NUSANTARA BIOSCIENCE
Vol. 9, No. 3, pp. 282-287
August 2017

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n090307

Isolation of anti-idiotype minor capsid *Human papillomavirus* type 16 (HPV 16 L2) IgY from egg yolk as immunogen of HPV vaccine

EKA NONENG NAWANGSIH[1], SAYU PUTU YUNI PARYATI[1], JUSUF S. EFFENDY[2], SUNARJATI SUDIGDOADI[3], EDHYANA SAHIRATMADJA[4], DIKI HILMI[5], EVI SOVIA[6]

[1]Department of Microbiology, Faculty of Medicine, Universitas Jenderal Achmad Yani. Jl. Terusan Jendral Sudirman, PO Box 148, Cimahi 40525, West Java, Indonesia. Tel.: +62-22-6642781, ♥email: eka.n.nawangsih@gmail.com
[2]Department Obstetric and Gynaecology, Faculty of Medicine, Universitas Padjajaran. Jatinangor, Sumedang 45363, West Java, Indonesia
[3]Department of Microbiology, Faculty of Medicine, Universitas Padjajaran. Jatinangor, Sumedang 45363, West Java, Indonesia
[4]Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Padjajaran. Jatinangor, Sumedang 45363, West Java, Indonesia
[5]Depatment of Immunology, STIKES Jenderal Achmad Yani. Cimahi 40525, West Java, Indonesia
[6]Department of Pharmacology, Faculty of Medicine, Universitas Jenderal Achmad Yani. Cimahi 40525, West Java, Indonesia

Manuscript received: 12 March 2017. Revision accepted: 4 July 2017.

Abstract. *Nawangsih EN, Paryati SPY, Effendy JS, Sudigdoadi S, Sahiratmadja E, Hilmi D, Sovia E. 2017. Isolation of anti-idiotype minor capsid* Human papillomavirus *type 16 (HPV 16 L2) IgY from egg yolk as immunogen of HPV vaccine. Nusantara Bioscience 9: 282-287.* A potential strategy for the production of safe protective vaccines for human papillomavirus (HPV) infection is to utilize anti-ids. Meanwhile, hyperimmunized hens could provide a convenient of specific immunoglobulin in their yolks (IgY). This study aimed to isolate IgY specific to anti-idiotype HPV 16 L2 from egg yolk which could be used for future alternative immunogen for HPV vaccine. Antibody anti-idiotype derived from chicken egg yolk that chickens immunized by HPV 16 L2 antibody. The antibodies were purified from immunized chicken egg yolk, then electrophoresis it using SDS-PAGE method to determine the molecular weight of IgY. To determine an amount of protein, we used fluorometer method and to confirm an existence of specific IgY using ELISA method. The results showed that the IgY preparation dissociated into three protein major bands with molecular weights of 180; 65; and 25 kD. The IgY and IgY specific concentration in eggs yolk increased until 8th week. After 8th week the levels decreased gradually. Samples derived from chickens immunized showed significantly higher concentrations of IgY and IgY specifics than control ($p<0.05$). These results suggested that chicken IgY could be a practical strategy in large-scale production of specific antibody anti-idiotype HPV 16 L2 for HPV Vaccine.

