



Evaluation of Different IgY Preparation Methods and Storage Stability as Potential Animal Feed Supplement

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ABSTRACT

Egg yolk can be used as feed additives to improve the immune status of animals and as passive therapy for veterinary diseases, and the egg yolk has proved to be a promising source of antibodies. However, the extraction methods and storage stability of IgY antibodies from egg yolk for feed supplements remain major problems in mass production. Based on Based on IgY antibody titer, IgY thermostability, IgY acid and alkali resistance, resistance to trypsin and pepsin, four different IgY purification methods (PEG extraction, water dilution method, the effect of liquid egg yolk and egg yolk powder preparations) on the activity of IgY antibodies was investigated. The results showed that IgY was stable below 70 °C, and the activity was significantly lost when it exceeded 70 °C; IgY was relatively stable in the range of pH 4-10, and the activity was significantly lost beyond this range; IgY had certain resistance to pepsin and trypsin. Among all four purification methods, the IgY antibody titer in liquid egg yolk and egg yolk powder was significantly higher than the other two methods. Moreover, egg yolk powder can prevent bacterial contamination and inactivation due to the formation of sediment. This study provide basic data for IgY application in animal feed supplements.

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Authors' Contribution

XZ and AM conceived and designed the study. SG and YY performed the experiments. SG and BC prepared the manuscript. BC, AM and FZ reviewed the manuscript and conducted the discussion.

Key words

Feed additives, IgY antibody, Stability, Yolk powder, Feed supplement

INTRODUCTION

Anutraceutical is a substance or food that can prevent or (and) treat a disease (isolated and purified from food), and generally exerts its physiological activity in the form of tablets, capsules, and syrups. The essence of medicinal nutrients is food, which is harmless to animals and human body. It is a substance with high safety, natural green color and both pharmacological and nutritional value, such as egg yolk immunoglobulin (IgY). In recent years, the abuse of antibiotics has led to serious drug residues in animal products, and drug-resistant pathogens have become more

frequent. Adding IgY to animal feed can play a role in disease prevention and treatment (Carlander *et al.*, 2000; Xu *et al.*, 2011). Because it is highly efficient, specific, and can be obtained in large quantities without slaughtering animals, it is regarded as a new generation of green and safe pharmacodynamics. The egg yolk antibody is a good feed additive because it is easy to extract, has no toxic residues, does not produce drug resistance, and does not cause harm to the body (Rose *et al.*, 1974; Schade *et al.*, 2005). In addition, bioactive substances such as yolk high-phosphorus protein, sialic acid, oligosaccharides and various amino acids contained in egg yolk can enhance the body's immune function. Therefore, the addition of specific IgY to animal feed can effectively prevent a variety of digestive and non-digestive diseases caused by bacteria or viruses. Antigen-specific antibodies has been revealed to be effective against a variety of enteric pathogens (in

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humans and animals) such as *Salmonella* spp., *Escherichia coli*, *Staphylococcus*, *Pseudomonas*, bovine and human rotavirus, bovine coronavirus, *Edwardsville tarda*, and *Yersinia ruckeri* (Xu *et al.*, 2011; Kovacs-Nolan and Mine, 2005).

In healthy animals, feeding IgY can improve the functioning of the immune system and achieve both nutrition and prevention, could suggestively improve growth rates in livestock (Kovacs-Nolan and Mine, 2012). However, there are still some problems to be improved in the application of IgY as an animal feed additive. For example, IgY's production process is still unstable and needs to be optimized. Because IgY is rich in nutrients and easily infected with bacteria, its anti-corrosion technology needs to be improved (Yegani and Korver, 2010). This study was based on the IgY extracted from *E. coli* and kanamycin-immunized chickens, and the effects of different extraction methods on the physical and chemical properties of IgY were studied by comparative studies. IgY is very stable during storage. The purified IgY maintains good structural integrity. The IgY dry powder formulation can be stored for more than one year at 4°C to minimize bacterial growth. The above biological characteristics make it possible to use IgY as a pharmacological nutrient in animal feed and disease prevention.

