

Production and characterization protein of anti HIV specific immunoglobulin Y for Immunotherapy

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ABSTRACT

In this study, an approach for large-scale production of immunoglobulin Y (Ig Y) specific anti Human Immunodeficiency Virus (HIV) for use as a potential immunotherapy for HIV disease. Lohman laying hens were immunized intramuscularly with HIV virus that had been inactivated using formaline. The immunizations were repeated two times with dose of each 1 ml of HIV antigen (20 copy/ml) with an interval of two week. The first immunizations were HIV antigen mixed with Freund Adjuvant Complete and subsequently mixed with Freund Adjuvant Incomplete. Egg yolk was separated from egg white and immunoglobulin Y (IgY) antibody was then purified by multiple polyethylene glycol (PEG) 6000 extraction and ammonium sulfate purification steps. Antibody response in yolk was detected by agar gel precipitation test (AGPT) and the protein pattern was detected using polyacrilamid gel electrophoresis (SDS-PAGE). Specific activity of the antibody was tested using commercial ELISA. Antibody of HIV was detected and produce a specific line of precipitation in AGPT beginning the second week after the first immunization. Analysis of results obtained with ELISA showed significant increase in the HIV-specific antibodies after two weeks from the primary immunization. Through the effect of boosting; the anti-HIV antibody levels reached a plateau at six weeks from the primary immunization and remained significantly higher till the end of observation period. SDS-PAGE revealed the IgY preparation to be pure and dissociated into protein bands with molecular weights of 164; 87; 68, and 37 kDa. These results suggested that chicken IgY could be a practical strategy in large-scale production of Ig Y specific anti HIV for immunotherapy and diagnostic KIT of HIV disease.

INTRODUCTION

Human Immunodeficiency Virus (HIV) is the causative agent of AIDS. Worldwide, 25 million patients have died so far and over 34 million people are currently infected. Combined antiretroviral therapy has transformed the treatment of HIV/AIDS and extended patients life expectancy. However, the long term-nature of this treatment is associated with severe toxic side-effects, limited compliance, development of resistance and high cost. The only hope to control the continuous spread of HIV is to develop a vaccine or therapy that it is not only effective but affordable. Recently the utilization of Immunoglobulin Y (IgY)

from eggs of chickens, which were immunized against certain pathogens, has been the focus of attention in immunotherapy and immunodiagnosis, since IgY antibodies are the predominant serum immunoglobulin in birds, reptiles and amphibia, and are transferred in the female from serum to egg yolk to confer passive immunity to embryos and neonates (Arasteh *et al.*, 2004; Chalghoumi *et al.*, 2009). Therefore, research and diagnostic community constantly demand new alternatives and procedures to produce cost-effective antibodies. The use of laying hens to produce polyclonal antibodies is an alternative to the use of mammals, such as rabbits and, since more than two decades, egg yolk antibodies (IgY) are a low cost and ethical alternative (Schade *et al.*, 2005; Rahimi *et al.*, 2007; Pauly *et al.*, 2009). Compared with the stressful bleeding of mammals to obtain serum, IgY can be easily obtained non-invasively from the egg yolk.

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From the economical point of view, the amount of antibodies produced by a single hen is similar to that of a large mammal such as a sheep or goats, whereas maintenance costs are much lower (Fu *et al.*, 2006; Schade *et al.*, 2005). IgY from serum is actively transferred into the yolk by a receptor-mediated process and the amount of the immunoglobulin varies between 100 and 250 mg per egg (Schade *et al.*, 2005). Thus, a substantial amount of antibody can be produced from just one hen (up to 40 g of total IgY per chicken per year), of which 1-10% is expected to be specific to the antigen of interest (Mine and Kovacs-Nolan, 2002). In contrast to mammalian IgG, IgY antibodies do not activate mammalian complement, do not cross-react with Fc receptors, mammalian rheumatoid factor, or human anti-mouse antibodies, thus eliminating false-positive results in serological assays (Schade *et al.*, 2005; Alexander *et al.*, 2009). Also, chickens are able to develop a better response against mammalian antigens, due to the phylogenetic distance between mammals and birds (West *et al.*, 2004; Schade *et al.*, 2005).