Keywords: Antibody anti-idiotype, egg yolk, HPV 16 L2, isolation

INTRODUCTION

Cervical cancer, the most common cancer affecting women in developing countries, is caused by persistent infection with “high-risk” genotypes of human papillomaviruses (HPV) (Castellsagué 2008). Two virus-like particles HPV VLP (virus-like-particle) vaccines aimed to prevent infection by high-risk HPV types are currently available: bivalent and quadrivalent vaccine (Cho et al. 2011; Clutt et al. 2007). They were a recombinant vaccine. Another potential strategy for the production of safe protective vaccines for HPV infection is utilized anti-ids method. This approach arose from Jerne’s idiotypic network theory (Ladjemi 2011). The theory stated that antigenic epitope elicits an immune response, resulting in the production of Ab1 antibody. The ab1 antibody can, in turn, trigger an anti-idiotypic response consisting of distinct subsets of Ab2 antibodies: α, β, γ, and ε. The β subtype of anti-Id Abs express the internal image of the Ag recognized by the Ab1 Ab and can, therefore, be used as Ag surrogates (Jerne 1974). The ab2β have successfully induced anti-pathogen and anti-tumor B-cell and T-cell response in several different species (Ladjemi 2012). The existing vaccines that made of capsid HPV major (L1) can induce high-titer antibody but type-restricted neutralizing antibodies. Meanwhile, L2 of genital HPV types contain broadly cross-neutralizing epitopes but low immunogenicity (Karanaam et al. 2009). To enhance immunogenicity IgY in eggs yolk was used as a carrier. The laying hens are an excellent source for the large-scale antibody production. Moreover, the phylogenetic distance of chicken from mammals caused it can produce high-titer antibodies against conserved mammalian antigens compared to other experimental animals commonly used (da Silva 2010).

This study aimed to produce IgY specific to anti-idiotype HPV 16 L2 from egg yolk which could be used for future alternative immunogen for HPV vaccine.

MATERIALS AND METHODS

Materials

The study used two specific pathogenic free white leghorn hens from Bio Farma as eggs source, one hen injected by antibody HPV 16 L2 (Gmab #2 from Santa Cruz) and another hen injected with physiological NaCl. Freund's adjuvant from Sigma-Aldrich used to increase

immunogenicity and for purification using solution A and solution B diagnostic kit from Promega (2004). Fluorometer reagent from Invitrogen used to measure IgY levels and to measure IgY specific using KPL reagent.

Methods

Hen immunization

Immunization procedure based on Thermo Fisher Scientific Inc (2012) method. The hen was immunized subcutaneously with antibody HPV 16 L2 to generate IgY of anti-idiotype HPV 16 L2 in eggs yolk. Booster injections performed at week 0, 2, 4 and 5 to raise the antibody anti-idiotype levels in eggs yolk (0.2 mg/dose/animal). For the first injection, the antibody was emulsified in the complete Freund's adjuvant and for three subsequent boosters in the incomplete adjuvant. Eggs were collected daily beginning before and after the fourth immunizations and stored at 4°C to be immediately purified with a diagnostic kit.

Egg Yolk preparation

Allow eggs to warm at room temperature before starting the preparation. Break the shell carefully and drain most ofalbumen. Transfer the yolk into a petri dish and remove the residual albumen with a pipette and with tissue or use the Egg Separator (Promega). Do not break the yolk sack. When the yolk was clean, puncture the yolk sack with a pipette tip and lets the yolk fluid drip into a tared 100 mL beaker glass. Hold back the yolk sack with the pipette tip. Determine the weight of the yolk to get the volume (1g = 1 mL). A yolk of an average egg was 10-15g.

Precipitation of egg by diagnostic kit

The antibodies were purified from immunized chicken eggs yolk by A and B solution diagnostic kit from Promega according to the established manual procedures from the manufacturer. The antibodies were purified from immunized chicken eggs yolk by diagnostic kit from Promega. Place the beaker with the yolk on a magnetic stirrer and mix for 1-2min to get a homogenous suspension. Added three volumes of precipitation A Solution (30 mL per 10 mL yolk) slowly and continue stirring for 5 min at room temperature (RT). Transfer to 50 mL centrifuge tubes and spin 10min 10000g/11500rpm at 4°C. Filter the supernatant through a 0.45μm filter into a tared 100 mL glass beaker and determine the weight to get the volume (1g = 1 mL). Discard the pellet. Added a stir bar and place on a magnetic stirrer. Added 1/3 volume Solution B precipitation (10 mL per 30 mL) filtered supernatant and continued stirring for 5 min at RT. Transfer to 50 mL centrifuge tubes and spin 10 min 10000g/11500rpm at 4°C. Discard the supernatant and dissolve the IgY pellet in 12 mL TBS by stirring on a magnetic stirrer with a small stir bar at RT. At this point, the protein concentration will be 10 mg/mL and IgY will be ca. 75% pure. This preparation of IgY was suitable for most applications and could be stored at -20°C or -75°C. For further purifications of IgY, place the centrifuge tube containing the redissolved IgY from the first precipitation with Solution B on a magnetic stirrer. Add 1/3 volume B solution precipitation slowly (4 mL per 12 mL redissolved pellet) and continue stirring for 5min at RT. Spin for 10min at 10000g/11500rpm 4°C. Discard the supernatant and dissolve the IgY pellet in 3 mL TBS by stirring on a magnetic stirrer with a small stir bar at RT. Filter the purified IgY through a 0.2μm filter.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-PAGE was done under denaturing conditions using Mini-PROTEAN II Cell (BioRad 2014) according to the instruction of the manufacturer. The purity of various IgY preparations was estimated using 10% SDSPAGE, and Coomassie brilliant blue R-250 (BioRad) was used to visualize the protein bands. Broad-range SDS-PAGE molecular weight standards of 15 to 225 kDa (BioRad) were used as markers.

