Name: Zhiyu Wei
Date: 11/15/2022
Lab Partner: Crina Maria Curca

Utilizing Specificity of Immune Response to Analyze Subunits of IgY

Introduction

There are 5 classes of antibodies present in humans, which are IgG, IgM, IgA, IgE, and IgD. The type of antibodies presents in egg yolk, IgY, is very similar to the human antibody, IgG. It contains 4 subunits in total, which are 2 light chains, and 2 heavy chains.[1] The 4 chains are held together by disulfide bonds.

The experiment had three main qualitative analysis methods, which were sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) Western blot, SDS-PAGE Coomassie Blue gel staining, and enzyme-linked immunoassay (ELISA). Western blot and ELISA were performed to analyze the quality of IgY whereas the SDS-PAGE Coomassie Blue gel was performed to analyze the purity of the purified protein.

To perform both the SDS-PAGE Western blot and Coomassie Blue gel staining, the disulfide bonds between the protein subunits must be reduced. The nitrocellulose membrane used in the Western blot would imprint the proteins present in the sample based on the binding of a specific agent. The positions of the bands on both SDS-PAGE Coomassie Blue stained gel and Western blot would tell us the weight of the targeting protein samples.

ELISA utilized the specificity of the binding of antigen to the antibodies to analyze the concentration of the antibodies present in the sample through the color change and the absorbance value of each well. Three main components were required to result in the color change. First, the capture antibodies need to attach to the wall of the wells. Then the biotinylated antigens need to attach to the capture antibodies. The Wall of each well needs to be blocked using bovine serum albumin (BSA) to prevent any unnecessary immobilization of the protein in the wells. After blocking, biotinylated antigens should only attach to the capture antibodies. streptavidin-alkaline phosphate (S-AP) is then added to attach to the biotinylated antigens to serve as the enzyme for the p-nitrophenyl phosphate (pNPP) to react and be transformed into p-nitrophenolate. The final step is adding the substrate to visualize the color change. The plate should have an intense to light color change from the left to the right of the plate. The absorbance result should also follow the trend of color intensity.

The overall aim of this experiment is to use several qualitative laboratory methods to analyze the quality of the IgY antibodies and use this information to calculate the unknown concentration of the given IgY sample.

Methods

Before performing the subsequent tests, the protein from the egg yolk must be separated to be used.[2] The first day of the experiment started with the precipitating of the protein in the egg

[1] Dias da Silva W, Tambourgi DV. IgY: a promising antibody for use in immunodiagnostic and in immunotherapy. Vet Immunol Immunopathol. 2010 Jun 15;135(3-4):173-80. doi: 10.1016/j.vetimm.2009.12.011. Epub 2010 Jan 7. PMID: 20083313; PMCID: PMC7126787.

[2] Pauly D, Chacana PA, Calzado EG, Brembs B, Schade R. IgY technology: extraction of chicken antibodies from egg yolk by polyethylene glycol (PEG) precipitation. J Vis Exp. 2011 May 1;(51):3084. doi: 10.3791/3084. PMID: 21559009; PMCID: PMC3197133.

yolk. The inorganic salt, sodium sulfate was used for this step. The final precipitated product was then used to perform dialysis to remove any unwanted small protein molecules.

On Day 2, the dialyzed sample was transferred into tubes to be biotinylated. Attaching the biotin to the IgY would allow the binding of S-AP in later steps for ELISA to work. The substrate, p-NPP used on the final day of the experiment would only bind to the S-AP to be transformed into the product that led to a color change.

To check the purity of the purified protein as well as the biotinylated and the non-biotinylated IgY, SDS-PAGE with Coomassie Blue Staining was performed. At the same time, the other half of the gel was used to perform a Western blot to check the presence of the targeting protein as well as the weight of each protein.

To perform the Western blot, the biotinylated IgY was first reduced to remove any disulfide bonds between the subunits of the protein. In this case, the light and heavy chains in the biotinylated IgY were separated and according to size, separated in SDS-PAGE. Since the targeting protein was the biotinylated IgY, the agent used was S-AP so that only the protein that was bound to the S-AP would be seen in the staining of bands.