MATERIALS AND METHODS

Immunization protocol

All animal experimental protocols were reviewed and approved by the Ethics Committee of Xinjiang Agricultural University for the use of Laboratory Animals. Two white Leghorn chickens (twenty-five-week-old) were immunized intramuscularly with 250 µL formalin killed *Escherichia coli* (1×10^9 cfu/mL) and additional two chickens were immunized with 250 µL Kanamycin conjugated to BSA (Kana-BSA; 1 mg/mL) as per standard protocol to enhance the immunogenicity (He *et al.*, 2016). For each immunization, 250 µL of immunogen (diluted with PBS buffer) was emulsified with an equal volume of Freund's complete and incomplete adjuvant (FCA/FIA; Sigma, St. Louis, MO, USA) for the first and following four booster immunizations respectively at two weeks' interval period. Eggs were collected from immunized chicken daily, marked and stored at 4°C for further process.

Four different methods for IgY preparation

From the hyperimmunized eggs, twelve eggs were randomly selected from the same immunization period (14 days). The shell was removed carefully, the yolks were separated from the albumin and pooled together. After thorough blending, liquid yolk was divided into four equal

parts and processed as follows for IgY preparations.

PEG-6000 precipitation method

IgY antibody was extracted by improved PEG-6000 method as described by POLSON and ZHANG (Polson *et al.*, 1980; Zhang *et al.*, 2008). Briefly, the egg yolk was diluted with sterile phosphate buffer solution (0.01 M PBS pH 7.4) at a ratio of 1:2. To eliminate lipids and lipoprotein, 3.5% (w/v) polyethylene glycol (PEG) 6000 was added to the diluted yolk and provided gentle shaking at room temperature for 20 min followed by centrifugation at 10,000g for 20 min at 4°C (HC-3018R, Anhui USTC Zonkia Scientific instruments, China). PEG-6000 to a final concentration of 12% (w/v) was added to the supernatant, mixed and the solution was centrifuged at 10,000g for 20 min at 4°C. Dissolved the pellet in 10 mL of PBS (0.01 M, pH 7.4) and PEG 6000 was added to a final concentration of 12% (w/v), mixed and centrifuged as above. Finally, the precipitated pellet was dissolved in 1.2 mL of PBS, transferred into a micro-dialysis device and dialyzed against PBS for overnight at 4°C.

Water dilution method

Egg yolk was diluted with nine volumes of cold distilled water (pH 3.5) and stored at 4°C overnight. After centrifugation at 12,000g for twenty minutes at 4°C, Supernatant was collected and ammonium sulphate was added to final saturation of 35%. Finally, precipitate was dissolved in 1.2 mL PBS and transferred into a micro dialyzer and dialyzed with PBS overnight at 4°C.

Liquid yolk preparation

The yolk separated from albumin was thoroughly mixed and sealed in the 50 mL test tube, labelled, and stored at 4°C.

Yolk powder preparation

Separated Egg yolk was diluted with an equal volume of cold (4°C) distilled water and frozen at -80°C for twelve hours. The frozen yolk was then lyophilized at -70°C, yolk powder preparation was labelled and stored at room temperature in a dark and cool condition.

SDS-PAGE

IgY in four different purification methods was identified by SDS-PAGE.

Determination of specific IgY titre

The titre of specific IgY antibody was measured by indirect ELISA, ELISA was performed as previously described (Li *et al.*, 2017). Briefly, 1: 2,000 dilutions of antigens (*E. coli* and Kana-BSA) in 0.05 M carbonate buffer (pH 9.6) was added to 96-well microtiter plates and

incubated overnight at 4°C. Following incubation, the plate was washed with PBST (PBS contains 0.5% Tween-20) for three times. Unbound sites were blocked with PBS containing 5% skim milk powder for 2 h at 37°C. Plate was again washed three times with PBST, followed by addition of IgY preparations diluted in blocking buffer (100 µL per well) and incubated for 1 h at 37°C. After washing three times with PBST, 100 µL of 1:5000 dilution of Goat anti-chicken IgY conjugated with horseradish peroxidase (Abcam, Canada) was added and incubated at 37°C for 1 h. After washing three times with PBST, Tetra methyl benzidine (TMB) substrate (Sigma) solution containing 0.1 mM citrate-phosphate buffer and 1 µL/mL H₂O₂ (100 µL) was added to each well and incubated for 15 minutes at 37°C. The reaction was terminated by addition of H₂SO₄ (2 M). The plate was read directly on micro plate reader at 450 nm (EL800, BioTek, USA). The titre of IgY were determined by maximum dilution of sample (P/N ≥ 2.1, positive control and negative control, nonspecific control, with three replicates each).