Fluorometer

By using fluorometric detectors, sample concentrations can be measured without the interference of contaminants by only emitting fluorescence when bound to the specific target molecules. Qubit fluorometer used because the Qubit Fluorometer accurately detects very low concentrations protein. Measurement of IgY levels according to manual part no. MP32866 MAN0003231 Qubit®2.0 fluorometer from Invitrogen.

Enzyme-linked immunosorbent assay (ELISA)

The titer of IgY against HPV 16 L2 virus was measured by an indirect noncompetitive enzyme-linked immunosorbent assay (ELISA) according to previously methods with modifications (Fischer and Hlinak 1996). First, microtiter plates were coated with the catching antibody. Monoclonal antibody HPV 16 L2 (G mab#2 Santacruz) was diluted 1: 800 in coating buffer and 50 µI per well was added. The plates were incubated for 2 hours at room temperature and after that overnight at 4°C. The plates were washed three times with washing buffer (8,0 g NaCl; 0,2 g KH2P04; 2,9 g Na2HP04 x 12 H20; 0,2 g KCL; 0,5 mL Tween20; add 1000,0 mL Aquadest., pH 7,4). All further washing steps were carried out with this buffer. Then plate were added blocking solution from KPL. The plates were washed three times with washing buffer.Samples containing the IgY were added in well following 4-hour incubation at 37°C, the plates were washed three times, and 50 µI per well POD-labeled anti-chicken IgY (Abcam)were added. Plates were incubated for 3 hours at 37°C. After this time the plates were washed and 50 µL of substrate chromogen solution were pipetted into each well. The reaction was stopped solution (50 ul/well) after 15 min. The absorption was measured at 450 nm using ELISA reader. The reproducibility of the experiment was ascertained by including a blank control (PBS) and a negative control (IgY derived from hen immunized by NaCl) in each plate. Positive results are determined based on three times the value of the negative control. The substance concentration contained in the sample is expressed by optical density. Measuring the optical density (OD) is a common method to quantify the concentration of substances (Beer-Lambert law), since the absorbance is

proportional to the concentration of the absorbing substances in the sample (David W, 2001).

Data analysis

All the values will be expressed as the mean±standard error mean and analyzed by unpaired Student's T test. The level of statistical significance will be set at $p<0.05$

RESULTS AND DISCUSSION

Nowadays, the anti-idiotypic vaccine approach has been successfully used for various aspects of vaccinology, especially for tumor immunotherapy (Thomas 2012). Recently, immunoglobulins obtained from avian eggs yolk were preferred to offer new fields of IgY application, particularly for therapeutic and/or prophylactic use in human and veterinary medicine. The use of chickens for the production of polyclonal antibodies provide several advantages over the traditional method of producing antibodies in mammals (Schade et al. 2005). Chicken eggs yolk as a source for antibody production represents a reduction in animal use since chickens produce larger amounts of antibodies than laboratory mammals. It also possible to eliminate the collection of blood, which is painful for the animal. The European Centre for the Validation of Alternative Methods (ECVAM) recommends that yolk antibodies should be used instead of mammalian antibodies for animal welfare reasons (Schade et al. 1996).

