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Production of immunoglobulin Y (IgY) antibodies against *Pseudomonas aeruginosa* from egg yolk

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Abstract

In the immunodiagnostic technologies, IgY is an excellent antibody for using in immunological assays involving mammalian sera, due to discriminative properties of IgY compared to mammalian IgG. Hens can be immunized by means of intramuscular vaccination (*Musculus pectoralis*). IgY was achieved after 3 boosts immunizations by *Pseudomonas aeruginosa*. The extraction of total IgY from egg yolk was carried out using the precipitation by polyethylene glycol 6000 and dialysis. Following precipitation with 3.5% PEG, different bands, apparently with high concentrations, eluted, then started to dilute with increased concentration of PEG to 8.5%, then some of these bands totally disappeared when increasing the concentration to 12% PEG. After dialysis, the purity and yield of IgY samples were 5.27 mg/mL and 28.33%. Electrophoretic separation of egg yolk IgY showed other bands that appeared between the heavy and light chains of IgY corresponding to molecular weights between 65 and 27 kDa.

Introduction

Immunoglobulins are glycoproteins called antibodies, which are secreted by plasma cells in response to antigen exposures and are considered a product that majorly effects humoral immunity. The property of antibodies is recognized small specific structures on other molecules which let them an indispensable tool in laboratory in various applications such as research, diagnostic and therapy. Antibodies presently available for these purposes are mostly mammalian monoclonal or polyclonal antibodies. The production of these antibodies requires normally the use of laboratory animals. Nowadays, most classical chosen mammals for polyclonal and monoclonal antibodies are rabbits and mice, respectively. The procedure involves two steps, each of which causes distress to the animals: the immunization itself and repeated bleeding or sacrifying for spleen removal, which is a prerequisite for antibodies preparation. The commercially available immunoglobulins play critical roles in diagnostic assays, therapy, and purification of specific target compounds.[1]

Hens egg yolk immunoglobulins or IgY have been studied intensively due to their importance. Serum IgG of the hen is transferred from the mother hen to the offspring egg yolk to acquire immunity. Antibodies are transferred from hen to chick via the latent stage of the egg, and play an important role in immunological function for the relatively immunoincompetent chick to resist various infectious diseases. Immunoglobulin is called IgY because it is present in the egg yolk and due to the differences in protein nature compared to that of the mammalian immunoglobulins.[1] The greater molecular mass of IgY is due to an increased number of heavy-chain constant domains and carbohydrate chains. In addition, the hinge region of IgY is much less flexible compared to that of mammalian IgG. Recently, it has

also been suggested that IgY is a more hydrophobic molecule than IgG.[2] Therefore, IgY has been applied successfully in scientific, diagnostic, prophylactic, therapeutic purposes, immunochemical reagents, and in food formulation or supplements due to the stability of IgY under food processing conditions.[3]

In the immunodiagnostic technologies, IgY is an excellent antibody for using in immunological assays involving mammalian sera, due to discriminative properties of IgY compared to mammalian IgG, as IgY does not react with the rheumatoid factor and human anti-mouse IgG antibodies do not activate the complement system and do not bind to Fc receptor.[4] Also, they have poor cross reactivity to mammalian IgG due to immunological differences. IgY is usually low-cost and can be generated through convenient production processes that make it an attractive antibody for research and diagnosis.[5] The advantages can be concluded as; (1) IgY is produced in egg yolk; so there is no need to bleed animals, (2) considerable amounts of antibodies can be obtained in a fairly low cost, (3) usually rapid production process, (4) IgY can be stored in eggs at 4 °C for at least one year, (5) it is achievable to produce a specific antibody to small amounts of antigen that is poorly immunogenic in mammals.[5] There are several IgY isolation methods available, but mostly based on using polyethylene glycol (such as PEG 6000) for precipitation from the supernatant extracts, which usually yield protein impurities.[6,7]

Pseudomonas aeruginosa is a ubiquitous organism present in many diverse environmental settings, and it can be isolated from various living sources, including plants, animals, and humans. The ability of *P. aeruginosa* to survive on minimal nutritional requirements and to tolerate a variety of physical conditions has allowed this organism to persist in both community and hospital settings.[8]

Specific IgY development and production can be achieved by immunizing laying hens with the target antigen. Immunized hens with a specific antigen such as *P. aeruginosa* can give a specific IgY produced in the egg yolk against the given antigen. The aim of this study was to optimize the production and purification of IgY antibodies from chicken egg yolk to achieve high yield of production for research and commercial uses.

