

ARTICLE

Investigation of Two Precipitation Methods for Extracting Immunoglobulin Y (IgY) from Egg Yolks

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ABSTRACT

Two groups of hens (control and immunization group) were arranged in an experimental design with an immunization schedule of 3 injections of BSA antigen. IgY antibodies were extracted from egg yolks by two precipitation processes (chloroform and polyethylene glycol precipitates) and quantified using a standard curve of protein concentration. The purification of IgY was confirmed by SDS-PAGE. Total protein extracted from egg yolks were less contaminated with yellow pigments (lutein and zeaxanthin) by using chloroform precipitate. The 2nd week post-immunization, IgY concentration increased respectively to $3903 \pm 726 \mu\text{g}\cdot\text{ml}^{-1}$ (chloroform extraction process) and $2937 \pm 294 \mu\text{g}\cdot\text{ml}^{-1}$ (PEG extraction process) ($P < 0.01$). After 3rd immunization, IgY level obtaining from in immunization group extracted by chloroform process ($6633 \pm 1166 \mu\text{g}\cdot\text{ml}^{-1}$) increased 2.7 times higher than that in control group ($2482 \pm 414 \mu\text{g}\cdot\text{ml}^{-1}$). Whereas IgY concentrations obtained from PEG extraction process were not significantly different between the experimental group and control group. Chloroform and PEG precipitation methods had the same protein profile on the SDS-PAGE. IgY antibody was identified by the presence of bands corresponding with IgY heavy chain (67-70 kDa) and IgY light chain (25 kDa) for both precipitation processes.

1. Introduction

To deal with challenges from many diseases in livestock husbandry and antibiotic resistance, the research focus approaches the therapeutic alternatives in terms of animal welfare and suitable environment as well as

reduction of undesirable effects on animals. Besides various solutions, the protective effect of egg yolks obtaining from hens immunized by specific antigen has been applied in many studies.

The collection of IgY antibodies from egg yolk is more advantageous in comparison to the collection of

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antisera from mammals because IgY antibodies can be extracted out of animals (via egg yolk thereby ensuring the animal welfare and having a lower cost compared with antisera). The antibodies extracting from chicken eggs is higher level of purity by avoiding contamination of other antibodies such as IgA or IgM generated by the humoral immune responses^[13,22]. In addition, the IgY concentration extracted from egg yolks increases significantly overtime throughout the life - span of hens^[3]. Successful extraction and purification of IgY from egg yolks will provide the basis for producing specific types of IgY antibodies against various antigens, which have been clinically tested as functional foods and protective agents for the body, etc.^[10,25]. Powered whole eggs or yolks have been used in veterinary medicine of immunoglobulin Y (IgY) source for the treatment of enteric diseases^[2]. The activity of IgY against gastrointestinal pathogens such as Enterotoxigenic *Escherichia coli* in vitro^[16] and *Salmonella* Typhimurium on mice^[15] showed the specific binding of these IgY to pathogens thereby reducing the mortality of infected animals. The efficacy of IgY against *Clostridium difficile* spore in the gastrointestinal tract of mice was also recorded^[18]. In addition, the application of IgY in practice to replace antibiotics in prevention and treatment promises to reduce antibiotic resistance in animal husbandry^[21]. Furthermore, other studies showed that the use of yolk antibodies to treat against group A Rotavirus in cattle^[26], porcine epidemic diarrhea virus^[14] significantly reduced the severity and mortality of infected animals. Moreover, IgY antibodies are also applied in the techniques to diagnose infections of viruses, bacteria, parasites such as ELISA, immunochromatography, Western blot^[27,29].