There were several methods for purifying IgY based on the strategy of separation of proteins from lipoproteins and the rest of the yolk lipids. Purification methods based on organic solvents like chloroform remain in use. Other methods are based on affinity chromatography or on dilution of the yolk followed by a freezing-thawing process. Ion exchange is also often used for purification and is usually combined with the number of salts precipitation method e.g. polyethylene glycol (PEG), dextran sulfate, dextran blue, sodium sulfate, ammonium sulfate, caprylic acid or sodium citrate (Akita and Nakai 1992). Method selection was defended on the yield and purity desired, final use of the IgY as well as material cost and labor skills (Carlender 2002). In this study, diagnostic kits from Promega for purifying the harvested yolk were used, it contains 3,5% and 12% PEG .The purity level was 70-90%. The Polyethylene glycol (PEG) that used in this process have low toxicity and widely used in pharmaceutical production. PEG is an excellent method to precipitate a specific protein from a complex mixture proteins (Goldring and Coetzer 2003). In the current study, PEG precipitation technique was found to be simple, easy and economical (Polson 1990) for purification of Immunoglobulins. PEG has multiple hydroxyl groups, which become highly reactive in an aqueous phase. In the aqueous phase, PEG acts as an anion nucleophile and can attract positively charged substances, thereby this electrostatic produces changes in water. Therefore, in a PEG medium, the hydrophobic interaction of antibody molecules are considerably enhanced which separate them from the other proteins of the suspensions (Shafique 1996).

The IgY concentration in eggs yolk determined by fluorometer method. By using fluorometric detectors, sample concentrations can be measured without the interference of contaminants by only emitting fluorescence when bound to the specific target molecules (Qubit 2010). The IgY concentration in eggs yolk increased during the immunization period and the titer began to rise dramatically in the 4th week. Antibody levels increased until the 8th week, after 8th week the levels decreased gradually. The results were accordance with the recommendations of Thermo Fisher Scientific Inc protocol that the eggs collection was done at 4th week and harvested at 8th week. The maximum concentration reached at the 8th week is 6,942 ± 0,041 mg/mL and the average of 12nd week is 6,076 ± 0,935 mg/mL. The results were significantly different ($p<0.05$) compared with the control group which the levels were stable relatively with the average value in 12 weeks was 4,348 ± 0,167 mg/mL. The statistical test results showed a significant difference between the treatment group and negative control group ($p<0,05$).

Serum IgG antibodies of immunized chicken were transported and accumulated in the egg yolk efficiently (Bar-Joseph and Malkinson 1980). Previous studies showed that isolation of IgY dengue results obtained 5,77 mg/mL (Sudjarwo et al. 2012), Other studies that isolated antibody anti-idiotype rabies IgY obtained 9,40 mg/mL (Paryati and Soejoedono, 2006). The concentration of isolated antibodies was varied. The variations of antibodies formation were influenced by several factors, including animal age, molecular antigen size, complexity of antigenic chemical structure, genetic constitution, the methods of antigen insertions and doses of antigen (Leeddell and Weeks 1995). To confirm the existence of specific IgY against HPV, ELISA examination was performed. Specific antibody anti-idiotype HPV 16 L2 IgY were detected positive result at 4th week after immunization with Optical Density value 0,575 ± 0,106. It is based on the value of the cut-off 0,475 calculated from 3 times the average value of the negative control. Following reimmunization, the level of specific antibodies continued to increase at 8th weeks after immunization and achieve maximum value with the value of OD 2,564 ± 0,168. After 8th week the levels decreased gradually to reach a level of 2,051 ± 0,014 (table 2). The decreasing of IgY levels in chicken eggs is a reflection of the loss of plasma cell populations that produce specific antibodies. Once fully differentiated, plasma cells die after three to six days and the resulting level of immunoglobulin would slowly decrease due to this process of catabolism (Tizard 2013). Meanwhile, on chicken that is not injected antibody HPV 16 L2 (control) showed negative results consistently. The statistical test results showed a significant difference between the treatment group and negative control group ($p<0,05$). The pattern of increased levels of specific antibodies in accordance with elevated levels of IgY (Table 1).