The rabbit anti-chicken antibody was used in ELISA to bind to the IgY in the sample. The first step of ELISA was coating each well using the ELISA coating buffer with chicken anti-rabbit antibodies. Instead of using antigens to bind to the capture antibodies, IgY antibodies were used in ELISA. After a day, several washes were performed to remove the unbounded protein in the wells and block the wall using bovine serum albumin (BSA). Both samples of biotinylated IgY with known concentration and unknown concentration were then diluted in series and added into the wells correspondingly. The dilution of S-AP was changed from 1:5000 to 1:7500 for better attachment to the biotinylated IgY. P-NPP was added as the substrate to visualize the intensity of color change to determine the concentration of biotinylated IgY in each well.

Results

Using the absorbance taken for the purified IgY sample solution, the yield of IgY could be calculated in each egg yolk based on the absorbance rate at 280 nm through Beer's law.

$$A = \varepsilon c l$$

$$\varepsilon = absorbance$$

$$c = concentration\ (M)$$

$$l = cuvette\ path\ length\ (cm), usually\ 1\ cm$$

$$\frac{A1}{\varepsilon c1 l} = \frac{A2}{\varepsilon c2 l}$$

$$\frac{A1}{c1} = \frac{A2}{c2}$$

The absorbance at 280nm for 10 mg/mL IgY = 13.8, which means A1 = 13.8, c1= 10mg/mL

Sample used in the experiment was diluted with a dilution factor 20.

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The absorbance obtained in the experiment for the purified IgY was 0.795

Volume of egg yolk obtained in the beginning of the experiment was 175 mL

$$\frac{13.8}{10\ mg/mL} = \frac{0.795}{c2/20}$$

c2 = 11.52 mg/mL

Yield of IgY in each egg yolk = 11.52 mg/mL × 175 mL = 2016 mg IgY = 2.016 g IgY

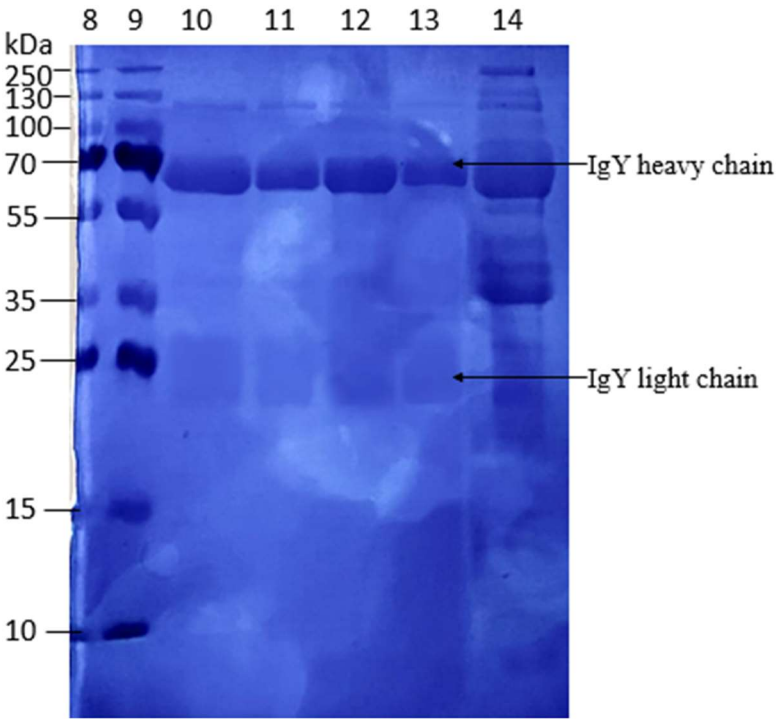

Figure 1 SDS-PAGE patterns of various IgY samples. Lane 8, lane loaded with prestained protein ladder that was cut; Lane 9, prestained protein ladder, size reference in kDa is labeled at the left of the get; Lane 10, non-biotinylated IgY A; Lane 11, non-biotinylated IgY B; Lane 12, Biotinylated IgY A; Lane 13, Biotinylated IgY B; Lane 14, crude IgY.