There is an increasing interest in the use of chicken egg yolk for polyclonal antibody production for practical and economical reasons (Zhen *et al.*, 2009; Wang *et al.*, 2011) and chicken egg yolk antibodies have been applied successfully for scientific, diagnostic, prophylactic and therapeutic purposes (Lee *et al.*, 2009; Liou *et al.*, 2010; Sui *et al.*, 2011). Because of the phylogenetic distance between birds and mammals, mammalian proteins are often more immunogenic in birds than in other mammals and antibody synthesis readily stimulated in hens. In addition, because of the phylogenetic distance bird antibodies against a mammalian protein may often react with analogous proteins in other mammalian species (Alexander *et al.*, 2009; Lu *et al.*, 2009).

This study aimed to produce IgY specific to HIV virus which could be used for future alternative diagnostic kit, prophylactic and therapeutic medicines for HIV.

MATERIALS AND METHODS

Preparation of viral antigen

HIV virus was obtained from the Institute of Tropical Disease Airlangga University (Surabaya, Indonesia). The virus was then inactivated by treatment with 1 % (v/v) formaldehyde at 32°C for 5 days. This viral sample was used to immunize the hens (Pellegrini, 1993).

Immunization of hens with HIV virus

Lohman laying hens were immunized intramuscularly with HIV virus that had been inactivated using formaldehyde with 1 % (v/v) at 32 °C for 5 days. The immunizations were repeated two times with dose of each 80 µg of antigen (viral protein) of HIV with an interval of two week. The first immunizations were antigen mixed with Freund Adjuvant Complete and subsequently mixed with Freund Adjuvant Incomplete. Eggs were collected daily, beginning before and after the first immunizations, and stored at 4 °C until analysis.

Isolation and purification of IgY

A rapid and simple method adapted from previous studies (Almeida *et al.*, 2009) was used to extract IgY from yolk. Briefly, the yolk was separated from the white by egg separators, and a volume of buffer containing 14% PEG6000 (w/v) equivalent to three volumes of yolk was added. The mixture was stirred at room temperature (RT) for 30 min and was centrifuged at 5000g for 20 min at 10 °C. The supernatant was collected and filtered through four layers of sterile gauze. The volume of the filtrate was measured, and PEG6000 was added by gentle stirring to adjust the final polymer concentration to 12% (w/v). The material was centrifuged at 5000g for 20 min at 10 °C. The pellet was dissolved to the original volume of yolk in phosphate buffer, solid ammonium sulfate was added to reach 50% saturation, and the mixture was stirred overnight at 4 °C. The precipitate was collected by centrifugation and washed with 33% saturated ammonium sulfate. The precipitate was dialyzed against PBS and freeze-dried, and the powder obtained was stored at -20 °C. The purified IgY concentration in egg yolk determined by spectrophotometer (Biorad, USA) and Bradford method. Finally, the IgY antibodies were stored at -20°C until use (15).

Agar gel precipitation test (AGPT)

AGPT was carried out on the immunoglobulin Y samples using the methods described by Okworl (2011) It was performed using immunodiffusion plates with 10 ml of 1% agar noble gel containing 8% sodium azide at PH 7.2 ± 0.1. Using a template and cutter wells of 4 mm diameter and 4 mm interspace (apart) were cut, the plates were set up with groups of 6 wells in a circle surrounding a centre well. The peripheral wells were filled with the immunoglobulin Y samples to be tested, while the centre well was filled with the antigen. The plates were incubated at 37°C and read at 24 h under diffused light. The observations were compared with before and after immunization. Positive IgY anti HIV samples showed a line of precipitation between the IgY and antigen wells, while negative IgY anti HIV samples showed no line of precipitation.