Table 1. Concentration of IgY in egg yolks

Week	Concentration of IgY sample (mg /mL) X ± SD	Concentration of IgY control (mg /mL) X ± SD
0	4.061 ± 0.098	4.010 ± 0.099
1	4.386 ± 0.076	4.280 ± 0.071
2	4.511 ± 0.044	4.190 ± 0.099
3	4.766 ± 0.062	4.410 ± 0.099
4	5.054 ± 0.076	4.320 ± 0.035
5	6.050 ± 0.057	4.278 ± 0.088
6	6.314 ± 0.079	4.530 ± 0.071
7	6.410 ± 0.071	4.590 ± 0.085
8	6.942 ± 0.041	4.540 ± 0.057
9	6.546 ± 0.079	4.420 ± 0.085
10	6.040 ± 0.028	4.490 ± 0.099
11	5.995 ± 0.640	4.240 ± 0.071
12	5.841 ± 0.960	4.220 ± 0.099
Average	6.076 ± 0.935	4.348 ± 0.167

Table 2. Optical density of antibody anti-idiotype of HPV 16 L2 IgY

Week	Optical density (OD) sample X ± SD	Inter-pretation	Optical density (OD) control X ± SD	Inter-pretation
0	0.172 ± 0.056	-	0.178 ± 0.020	-
1	0.206 ± 0.089	-	0.176 ± 0.014	-
2	0.273 ± 0.107	-	0.175 ± 0.010	-
3	0.46 ± 0.106	-	0.171 ± 0.016	-
4	0.575 ± 0.106	+	0.171 ± 0.004	-
5	0.865 ± 0.104	+	0.175 ± 0.010	-
6	1.401 ±0.087	+	0.175 ± 0.011	-
7	2.158 ± 0.281	+	0.178 ± 0.010	-
8	2.564 ± 0.168	+	0.177 ± 0.011	-
9	2.254 ± 0.116	+	0.179 ± 0.015	-
10	2.142 ± 0.103	+	0.116 ± 0.011	-
11	2.137± 0.202	+	0.115 ± 0.014	-
12	2.051 ± 0.014	+	0.115 ± 0.012	-
Average	1.424	+	0.158	-
SD	0.890		0.026	
Cut off (3x control average)	0.475			

