgY (mg/g egg), whereas MLW showed the lowest (4.034 ± 1.065). The yolk IgY (mg/g egg) was similar between MLC, MLW2 and FLW (P>0.05). The total amount of IgY per millilitre of yolk was the highest in FLW (30.904 ± 7.621) and the lowest in MLW (16.753 ± 5.282). However, the calculated total amount of yolk IgY (mg/egg) contained by the entire egg yolk was varied between 206.015 and 392.030 in the studied lines of chicken. The lowest amount of yolk IgY (mg/egg) was found in FLC (206.015 ± 61.058) and the highest was in MLW2 (392.030 ± 136.185). The amount of total yolk IgY (mg/egg) was almost similar between MLW, MLC, MLW2, FLW and MLW × FLW. The total egg yolk IgY determined in this study is greater than the 5.2 mg/mL and 42 to 105 mg/yolk reported by Carlander et al. [11] in 50-wk-old Single-Comb White Leghorn (SCWL) hens. It was also greater than the 1.15 and 2.26 mg/mL and 22.5 and 43.9 mg/yolk reported by Hamal et al. [1] in 39-wk-old hens of 2 meat lines. The differences observed between the results could be a consequence of the different IgY extraction and quantification methods used in each study [29]. Gadde et al. [30] reported that the concentration of IgY in egg yolk is always higher (∼8– 25 mg mL−1). By our measurements, the yolk IgY concentration (mg/mL yolk) of six lines of chickens ranged from 16.753 to 30.904 mg/mL yolk, which agreed well of the previously reported yolk IgY concentrations.

Table-1, Egg and yolk parameters of different lines of chicken during experimental period

Chicken lines	Number of samples	Egg weight (g)	Yolk weight (g)	Yolk and egg weight ratio	Yolk volume (mL)	Yolk(%, w/w)
MLW	7	65.97 ± 4.53^{a}	16.25 ±1.03^{ab}	0.25 ± 0.01^{bc}	16.13± 1.14^{ab}	24.65± 1.02^{bc}
MLC	7	47.44 ± 0.91^{b}	12.83± 0.33^{cd}	0.27 ± 0.01^{ab}	12.63± 0.22^{cd}	27.04± 0.57^{ab}
MLW2	7	59.78 ± 7.93^{a}	17.40 ± 3.11^{a}	0.29 ± 0.02^{a}	17.25 ± 2.77^{a}	28.93 ± 1.63^{a}
FLW	7	48.94 ± 1.49^{b}	10.72 ± 1.15^{d}	0.21 ± 0.03^{c}	10.50 ± 0.89^{d}	21.91 ± 2.30^{c}
FLC	7	47.31 ± 2.84^{b}	11.10 ± 0.42^{d}	0.24 ± 0.02^{c}	11.13 ± 0.49^{d}	23.55 ± 2.08^{c}
MLW × FLW	7	62.55 ± 3.07^{a}	14.13± 1.27^{bc}	0.23 ± 0.01^{c}	14.20± 1.60^{bc}	22.55 ± 1.14^{c}
P value		4.383 × 10^{-05}	1.504 × 10^{-05}	5.78 × 10^{-05}	1.038 × 10^{-05}	5.775× 10^{-05}

Data are mean ± standard deviation. Values with common superscript within a column do not differ significantly (P ≥ 0.05)

Table-2, Egg yolk IgY concentration of different lines of chicken

Chicken lines	Absorbance[1]	IgY (mg/g egg)	IgY(mg/mL yolk)	Total IgY (mg/egg)
MLW	0.448± 0.149^{ab}	4.034 ± 1.065^{b}	16.753 ± 5.282^{c}	269.624 ± 89.606^{a}
MLC	0.539 ± 0.188^{b}	6.822 ± 2.309^{a}	25.651±8.819^{abc}	324.511 ± 113.572^{a}
MLW2	0.625 ± 0.226^{a}	6.454 ± 1.594^{a}	22.532±5.875^{abc}	392.030 ± 136.185^{a}
FLW	0.535 ± 0.126^{a}	6.580 ± 1.538^{a}	30.904 ± 7.621^{a}	322.165 ± 75.966^{a}
FLC	0.343 ± 0.102^{a}	4.345 ± 1.206^{b}	18.590 ± 5.710^{bc}	206.015 ± 61.058^{b}
MLW × FLW	0.652 ± 0.040^{a}	5.891 ± 0.359^{ab}	26.197 ± 2.998^{ab}	368.120 ± 24.071^{a}
P value	1.174× 10^{-03}	2.596 × 10^{-03}	9.403 × 10^{-04}	1.174× 10^{-03}

NOTE: 1280 nm, 40 times diluted. Data are mean ± standard deviation. Values with common superscript within a column do not differ significantly (P ≥ 0.05).

CONCLUSION:

A high purity of IgY preparation is desirable for many immunoassays and for the production of antibodies. There are several advantages of PEG-precipitation method, such as: eggs are cheap and readily available, antibody levels in egg yolks are high and one can easily avoid bleeding and other invasive techniques normally required to obtain antibodies from animals. The PEG-precipitation method is simple, requires few steps, and yield is high. In this optimized method of PEG, the yield of IgY could reach 30.904 mg/mL, 6.82 mg/g egg and 392.030 mg/egg in the studied lines of chickens. Extraction of IgY by this optimized PEG-precipitation method is cost effective, avoiding energy and time-consuming. The isolated IgY should not be frozen and can be stored at 4°C for several years. It can be used as source material for protein and immunological studies. Isolated IgY in this optimized protocol, might be applied for large-scale production and it opens the venues for using IgY in human and veterinary medicine for therapeutic and prophylactic purposes.

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