Enzyme-linked immunosorbent assay

The titer of IgY against HIV virus was measured by an indirect noncompetitive enzyme-linked immunosorbent assay (ELISA) according to previously reported methods with modifications (Zhen *et al.*, 2009). A 96-well micro-titer plate was coated with inactivated HIV virus containing 0.58 mg/mL protein in carbonate-bicarbonate buffer (0.05 M, pH 9.6) at 100 uL/well. Serial dilutions of specific IgY were incubated in the pre-coated and blocked plate for 1 h before the bound IgY was detected with 100 uL/well HRP-conjugated rabbit anti-chicken IgY (1:5000) (Promega, USA). After incubation for 1 h at 37 °C, the plate was washed four times with PBS containing 0.05% Tween 20 (PBST). Next, 100 uL/well 3,3',5,5'-tetramethylbenzidine (Amresco, USA) substrate was added and incubated for 15 min at 37 °C. The color development was stopped with 2 M sulfuric acid (50 uL/well), and the optical density (OD) was measured on a micro-

titer plate reader (Tecan f200, Switzerland) at 450 nm. The reproducibility of the experiment was ascertained by including a blank control (PBS) and a negative control (IgY derived from non-immunized hens) in each plate. HIV-specific IgY titer was defined as the maximum dilution multiple of the sample with an OD value that was 2.1 times that of the negative control.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the purity of IgY. A 15% polyacrylamide gel was used with a Mini-Protean 3 cell (Bio-Rad Laboratories, USA). The analysis was conducted under reducing conditions; the sample was mixed with sample buffer and held for 5-8 min at 100 °C. Ten microliters of the sample was loaded into each well. Pre-stained protein standard (Fermentas, Lithuania) was used as a molecular weight marker. The protein bands were visualized with Coomassie Brilliant Blue R250 (Fluka USA). The gel was analyzed using Bio-Rad image analysis software.

Statistical analysis

Data were reported as means \pm standard deviation (SD) and levels of significance were evaluated using one-way ANOVA with Least Significant Difference test. The differences were considered significant at the level of $p < 0.05$.

RESULTS

The IgY concentration in egg yolk.

The IgY concentration in yolk from eggs before immunizations was 3.37 ± 0.44 mg/ml of egg yolk. Whereas the IgY concentration in egg yolk demonstrated significantly increased beginning the second week after the first immunization 3.76 ± 0.39 mg/ml and reached maximum of 4.07 ± 0.36 mg/ml at 4 week after immunization. After week 6 the levels decreased gradually to reach a level of 3.97 ± 0.38 mg/ml (Table 1).

Table 1: Concentration of immunoglobulin Y (IgY) content before and after immunization

Group	Concentration of IgY(mg/ml) $\bar{X} \pm SD$
Before immunization	$3.37^a \pm 0.44$
At 2 weeks after immunization	$3.76^b \pm 0.39$
At 4 weeks after immunization	$4.07^b \pm 0.36$
At 6 weeks after immunization	$3.97^b \pm 0.38$

The data represent the average from 15 eggs. Superscript within each column indicate significant difference between the means ($p < 0.05$).

Agar gel precipitation test of IgY

AGPT reactions between HIV antigen and IgY before or after immunization are shown in Figure 1. IgY positive anti HIV if the precipitation lines from the IgY into the well. One line of precipitation was produced by IgY from 2; 4 and 6 week after immunization but not before immunization. The AGPT test was a satisfactory and uncomplicated technique for detecting precipitating antibodies against HIV virus.

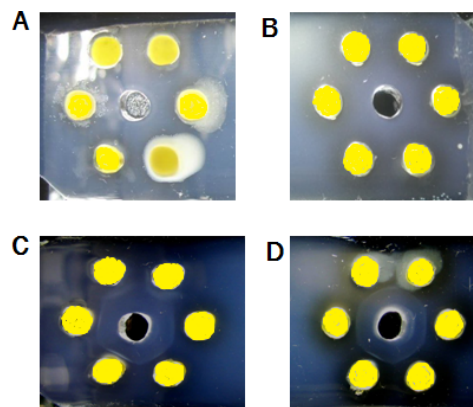


Fig. 1: AGPT reactions between HIV antigen and IgY before immunization (A); at 2 weeks (B); 4 weeks (C) and 6 weeks (D) after immunization .