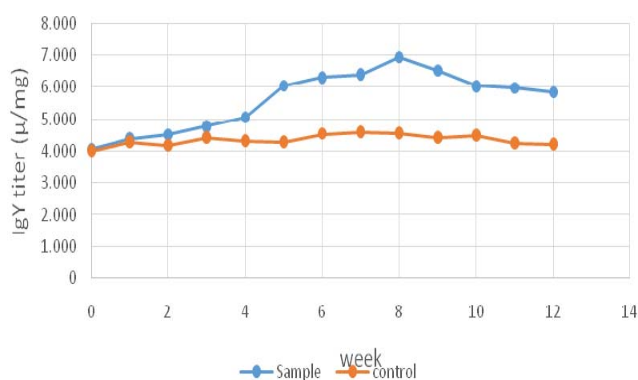

Figure 1. IgY titer pattern

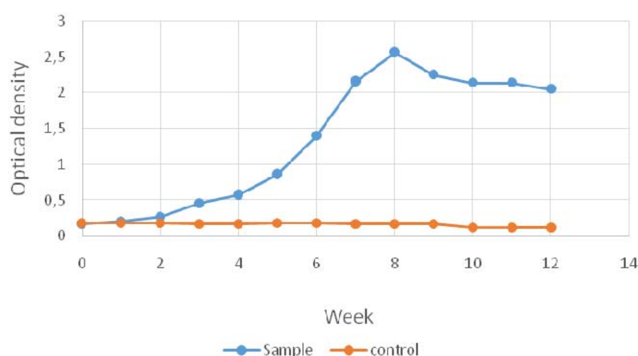

Figure 2. IgY specific for anti-idiotype HPV 16 L2 pattern

IgY and specific IgY were successfully obtained by immunizing the hens with antibody HPV 16 L2 emulsified in Freund's adjuvant. The use of adjuvants to stimulate the immune system and to enhance the immune response is routine in antibody production. Most protein antigens, especially small polypeptides (<10 kDa) and non-protein antigens, usually need to be conjugated to a large immunogenic carrier molecule to become good immunogens. The administration of these antigens would need an adjuvant especially if it administered in small quantity, to assure a high quality/high quantity antibody response by the immunized animal (Mayo 2009). According to Schade et al. (2005) experiment, Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) remain the most effective adjuvant to enhance antibody levels. Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (FIA) are the most common adjuvants used for parenteral immunization. These adjuvants are a mixture of mineral oil, surfactant, and heat-killed *Mycobacterium tuberculosis* or *M. butyric* (FCA) or without mycobacteria (FIA) (Freund et al. 1937).The water-in-oil emulsion is prepared by mixing one volume of the adjuvant (FCA or FIA) to one volume aqueous antigen solution. In the emulsion, the antigen is distributed in oil droplets which disperse widely after injection in the body, hence increasing the potential for interaction with relevant cells. Antibody production was enhanced by Freund's adjuvant primarily because of the depot effect, a depot of antigen forms at the injection site resulting sustained release of small quantities of the antigen over a long period of time and non-specific immunopotentiation of macrophages by surfactants and the mycobacteria (Stills 2005). FIA is frequently used to boost animals that received a primary antigen injection with FCA

but it also can be used as an adjuvant for primary injection. It has adjuvant properties that favor humoral immunity without cell-mediated-immunity, although less potently than FCA. Our experiment used subcutaneous (s.c.) injection method. The most common route for antigen injection in hens is the intramuscular (i.m.) route, but the extensive study by Swarzkoft et al. (2000) showed that the s.c. injection method provokes higher titer than the intramuscular (i.m.) injection.

Positive results in the ELISA examination showed chicken's immune response to HPV 16 L2 antibody (Ab1). Antibodies that produced are anti-idiotype that are expected to have the same serological characteristics with the original antigen and can be used to replace antigens in immunization. The ability to mimic the structure of the original antigen (internal image) is the cornerstone of its use as a replacement antigen. As with antigens, anti-idiotype antibodies have the ability to bind competitively with specific antibodies to the original antigen (Fields et al. 2002).

SDS-PAGE electrophoresis performed to illustrate the presence of IgY in the sample based on their molecular weight. Samples at 8th week the highest titer is checked by electrophoresis. There were three major bands of 180, 65 and 25 kDa, and three minor bands of 100; 70 and 35 kDa. The electrophoresis pattern was in accordance with the standard IgY (figure 2). Narat (2003) stated that IgY had the molecular weight greater than IgG, which is about 180 kDa or greater.). IgY is the major low molecular weight immunoglobulin in egg yolk (Michael 2010). The general structure of the IgY molecule is the same as the IgG molecule with two heavy (H) chains and two light (L) chains. The molecular mass of the H chain in IgY is larger than the H chain from mammals. 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 (Cγ1-Cγ3), while IgY has 4 C regions (Cυ1-Cυ4) and the presence of one additional C region with its two corresponding carbohydrate chains (Charlender 2002) logically results in a greater molecular mass of IgY compared with IgG. SDS sample buffer containing betamercaptol divided disulfide bonds of IgY that lead to the separation of heavy chains and light chains. Davalos et al. (2000) stated that the IgY consists of 65-68 kDa for each H chain (heavy chain) and 25 kDa for each of the L chain (light chain). Meanwhile band of 70 and 100 kDa suspected the Fc and Fab fragment was not assembled into IgY yet. According to War et al. (1995), IgY Fc fragment has a molecular weight of 76,542 kDa. Sun et al. (2001) also stated the molecular weight of IgY Fab fragment was 90,718 kDa. Another protein bands were suspected the contaminant proteins.