The extraction and purification of IgY antibodies from egg yolk are increasingly attracting the interest of the scientific community as demonstrated by the significant growth of interest regarding IgY in literature. However, the purification and the quantity of IgY antibodies collected after extraction varies greatly depending on the method of extraction. Therefore, standardizing the extraction procedure is a fundamental step in IgY application^[8]. The most important of purifying IgY is the separation of proteins (levitins) from lipoproteins (lipovitellins) and the rest of the yolk lipids using various chemical substances^[5]. The yolk lipids must be removed, leaving the IgY antibody in the hydrophilic protein fraction (HPF). The different components of HPF include IgY, α - and β -livetin, low density lipoproteins, and albumin^[9,12]. The precipitation of lipid from HPF depends on type of precipitating agent applied^[6].

The purpose of this study was to evaluate the production of IgY antibodies in hens and to determine IgY

extraction processes based on two types of precipitating agent (chloroform and PEG).

2. Materials and Methods

Immunization schedule and egg collection

A total of six hens (19 weeks old, non-clinical signs of disease) purchased from a healthy commercial layer farm were raised in cages with temperature control (25 – 32 °C) and fed with a commercial feed. Hens were divided into experimental group and control group, each group included 3 hens which were raised in individual cage and labeled as A1-A3 (immunization group) and C1-C3 (control group). Bovine serum albumin (BSA, 20 mg.ml⁻¹, AJ642, Bio Basic Inc., Canada) was used as antigen to immunize hens. BSA was diluted in PBS 1X solution and emulsified with an equal volume of complete Freund's adjuvant (F5881, Sigma-Aldrich Inc., USA) to obtain the final solution having 1 mg.ml⁻¹ BSA and then stored at 4 °C.

The immunization process based on the description of Sunwoo et al. (1996)^[24]. Briefly, all of hens of the immunization group were injected with the immunization schedule of three times. In the first immunization, hens of immunization group were injected with 1 mg BSA into chest muscles at 4 sites (IM, 0.25 mg/site). Hens were treated with the same antigen dose in second immunization (2 weeks post 1st immunization) and last immunization (3 weeks post 1st immunization). Hens of the control group were injected with sterile normal saline (NaCl 0.9%) in the same schedule as the immunization group. Each hen's eggs were collected daily from 1 week before the first immunization to 6 weeks post 1st immunization. Eggs were collected and marked according to the order of egg collection date and stored at 4 °C for IgY extraction.

IgY extraction and precipitation from egg yolks

A total of 126 eggs (3 eggs /hen/week, from 1 week before the 1st immunization to 6 weeks post 1st immunization) were assessed to IgY extraction. Eggs were broken carefully to discard egg white, then egg yolks were rolled gently on tissue paper to remove egg white residues to obtain egg yolks. The yolk membranes were punctured by pipette tip and egg yolks were transferred to a graduated cylinder and recorded egg yolk volume. The IgY extraction process was carried out using two separate precipitating agents, i.e., chloroform and polyethylene glycol (PEG).

For IgY extraction using chloroform, 15 ml egg yolk was mixed vigorously with 25 ml PBS 1X solution, (PD0100, Bio Basic Inc., Canada), then 20 ml chloroform

(67-66-3, VN-Chemsol Co., Ltd., Vietnam) was added and mixed for 30 seconds to obtain a homogenous mixture. The mixture was continuously centrifuged at 2,000 rpm for 30 minutes. The supernatant was filtered through filter paper and decanted to another tube for precipitation of IgY.

For IgY extraction using PEG, 15 ml egg yolk was mixed vigorously with 7.5 ml PBS 1X solution and then 7.5 ml PEG 14% solution prepared by diluting PEG 6000 powder (Polyethylen glycol 6000, PB0432, Bio Basic Inc., Canada) with PBS 1X solution was added and mixed for 30 seconds to obtain a homogenous mixture. The final mixture was centrifuged at 4 °C for 15 minutes (Hettich Rotina 35R, 9,990 × g). The supernatant was filtered through filter paper and decanted to another tube for precipitation of IgY.