Methodology

Immunization of chicken and egg collection

For the immunization, $1x10^8$ cells/mL of *Pseudomonas aeruginosa* was used. The three inoculations were administered in the intervals of one week, intramuscular in the *Musculus pectoralis* of chicken (*Gallus gallus domesticus*) with a final volume of 1.0 mL, distributed in two points. After an interval of 7 days from the last inoculation, the eggs were collected daily during two weeks and stored at 4 °C until the next step, which was the extraction of the IgY from the immunized egg yolk.

Extraction of total IgY

The extraction of total IgY from egg yolk was carried out using the precipitation by polyethylene glycol 6000 (PEG 6000). The method involves two important steps. The first one is the removal of lipids and the second is the precipitation of total IgY from the supernatant of step one.[6]

The egg shell was carefully cracked and the egg yolk was transferred to a filter paper to remove as much egg white as possible, then the yolk skin was cut with a pipette tip. The yolk was poured into a 50 ml tube and the egg volume was registered. Twice the egg yolk volume of phosphate buffer saline (PBS), containing 10 mM phosphate buffer, 137 mM sodium chloride, 2.7 mM potassium chloride, pH 7.4 (Amresco, USA), was mixed with the yolk, thereafter 3.5% PEG 6000 (Ajax-Finechem, Thermo Fisher Scientific, USA). (in gram, pulverized) of the total volume was added and mixed, for 10 min. The extraction procedure separates the suspension in two phases. One phase consisted of "yolk solids and fatty

substances" and a watery phase containing IgY and other proteins. The tubes were centrifuged (13,000xg) at 4°C for 20 min. The supernatant was poured through a folded filter and transferred to a new tube. 8.5% PEG 6000 in gram (calculated according to the new volume) were added to the tube, mixed and centrifuged (13,000xg) at 4°C for 20 min. The supernatant was discarded. The pellet was carefully dissolved in 1 ml PBS by means of a vortexer. PBS was added to a final volume of 10 ml. The solution was mixed with 12% PEG 6000 (w/v, 1.2 gram) and mixed. The extract was dialysed overnight in 1,600 mL phosphate buffer saline (PBS) and gently stirred by means of a magnetic stirrer. The sample was pipetted from the dialysis bag and stored at -20°C.

The protein content (mg/mL) of the samples was measured photometrically at 280 nm (1:50 diluted with PBS) using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and calculated according to the Lambert-Beer law with an extinction coefficient of 1.33 for IgY.

IgY characterization by SDS-PAGE

After the isolation and purification procedures, IgY was characterized by the SDS PAGE gel, using a Protean II electrophoresis system (Bio-Rad) with the discontinuous buffer system of Laemmli (1970).[9] The IgY was diluted at 1:10 and examined in 15% SDS-PAGE under reduction conditions. The electrophoretic run was performed at 200 volts for 80 min. For visualization, the Coomassie Blue staining solution was used.

Results and Discussion

Hens were immunized by means of intramuscular vaccination (*Musculus pectoralis*) with $1x10^8$cells/mL of *P. aeruginosa*. The three inoculations were administered in the intervals of one week. During the immunization process, the hens remained healthy without abnormality in the development. No deaths occurred and all the animals presented normal behavior of the species. At the inoculation sites, there was neither pain nor discomfort, based on the reaction of the animal to palpation, or edema, and no tissue damage was evident. After an interval of 7 days from the last inoculation, the eggs were collected daily during two weeks and stored at 4 °C. The extraction of total IgY from egg yolk was carried out using the precipitation by polyethylene glycol 6000 and dialysis. The method involves two important steps. The first one is the removal of lipids and the second is the precipitation of total IgY from the supernatant of step one. After dialysis against a buffer (normally PBS) the IgY-extract can be stored at -20°C for more than a year.

Measurements of the purity and yield for each stage of purification from immunized egg yolk were shown (Table 1). After dialysis, the purity and yield of IgY samples were 5.27 mg/mL and 28.33%.