Temperature measurement

IgY samples were diluted to 100-fold with PBS (pH 7.4) in test tubes and incubated at 37°C, 50°C, 60°C, 70°C, 80°C and 90°C with control samples at room temperature (25°C). After incubation for fifteen minutes, the different samples were cooled in an ice bath and the remaining antibody activity after heat treatment was measured by indirect enzyme-linked immunosorbent assay (iELISA). Antibody activity retention rate was calculated according to the formula below:

$$\text{Remaining activity (\%)} = T/T_0 \times 100\%$$

Where, T₀ is antibody titres before treatment, T is antibody titres after treatment.

Acid and alkali determination

IgY samples were diluted to 100-fold with PBS in test tubes and incubated at different pH ranges (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12). Samples incubated at pH 7.4 served as control. After incubation for thirty minutes, the mixtures were cooled in an ice bath and the remaining antibody activity after acid/alkali treatment was measured by iELISA. Antibody activity retention rate was calculated according to the formula described previously.

Detection of IgY antibody stability towards digestive enzymes

Pepsin was dissolved in 0.07 M of sodium acetate buffer solution (pH 2.0 with 1 M hydrochloric acid to adjust) to a final concentration of 5 µg/mL, and trypsin in 50 mM of Tris-HCl buffer solution containing 10 mM CaCl₂, pH 8. IgY samples were diluted in two digestive enzyme solutions to a final concentration of 5 mg/mL respectively, and the mixture (pepsin/yolk antibody =

1/100) was incubated in roller-shaker at 80rpm in 37 °C for different time intervals. After incubation for (0 h, 0.5 h, 1 h and 4 h), 0.45 mL aliquot of sample was mixed with 0.05 mL sodium carbonate solution (5.0%) to terminate the pepsin digestion reaction. The remaining antibody activity after pepsin treatment was measured by ELISA. Antibody activity retention rate was calculated according to the formula previously mentioned.

Statistical analyses

The statistical analyses were performed using Prism software (Graphpad Software, version 6.0). The unpaired two-tailed Student's t-test was used to compare differences between two independent groups. The comparison of three and more groups one was performed with one-way ANOVA. A difference was considered to be statistically significant for p values of lower than 0.05 ($P < 0.05$).

RESULTS

Anti-*E. coli*-IgY and Anti-Kana-IgY

Immunized chickens were reared until the completion of research work and eggs were collected after the first immunization. The titre level of specific IgY against *E. coli* and Kana in immune egg yolk was determined by iELISA. Initially, the titres of anti-*E. coli*-IgY and anti-Kana-IgY were found to be ≥ 1: 4,000 and ≥ 1:8000 respectively. Post first booster immunization, the titre attained the peak of about ≥1: 64,000 for anti-*E. coli*-IgY and ≥1: 256,000 for anti-Kana-IgY. After the fourth booster injection it was found to be stable reaching the highest titre (≥ 1:64,000 for anti-*E. coli*-IgY; ≥ 1: 256,000 for anti-Kana-IgY) for more than one month following gradual decrease in titre to a level of about 1: 28,000 for anti-*E. coli*-IgY and 1: 64,000 for anti-Kana-IgY and was maintained till the end of the experiment.

Analysis of IgY purity by SDS-PAGE

SDS-PAGE analysis of purified IgY preparations in reducing conditions showed two major bands viz., a heavy chain (66 KDa) and light chain (27 KDa) of the antibody. Besides, minor impurities band around 40 kDa was seen which probably the C-terminal fragment of vitellogenin II precursor might be (Klimentzou *et al.*, 2006) (Fig. 1).