The supernatant containing IgY antibodies from both extraction processes was precipitated by adding an equal volume of PEG 24% solution, then mixed for 30 seconds and centrifuged at 4 °C for 15 minutes at high speed (Hettich Rotina 35R). The supernatant was discarded, and the pellet was dissolved again in PBS 1X solution to reach the original volume of egg yolk, an equal volume of PEG 24% solution was added and then centrifuged (10,000 × g at 4 °C for 15 minutes) for the second precipitation. After centrifugation, the supernatant was discarded and the pellet was centrifuged two more times to remove residues of PEG, then dissolved in 10 ml PBS 1X and stored at 4 °C.

IgY quantification

The IgY concentration of extracting samples was determined by using protein quantification KIT-rapid (51254, Sigma-Aldrich Inc., USA). The standard curve of protein concentration was established as the manufacturer's procedure and used to determine the IgY concentration in extract samples.

IgY confirmation by SDS-PAGE

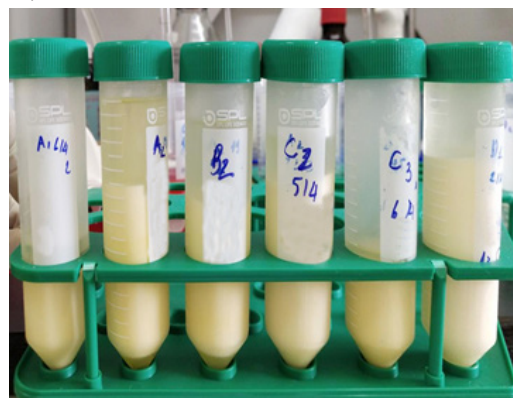
Extraction of chicken IgY samples were checked by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method. Briefly, 8% separating gel was prepared vertically in a chamber and then 5% stacking gel was added on the top. One ml of IgY extraction sample (stored in PBS 1X) was centrifuged at 12,000 rpm for 10 minutes and then discarded the supernatant to obtain the pellet of protein. SDS 10% and loading dye (20 µl and 10 µl, respectively) were added to the pellet, mixed, and then incubated at 95°C for 10 minutes. After that, Molecular weight marker (P7703S, BioLabs Inc., USA) and samples were loaded into wells

of stacking gel and the whole gel was put in a vertical electrophoresis system (Apelex Mini-Vertigel 2 PC, Cat. No. 400800, France). The gel was run under the electric field of 50 volts for 30 minutes and then 80 volts for 120 minutes. When the electrophoresis procedure was finished, the gel was treated with isopropanol for 30 minutes to fix proteins then stained with Coomassie for 3 hours and destained by 10% acetic acid until the bands on the gel were clear to observe. In total, 18 and 14 samples extracting by chloroform and PEG methods, respectively, were checked by SDS-PAGE.

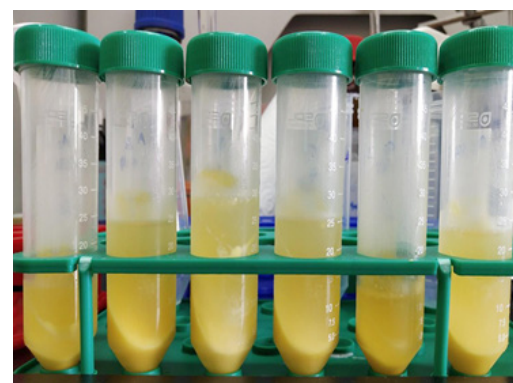
3. Results

Extraction and precipitation process

The total of 120 eggs was qualified to extract IgY using either chloroform or PEG precipitate agent. In this study, the supernatant (after lipid removal) obtained from chloroform extraction process was more homogenous opaque than that from PEG extraction process (Figure 1A and 1B). Extract proteins from egg yolks were less contaminated with yellow pigments (lutein and zeaxanthin) by using chloroform precipitation (Figure 2A and 2B).