Table 1. Measurements of the purity and yield for each stage of purification from immunized egg yolk

	Measurements	**Total volume**	**Protein concentration**	**% yield**
Egg weight	45.16 g			
Yolk volume	15 mL			
3.5% PEG 6000 precipitate	22.5 mL	22.5 mL	8.27 mg/mL	100
Pellet precipitate	1 mL			
Pellet dissolved in PBS	10 mL			
After dialysis ((A280/1.33)/mL)	mg/mL	10 mL	5.27 mg/mL	28.33

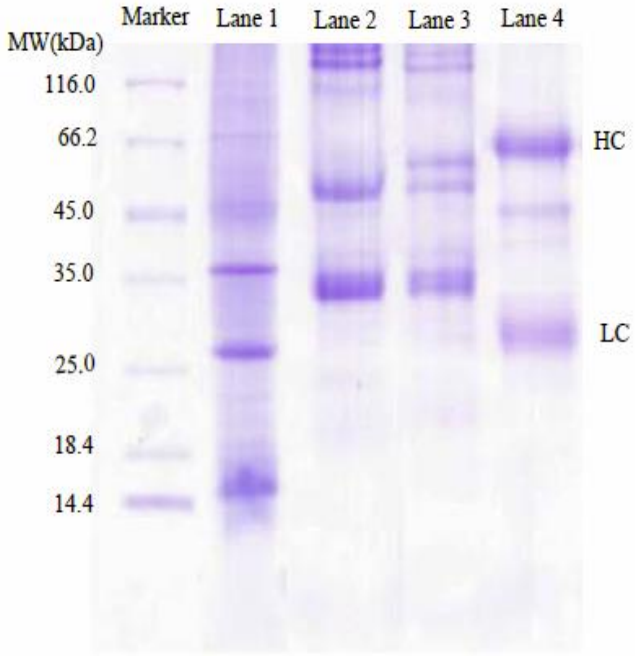

Figure 1. The SDS-PAGE profile of IgY. The two IgY chains appeared on the SDS PAGE by using 15% resolving SDS-PAGE gel. The HC heavy chain with 65 kDa, LC the light chain with 27 kDa. Marker = prestained Protein Ladder, lane 1 = purified IgY after 3.5% PEG 6000 precipitation, lane 2 = purified IgY after 8.5% PEG 6000 precipitation, lane 3 = purified IgY after 12% PEG 6000 precipitation, lane 4 = purified IgY after dialysis, HC - heavy chains (65 kDa), LC - light chains (27kDa)

SDS-PAGE gel electrophoreses was used to determine the molecular weight of the purified chicken IgY antibodies. Electrophoretic separation of egg yolk IgY antibodies in a 15% resolving SDS-PAGE gel was shown (Figure 1). PEG6000 precipitation is a method to precipitate IgY from mixture of proteins to remove lipid substances which are present in high quantities in chicken eggs. This effective method consists of three steps precipitation, starting with 3.5%, followed by 8.5% PEG to remove lipid substances and 12% PEG to precipitate the IgY. Following precipitation with 3.5% PEG, apparently bands with high concentrations, eluted, then started to dilute with increased concentration of PEG to 8.5%, then some of these bands totally disappeared when increasing the concentration to 12% PEG. Other bands that appeared between the heavy and light chains of IgY were minor impurities corresponding to molecular weights between 65 and 27 kDa, and have been removed by dialysis. Dialysis of the extracted IgY against PBS will give pure extracted IgY. The results indicate that the combination between PEG and dialysis methods is quite important to improve purity.

Our results show that it is possible to generate IgY antibodies from chicken eggs with chicken immune system boosted by vaccination with *Pseudomonas aeruginosa*. Compared to antibody production in rabbits, the IgY technology offers several advantages; no blood sampling, only eggs are needed following immunization and low quantities of antigen are required to obtain high and long-lasting IgY titers in the yolk of immunized hen eggs.[5] Therefore, the production of polyclonal antibodies through the chicken immunization makes IgY an excellent alternative, producing the antibodies in large amount and quality from simple methods of production without the need for invasive techniques.

We have successfully purified chicken IgY antibody in egg yolks of hyperimmunized hens, which can be used for development of oral passive immunotherapy as well as a diagnostic reagent for *Pseudomonas* spp. detection and has the potential of expanding to other diseases. PEG 6000 precipitation is a cheap and rapid method to precipitate IgY from mixture of proteins,

and it is followed by a dialysis step due to the problem in the removal of lipids which are present in high quantities in chicken eggs.

Conclusion

The production of polyclonal antibodies through the chicken immunization proved to be an excellent alternative, producing the antibodies in large amount and quality from the simple methods of production without the need for invasive. IgY should be used as an alternative to mammalian antibodies, and it is better to immunize chickens before they begin to produce eggs, since the stress induced by handling them could have an adverse effect on egg production.

The benefits of IgY technology and its universal application in research and medicine is expected to expand at a large-scale. It is expected that IgY will play an increasing role in research, diagnosis, and immunotherapy in the future.

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