Stability of antibodies towards heat and acid-alkali treatment

At temperatures below 70°C, the IgY antibody maintains a relatively high activity of about 70%; at temperatures above 70°C, the IgY activity decreases significantly with increasing temperature (Fig. 2A). IgY was relatively stable in the range of pH 4-10, and the activity was significantly lost beyond this range (Fig. 2B). The results show that high temperature and strong acid or strong

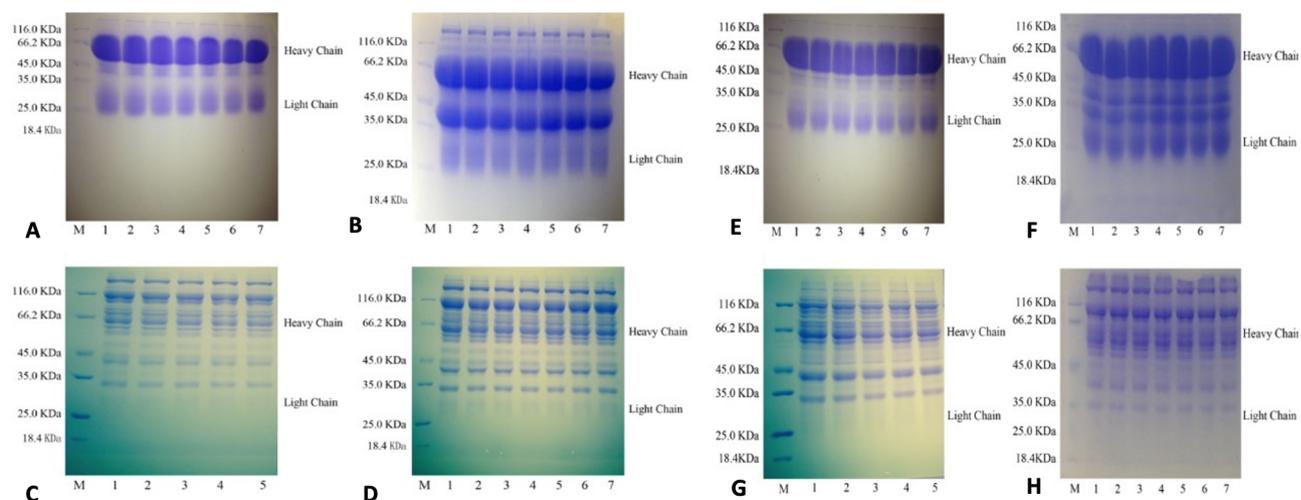


Fig. 1. SDS-PAGE analysis of IgY among different preparations and storages. A, B, C and D represent four different IgY preparations (PEG-extraction, WD, liquid yolk, yolk power) against *E. coli*; E, F, G, and H represent four different IgY preparations (PEG-extraction, WD, liquid yolk, yolk power) against Kanamycin, respectively. M, Marker; Lane 1: Fresh antibody preparation; Lane 2 to 7: every month storage monitoring for 6 months period (liquid yolk preparation only 4 months).

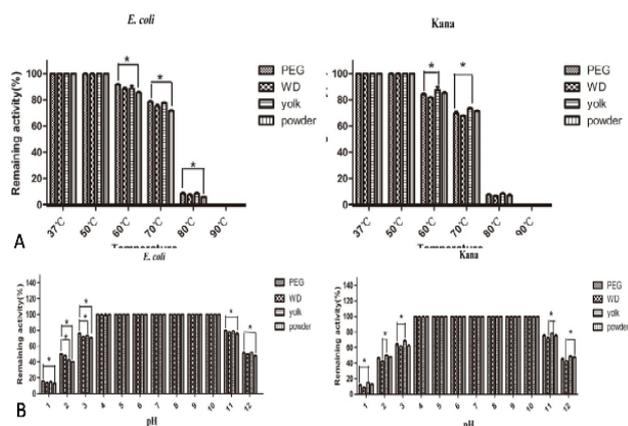


Fig. 2. Activity of IgY at various temperatures (A) and pHs (B). The remaining IgY activity was measured by ELISA and was expressed as % activity relative to an untreated sample ($P \leq 0.05$).

base will change the conformation of the Fab part of the antigen binding site on IgY, resulting in inactivation.

Stability of antibodies towards digestive enzymes treatment

With the delay of time, IgY resistance to pepsin and trypsin decreases, but IgY is more sensitive to pepsin, and during the same incubation time, pepsin can rapidly decompose IgY and lose activity (Fig. 3). Although the partial structure of the IgY antibody can be decomposed by trypsin in a short time, IgY still has its antigen-binding activity and cell agglutination activity.