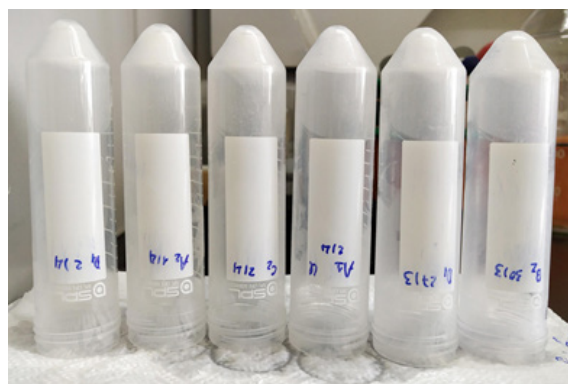


A

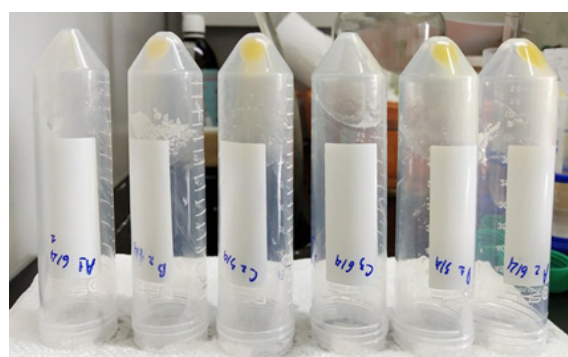


B

Figure 1. Lipid removal by (A) chloroform precipitation process and (B) PEG precipitation process



A



B

Figure 2. Protein obtained after lipid removal at 1st precipitation with (A) chloroform precipitate and (B) PEG precipitate

IgY concentration

IgY concentration collected from chloroform extraction process ranged from 536 – 7805 $\mu\text{g}\cdot\text{ml}^{-1}$ (n=62), and it ranged from 412 – 5874 $\mu\text{g}\cdot\text{ml}^{-1}$ (n=58) in PEG extraction process. One week before immunization, IgY concentration obtained from both extraction methods ranged from 592 $\mu\text{g}\cdot\text{ml}^{-1}$ to 883 $\mu\text{g}\cdot\text{ml}^{-1}$ ($P > 0.05$). The 1st week post immunization, IgY concentration was no significant difference between immunization group and control group. Otherwise, 2nd week post-immunization, IgY concentration increased respectively to 3903 \pm 726 $\mu\text{g}\cdot\text{ml}^{-1}$ (chloroform extraction process) and 2937 \pm 294 $\mu\text{g}\cdot\text{ml}^{-1}$ (PEG extraction process) ($P < 0.01$). After 3rd immunization, IgY level obtaining from in immunization group extracted by chloroform process (6633 \pm 1166 $\mu\text{g}\cdot\text{ml}^{-1}$) increased 2.7 times higher than in control group (2482 \pm 414 $\mu\text{g}\cdot\text{ml}^{-1}$) (Figure 3).

Meanwhile, our results showed that the IgY concentration obtained from PEG extraction process were not significantly different between the experimental group and control group (Figure 4).

Identification of IgY by using SDS-PAGE

Total protein obtained from extraction process was separated on polyacrylamide gel and IgY was detected using SDS-PAGE (Figure 5) under reducing conditions. SDS-PAGE results demonstrated that IgY extracted with