In conclusion, the result of this study indicate that immunization white leghorn hen with antibody HPV 16 L2 could be novel and promise strategy to produce specific antibody anti-idiotype HPV 16 IgY in large-scale with animal care and also could be source of low-cost antibody anti-idiotype for HPV vaccine as immunogen

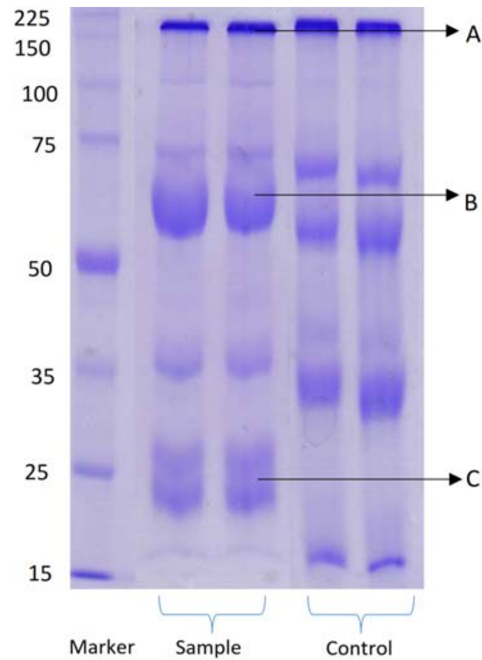

Figure 3. Proteins isolation of immunoglobulin(IgY) from egg yolk analyzed on SDS-PAGE. Patterns of sample fractions obtained from solution A and solution B purification

ACKNOWLEDGEMENTS

This study financially supported by Department of Ristekdikti Indonesia. The authors gratefully acknowledge the help given by experts and staff of the Research and Development Department of Microbiology Faculty of Medicine Jenderal Achmad Yani University and Laboratory of Genetic and Biomolecular Faculty of Medicine Padjajaran University.

REFERENCES

Akita EM, Nakai S. 1992. Immunoglobulins from egg yolk: Isolation and purification. J Food Sci 57: 629-634.

Alexander IT, Stella MF, Brian JS, Rosaleen AC. 2009. The crystal structure of an avian IgY-Fc fragment reveals conservation with both mammalian IgG and IgE. Biochemistry J 48: 556-562.

Bar-Joseph M, Malkinson M. 1980. Hen egg yolk as a source of antiviral antibodies in the enzyme-linked immunosorbent assay (ELISA): a comparison of two plant viruses. J Virol Meth 1: 79-184.

BioRad. 2014. Guide to Polyacrylamide Gel Electrophoresis and Detection. Bulletin 6040 Rev A US/EG. BioRad. www.bio-rad.com

Carlender D. 2002. Avian IgY antibody: in vitro and in vivo. [Dissertation]. Faculty of Medicine Uppsala Universitet, Uppsala. [Sweden].

Castellsagué X. 2008. Natural history and epidemiology of HPV infection and cervical cancer. J Gynecol Oncol 110: S4-S7.

Cho HJ, Kyoung OY, Bong KY. 2011. Advances in human papillomavirus vaccines: a patent review. Expert Opin Ther Patents 21(3): 295-309

Cutts FT, Franceschi S, Goldie S, Castellsague X, de Sanjose S, Garnett G, Edmunds WJ, Claeys P, Goldenthal KL, Harperi DM, Markowitz L. 2007. Human papillomavirus and HPV vaccines. Bulletin of the World Health Organization: 85 (9). WHO, Rome.

Da Silva W, Tambourgi DV. 2010. IgY: A promising antibody for use in immunodiagnostic and in immunotherapy. Vet Immunol and Immunopathol. DOI: 10.1016/j.vetimm.2009.12.011

Davalos PL, Ortego VJL, Bastos GD, Hidalgo AR. 2000. Colloidal stability of IgG and IgY-coated latex microspheres, colloids and surfaces B. Biointerfaces 20: 165-175.

David W, 2001. Immunoassay Handbook, Nature Publishing Group, London.

Fields BA, Goldbaum FA, Ysern X, Poljak RJ, Mariuzza RA. 2002. Molecular Basis of Antigen Mimicry by An Anti idiotope. Nature 374: 739-742.