Stability of different forms of IgY at various storage conditions

The results of iELISA presented that, IgY preparations from PEG extraction, Water dilution method and yolk powder was quite stable at 4°C when stored for six months. There were no significant changes in terms of IgY titre and stability in yolk powder after six-month storage at room temperature. The comparative analysis showed the IgY titre was higher in liquid yolk and yolk powder than the preparations obtained from WD and PEG extraction methods. Relatively, the higher IgY titre was observed in liquid yolk without protective agent, probably the

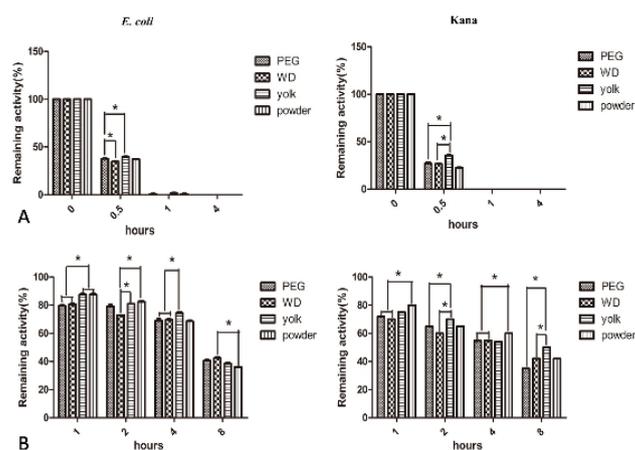


Fig. 3. Effect of pepsin (A) and trypsin (B) on the activity of IgY antibodies. The remaining IgY activity was measured by ELISA and was expressed as % activity relative to an untreated sample ($P \leq 0.05$).

freeze-drying cause certain damage to proteins in the lyophilized samples. Yet, in liquid yolk (without any preservatives), the bacterial contamination and

solidification occurred after twelve weeks storage which imposes a risk when using the IgY preparation of feed supplementation in livestock industry (Fig. 4).