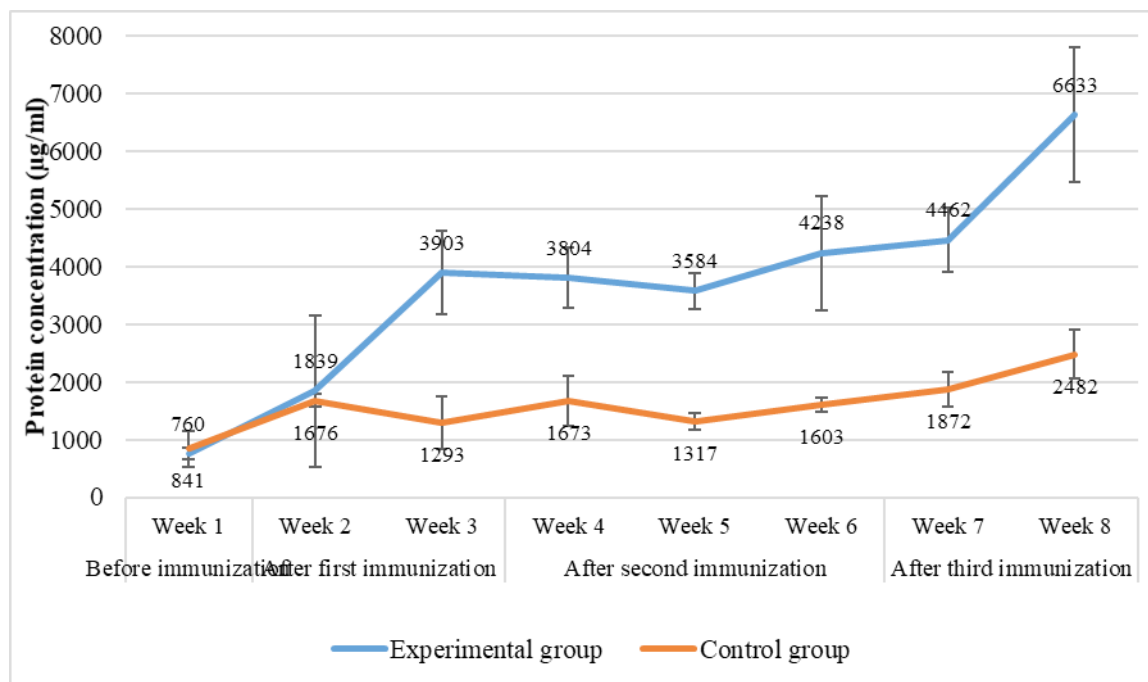


Figure 3. IgY concentration ($\mu\text{g}\cdot\text{ml}^{-1}$) from egg yolks with chloroform precipitation method