Fischer M, Hlinak A. 1996. An ELISA for the quantification of chickens immunoglobulin (IgY) in various liquid media. ALTEX 13: 181-182

Freund J, Casals, J, Hosmer EP. 1937. Sensitization and antibody formation after injection of tubercle bacilli and paraffin oil. Exp Biol Med 7: 509-513.

Goldring JP, Coetzer THT. 2003. Isolation of Chicken Immunoglobulins (IgY) from Egg Yolk. Biochem Mol Biol Edu 31: 185-187.

Jerne NK. 1974. Towards a network theory of the immune system. Ann Immunol 125: 373-389.

Karanam B, Jagul S, Huh KW and Richard BS. 2009. Developing vaccines against minor capsid antigen L2 to prevent papillomavirus infection. Immunol Cell Biol 87: 287-299

Ladjemi MZ. 2012. Anti-idiotypic antibodies as cancer vaccine achievements and future improvements. Review article. Frontier Oncol 2 (158) : 1-4.

Leeddell E, Weeks I. 1995. Antibody Technology. In: Graham JM, Billington D. (eds). BIOS Scientific Publisher Ltd, Oxford, UK.

Mayo SL. 2009. Non-Invasive Antibody Production. [Dissertation]. Faculty of Medicine Uppsala Universitet, Uppsala. [Sweden].

Michael A, Meenatchisundaram S, Parameswari G, Subbraj T, Selvakumaran R, Ramalingam S. 2010. Chicken egg yolk antibodies (IgY) as an alternative to mammalian antibodies. Indian J Sci and Technol 3(4): 468-71

Narat M. 2003. Production of antibodies in chickens. J Food Technol. Biotechnol: 41(3): 259-67.

Paryati SPY, Soejoedono RD. 2006. Antibodies Anti-idiotype as Vaccine Candidate for Rabies Prevention in Animals. [Dissertation]. Bogor Agriculture Institute, Bogor [Indonesia]

Polson, A. 1990. Isolation of IgY from the yolks of eggs by a chloroform polyethylene glycol procedure. Immunol Invest 19: 253-8.

Promega. 2004. EGG stract IgY purification system. IgY purification protocol. Technical Bulletin No. 188. Promega. www.promega.com.

Qubit® 2.0 Fluorometer. 2010. User manual. Catalog no. Q32866. Manual part no. MP32866. MAN0003231

Schade R, Calzado EG, Sarmiento R, Chacana PA, Porankiewicz-Asplund J, Terzolo HR. 2005. Chicken egg yolk antibodies (IgY-technology): a review of progress in production and use in research and human and veterinary medicine. Altern Lab Anim 33 (2): 129-154.

Schade R, Staak C, Hendriksen C, Erhard M, Hug H, Koch G et al. 1996. The production of avian (egg yolk) antibodies: IgY. Alter Lab Anim 24: 925-934.

Shafique, M.K. 1996. Purification of immunoglobulins against infectious bronchitis from egg yolk by chloroform polyethylene glycol technique in broiler breeds. [Thesis]. University of Agriculture, Faisalabad, Pakistan

Stills HF. 2005. Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. ILAR J 46(3): 280-293.

Sudjarwo SA, Sudjarwo KE, Koerniasari. 2012. Purification and characterization protein of anti-dengue specific immunoglobulin Y for a diagnostic kit of dengue. J Appl Pharm Sci 2 (12): 007-012.

Sun S, Mo W, Ji Y, Liu S. 2001. Preparation and mass spectrometric study of egg yolk antibody (IgY) against rabies virus. Rapid Commun. Mass Spectrom 15: 708-712.

Thermo Fisher Scientific Inc. 2012. Custom chicken antibody production protocols. Thermo Fisher Scientific Inc. www.Thermofisher.com.

Thomas KE, Bejatohlah MK, Anastas P, Somdutta S, Ramachandran M and Heinz K. 2012. The promise of the anti-idiotype concept. Review article. Frontier Oncol 2 (196): 1-9.

Tizard IR. 2013. Veterinary Immunology. An Introduction, 9th ed. W.B. Saunders, Philadelphia, USA.

Warr GW, Magor KE, Higgins DA. 1995. IgY: clues to the origins of modern antibodies. Immunol Tod 16: 392-398.