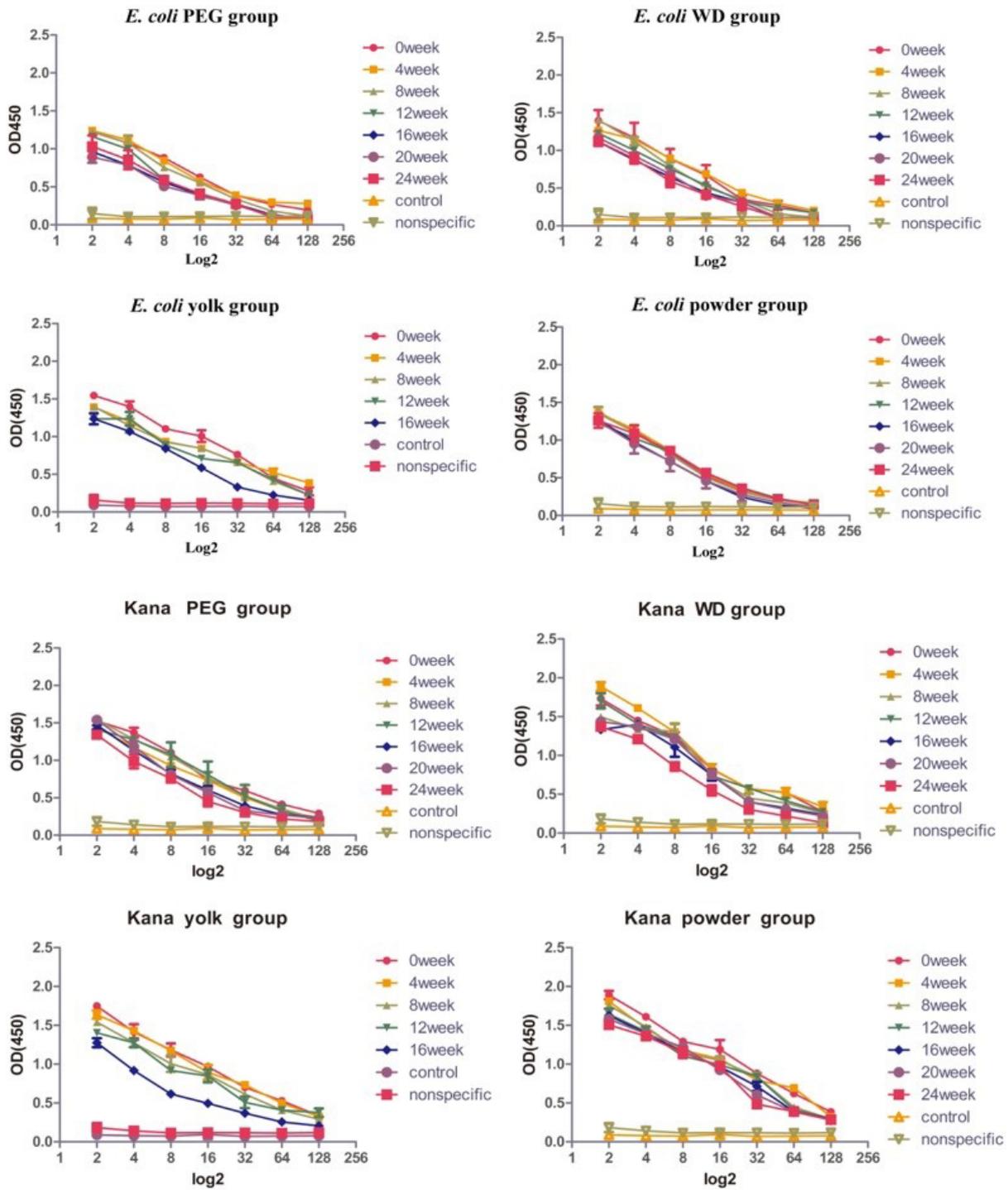


Fig. 4. Effects of *E. coli* and kanamycin on the titer of IgY storage. Data are presented as mean \pm SD. Notes: X-axis, Dilution factor of antibody; Y-axis, OD values.

DISCUSSION

Studies have shown that water-dilution is safe and that IgY is suitable as a functional food or feed because it does not require any chemicals (Akita and Nakai, 1993). However, the IgY extracted by the water dilution method is very sensitive to heat and acid and loses activity due to precipitation for a long time (Malik *et al.*, 2006). In order to overcome this obstacle, sugar, glycerol or glycine is added to the IgY preparation, so the water dilution method is significantly laborious and not very economical (Shimizu *et al.*, 2006; Chang *et al.*, 1999). At the same time, the addition of a protective agent to the IgY formulation increases the cost of managing the livestock. Therefore, it is urgent to study other different IgY extraction methods. It has been reported that IgY after lyophilization can be stored at 4 °C for more than one year without significant loss of activity (Larsson *et al.*, 1993), and the addition of disaccharide to lyophilized IgY will result in higher stability (Nilsson *et al.*, 2008). It indicates that IgY after lyophilization is more conducive to storage.

Even though, the passive immunization using chicken IgY has been recognized as a means of controlling infectious diseases for more than two decades, still there are many limitations to commercialize this perception. Since the scale-up for industrial production, stability of IgY antibodies and storage. A series of IgY extraction methods have been developed and applied, amongst water dilution method (WD) has been recognized as a simple and inexpensive to obtain high-yield of IgY, PEG precipitation which is most commonly used and effective method (Polson *et al.*, 1980; Schade *et al.*, 2005). Based on the application of these antibodies in different field, different IgY extraction and purification strategies need to be handled for improved outcomes (Ren *et al.*, 2016).

This study aimed to compare four different methods for extracting IgY from egg yolk by chickens immunized with *E. coli* and kanamycin, and to screen for storage conditions with higher stability IgY. The results are similar to previous reports on the physicochemical properties of IgY: IgY is relatively stable at 70 °C, and IgY activity is significantly reduced at higher temperatures; IgY exhibits good stability in the range of pH 4 and pH 10, beyond which activity is lost; IgY is more active against trypsin than against pepsin. The antibody titer of IgY in liquid egg yolk and egg yolk powder is higher than that of the other two methods, especially the stability of egg yolk powder in the absence of preservatives for more than one year. Ultra-free egg yolk powder can be used directly as a functional feed additive in the livestock industry.

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Statement of conflicts of interest

The authors have declared no conflict of interest.

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