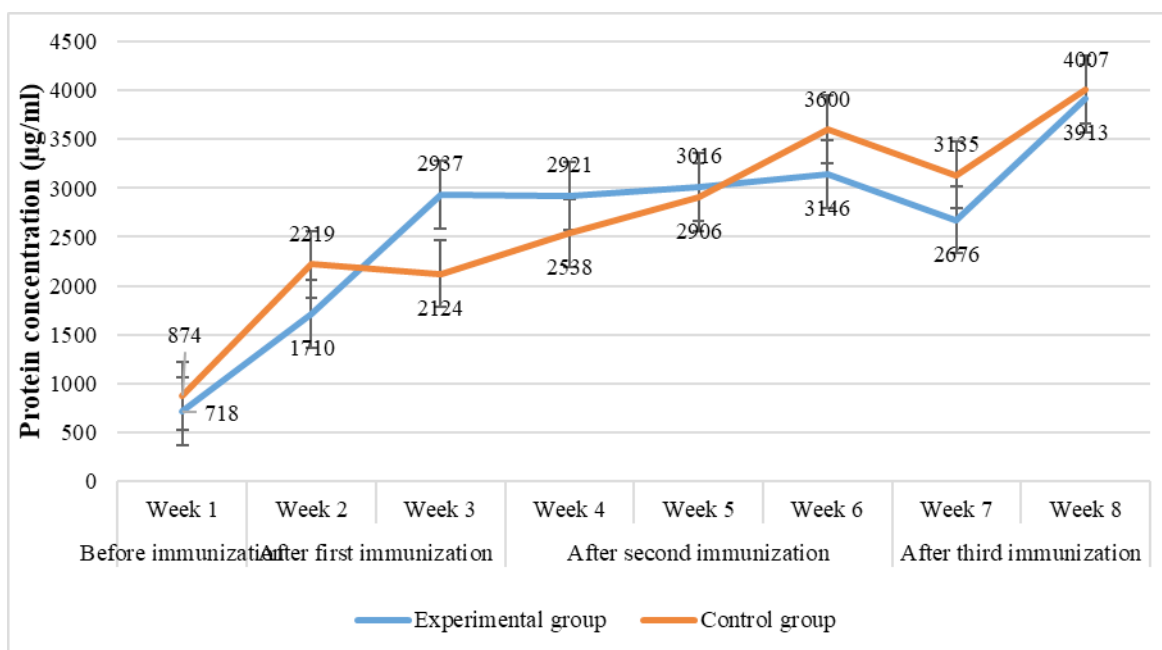


Figure 4. IgY concentration ($\mu\text{g}\cdot\text{ml}^{-1}$) from egg yolks with PEG precipitation method

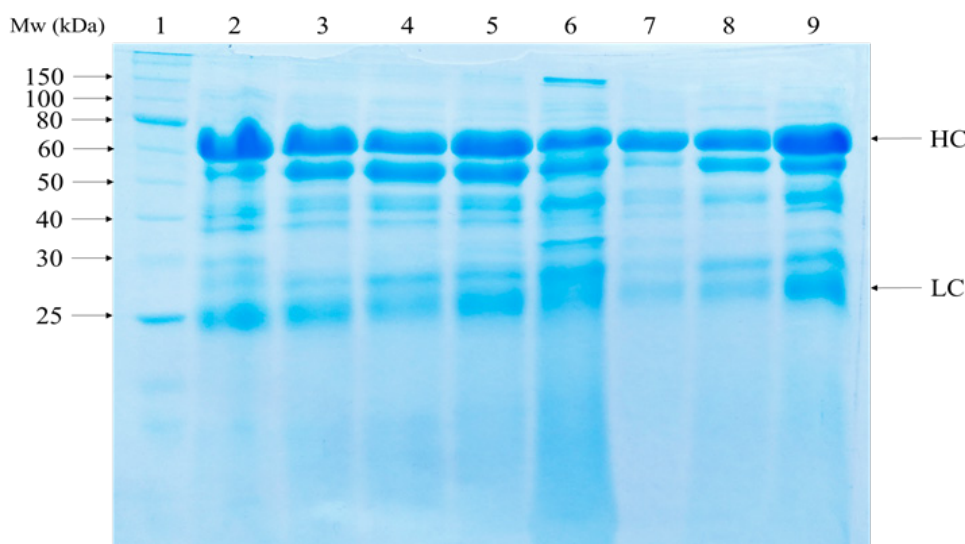


Figure 5. Identification of IgY by SDS PAGE. Lane 1: Molecular weight marker; Lane 2-7: Profile of IgY with PEG precipitation method; Lane 8-9: Profile of IgY with chloroform precipitation method; HC: heavy chain (67-70 kDa), LC: light chain (25 kDa).

PEG and chloroform containing two distinctive major protein bands which included the heavy (67-70 kDa) and light (25 kDa) chains of IgY antibodies. However, IgY purified with chloroform precipitation method contained 1 major protein band and 3 minor protein bands, also IgY purified by PEG precipitation method contained 1 major protein band and 3 minor bands.

4. Discussion

Our results showed that it is possible to generate IgY

antibodies from chicken eggs using a specific antigen inducing an immune response by booster antigen injection. Therefore, the production of polyclonal antibodies through the chicken immunization showed an excellent alternative that collect antibodies with a large and qualified amount from simple methods without the need for invasive techniques^[4].