IgY titres off egg yolk antibodies with specificity against HIV virus

Specific HIV IgY were detected at 2 week after immunization. Following reimmunization, the level of specific antibodies continued to increase at 4 and 6 week after immunization. 2; 4 and 6 weeks after immunization demonstrated significantly higher egg yolk antibody titers than that of before immunization (Table 2).

The optimal cut-off value for the optical density of an ELISA is 0.227. The Titer of anti HIV IgY more than 0.227 are classified as positive and below which they are considered negative.

Table 2: Titer of anti-HIV IgY in egg yolks were measured by ELISA.

Group	Titer of anti-HIV IgY (OD) $\bar{X} \pm SD$
Before immunization	$0.156^a \pm 0.015$
At 2 weeks after immunization	$0.299^b \pm 0.019$
At 4 weeks after immunization	$0.392^c \pm 0.029$
At 6 weeks after immunization	$0.419^d \pm 0.022$

The data represent the average from 15 eggs.

Superscript within each column indicate significant difference between the means ($p < 0.05$).

Characterization of IgY by SDS-PAGE

SDS-PAGE was applied to confirm isolation of immunoglobulin Y. Analysis of purified IgY by SDS-PAGE identified two major bands of 68 and 37 kDa, and two minor bands of 164 and 87 kDa. The electrophoresis pattern of gel filtration found in this procedure was in accordance with the standard IgY; showing 68 kDa heavy chain and 37 kDa light chain (Fig. 2).

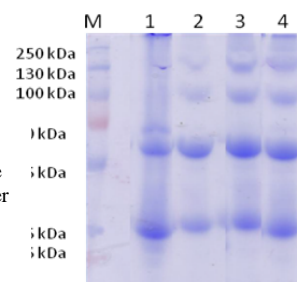


Fig. 2: SDS-PAGE of IgY. Protein markers (M). IgY before immunization (Lane 1). IgY after immunization at 2 weeks (Lane 2); 4 weeks (Lane 3) and 6 weeks (Lane 4).

DISCUSSION

In recent years, immunoglobulins obtained from avian egg yolks are increasingly finding favor to replace mammalian antibodies for diagnostic and therapeutic applications. The production of antibodies in chicken is less expensive, easy and non-invasive with high antibody yields (Fu *et al.*, 2006). IgY does not activate the complement system nor does it interact with rheumatoid factor. Not surprisingly, IgY antibodies also show far superior immunogenicity against highly conserved mammalian proteins making the production of IgY against mammalian proteins orders of magnitude more efficient than current IgG production using rabbits, donkeys and mice.

Lohman laying hens are easily raised and have high egg productivity. After immunization with a small dose of antigen, a chicken can continuously produce eggs containing antigen-specific antibodies in their yolks. A chicken usually lays 280 eggs/year and an egg yolk (12–15 ml) usually contains 150–200 mg IgY, of which 2–10% are specific antibodies (Nguyen *et al.*, 2010). Thus, a chicken is referred as a small “factory” for antibody production.

In this study, we adopted a PEG extraction method followed by several steps of ammonium sulfate precipitation. The method is simple, efficient, and safe compared with water dilution, chloroform extraction, and gel-filtration chromatography and therefore, it is suited to large-scale isolation of IgY from egg yolk. The PEG used in this process, which is of low toxicity, is widely used in pharmaceutical production. After purifying of IgY from egg yolk, the IgY concentration in egg yolk determined by spectrophotometer (Biorad, USA) and Bradford method. IgY was successfully elicited by immunizing the hens with formalin-inactivated HIV antigen emulsified in Freund’s adjuvant. The IgY concentration in egg yolk increased during the immunization period until week 6 where it began to increase dramatically at 2 weeks and it reached a plateau at 4 weeks after immunization. After week 6 the levels decreased gradually. These results indicated that chickens require about two weeks for antibody production and it also indicates that lohman hens, as the host for the production of anti HIV IgY, show the remarkable ability to rapidly and efficiently generate an abundant IgY and provide specific IgY in a noninvasive way. The injection of the antigen by the intramuscular route results in higher antibody levels by day 28 after immunization, and the resulting antibodies also exhibit higher specificity, being over 10 times more specific when compared with chickens immunized with the same antigen (Pauly *et al.*, 2009; Hirai *et al.*, 2010). Chickens, immunized by the intramuscular via, continue producing specific antibodies during more than 200 days (Sui *et al.*, 2011). Chickens can also tolerate the use of common immunological adjuvants, such as Freund’s adjuvant. AGPT are able to detect the presence or absence of antibodies to any virus. AGPT reactions between dengue antigen and IgY after immunization showed that IgY positive anti HIV if the precipitation lines from the IgY into the well. One line of precipitation was produced by IgY from 2; 4 and 6 week after

immunization but not before immunization. The AGPT test was a satisfactory and uncomplicated technique for detecting precipitating antibodies against dengue virus. AGPT is simple and economical and diagnostic results are obtained sooner. It is a well-known concept that the immune response is more potent when the distance between the antigen source and the immune system increases. Therefore, to obtain immunoreagents containing antibody titers against mammalian antigens, chickens are better and cheaper than mammals (Lu *et al.*, 2009; Wang *et al.*, 2011).

The anti HIV IgY produced by the immunized hens increased over time, as revealed by ELISA. The specific IgY titer increased starting the second week after the first immunization, with the titer peaking at the fourth week. After 6 weeks, the antibody titer slowly increased. Titer of anti HIV IgY in yolk at 2; 4; 6 weeks after immunization demonstrated significantly higher than that of before immunization. It indicates that lohman hens, as the host for the production of anti HIV IgY, show the remarkable ability to rapidly and efficiently generate an abundant IgY and provide specific IgY in a noninvasive way. Chickens, as a source of desired antibodies, represent an alternate animal system that offers some advantages with respect to animal care, high productivity and special suitability of avian antibodies for certain diagnostic purposes.

SDS-PAGE was applied to confirm isolation of immunoglobulin Y. The electrophoresis pattern of gel filtration found in this procedure was in accordance with the standard IgY; showing two major bands of 68 and 37 kDa, and two minor bands of 164 and 87 kDa. The electrophoresis pattern of gel filtration found in this procedure was in accordance with the standard IgY; showing 68 kDa heavy chain and 37 kDa light chain. The molecular mass (67-70 kDa) of the H chain in IgY is larger than the H chain from mammals (50 kDa). The greater molecular mass of IgY is due to an increased number of heavy-chain constant domains and carbohydrate chains (Alexander *et al.*, 2009). IgG has 3 C regions (C γ 1–C γ 3), while IgY has 4 C regions (C ν 1–C ν 4) and the presence of one additional C region with its two corresponding carbohydrate chains logically results in a greater molecular mass of IgY compared with IgG. The immunization of chickens provides an attractive alternative to using mammals as hosts for antibody production. IgY is the major low molecular weight immunoglobulin in oviparous animals. This type of antibody has distinctive properties which can be exploited in various ways in research, diagnostics and therapy. One important advantage arises from the phylogenetic distance and genetic background that distinguishes birds from mammals. This improves the likelihood that an immune response will be elicited against antigens or epitopes that may be non-immunogenic in mammals. The deposition of IgY into the egg yolks of the immunized bird then provides an elegant source of polyclonal immunoglobulins. Since polyclonal IgY can be recovered from the eggs of laying hens for prolonged periods, this approach provides a longterm supply of substantial amounts of antibodies.

CONCLUSION

The results presented in this study indicate that immunization of hens with HIV virus could be a strategy to obtain at low cost a relatively high concentration of anti HIV egg yolk IgY, could be an useful tool for research, diagnosis and therapy of HIV infection. Our results also suggest that egg yolk from immunized laying hens may offer a new large-scale source of low-cost antibody

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