Both chloroform and PEG precipitates were evaluated in some studies but their results fluctuating according to many factors. The selection of IgY crude extraction

method based on the recovery and purity of IgY levels obtained from extraction process. The selectivity of precipitation directly depends on the type of precipitating agent applied. However, several precipitation steps using organic solvents are necessary to obtain a purified protein^[5]. In this experiment, IgY concentration was higher in samples purified by chloroform precipitate than by PEG precipitate. This was in accordance to another study, in which, the chloroform precipitation method reached the highest protein concentration compared to Dextran-PEG, charcoal-PEG, and PEG extraction process. Therefore, the yield of protein obtained was also highest with chloroform method (61%) than various methods such as dextran-PEG, charcoal-PEG and PEG method (8%, 50% and 26% respective)^[3].

PEG was first described by Polson et al. (1980)^[20] for IgY extraction from egg yolks, and the PEG method can be seemed as the standard technique^[22] (Schade et al., 2001). However, this method results in a low titer of IgY and could be explained because of the loss of more than half IgY in lipid removal step by 3.5% PEG^[19]. Therefore, organic solvents as chloroform were used to increasing lipid removal step from egg yolk. Previous study showed the higher content of IgY obtaining from chloroform precipitating process comparing to PEG precipitating process^[3]. However, the IgY obtained by chloroform method couldn't be used to produce diagnostic reagents due to inadequate residue. Instead, these IgY is suitable for measuring antibody titers after infection or inoculation^[22]. However, the precipitation by using polyethylene glycol offered a cheap and easy methodology and could be used in laboratory practice^[6]. In another study, the PEG precipitation method gave several advantages such as simple, requires few steps, and yield is high by optimizing PEG precipitation method, 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. Therefore, PEG precipitation method might be applied for large-scale production, and it opens the venues of using IgY in human and veterinary medicine for therapeutic and prophylactic purposes^[11].

IgY purified with PEG method resulted in a significantly low total protein content compared with chloroform purification method, this result agrees with Akita and Nakai (1993)^[1]. Our results are in accordance with the conclusion from Al-Edany (2013)^[3] who found that the higher concentration of IgY obtained from chloroform precipitation method compared to PEG precipitation. The chloroform seems more effective than PEG in the precipitation step for the extraction of IgY from egg yolks. However, with the same schedule of immunization and egg collection, the IgY concentration

by both chloroform and PEG methods are higher than our results ($12.8 \pm 0.25 \text{ mg.ml}^{-1}$ and $4.4 \pm 0.36 \text{ mg.ml}^{-1}$, respectively)^[3]. This indicates that IgY levels could be influenced by the interaction of several factors (such as chicken breeds, hen ages, housing temperature, feed). The result of another study showed the possibility of generating large quantities of highly pure IgY from chicken eggs and concluded the large differences in yield of IgY production between the two studied breeds^[4].

SDS-PAGE results showed that both extraction methods had the same purification ability of IgY from egg yolks. SDS-PAGE analysis of IgY purified with chloroform extraction method appears to confirm previous observation by Bizhanov *et al.* (2004)^[5] who reported that the IgY extracted with chloroform is contaminated with 20% unwanted non-sense proteins.

The protein profile seems more purified due to the limitation of contaminant bands presented in our study (1 major band and 3 minor bands) in comparison with the result of Al-Edany (2013)^[3] (4 major protein bands and 3 minor bands with PEG method; 4 major protein bands and 5 minor bands with chloroform method). Otherwise, by optimizing the purification process of IgY (increasing the centrifugal speed at 13,000 x g and completing with the dialysis step, Pauly *et al.* (2011)^[17] showed IgY protein profile were more purified due to SDS-PAGE result presented only one minor contaminant band.

5. Conclusions

In conclusion, IgY antibodies were successfully purified from egg yolks by both chloroform and PEG precipitation methods, which were identified in IgY profile as using SDS-PAGE. However, IgY concentration extracted with chloroform precipitation method resulted in a significantly higher total protein content compared with PEG purification method. Isolated IgY with chloroform protocol might be evaluated for large-scale production by optimizing the purification step to obtain higher amounts of IgY antibodies and avoid contaminant proteins.

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for technical support of SDS-PAGE method.

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