The expected effect of β-mercaptoethanol (βME) was to reduce the disulfide bond between the subunits of IgY. In this case, the IgY's subunits are 2 heavy and 2 light chains. The disulfide bonds between the chains were reduced to separate the chains apart. On the gel, the separation of the chains was obvious. The heavy chain has the weight of 66 kilodaltons (kDa). From Lane 10 to Lane 13, there is one dark band for each lane that represents the heavy chain of IgY. The heavy chain weighs a bit less than 70 kDa. The light chain, which is the long pale bands represented in Lane 10 to 13, weigh a bit less than 25 kDa. According to the known value, the weights of both light and heavy chains were correct.[3]

[3] Shaik Abdool F, Coetzer THT, Goldring JPD. Isolation of Nile crocodile (Crocodylus niloticus) serum immunoglobulin M and Y (IgM and IgY). J Immunol Methods. 2020 Mar;478:112724. doi: 10.1016/j.jim.2019.112724. Epub 2019 Dec 16. PMID: 31837304.

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The heavy chains stained more intensely than the light chains. This is caused by the difference in the number of domains in the two types of chains. In a heavy chain, there are 4 domains of proteins, whereas in the light chain, there are only 2 domains.[4]

Overall, although Lane 12 and Lane 13 were loaded with the biotinylated IgY samples, Lane 10 to Lane 13 all look very similar in terms of the intensity of the bands and the position of the bands. This indicates that the change in apparent molecular weight of the biotinylated chains was not detectable.

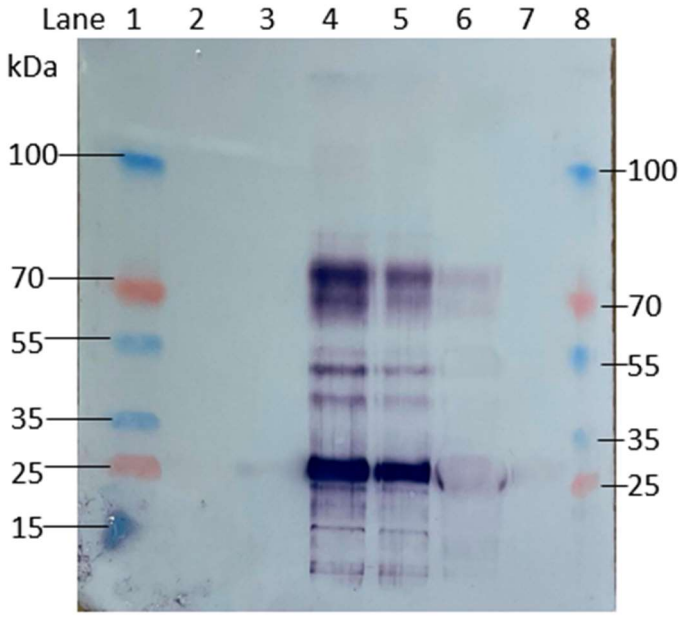

Figure 2 Western blot using S-AP to visualize the biotinylated IgY samples. Lane 1, prestained protein ladder; Lane 2, non-biotinylated IgY (non-visible); Lane 3, non-biotinylated IgY (non-visible); Lane 4, biotinylated IgY; Lane 5, biotinylated IgY; Lane 6, crude IgY sample; Lane 7, no sample loaded; Lane 8, half-cut lane with prestained protein ladder.

The prestained protein ladders were used in the Western blot to refer to the heavy and light chains' bands' positions so the position of the bands that represent the chain attached to the biotins could be identified. Since S-AP binds specifically to the biotins bound to the IgY, the non-biotinylated samples should not show any signals on the Western blot. In the result presented in Figure 2, the Western blot did not detect any signal from the non-biotinylated sample.

After the S-AP is bound to IgY, a darker band should appear for either the light chain or the heavy chain subunits. The Western blot result above shows two darkest bands under Lane 4 and Lane 5 which are around 25 kDa. This indicates that the biotin was bound to the light chain to appear in a darker color.

[4] Chailyan A, Marcatili P, Tramontano A. The association of heavy and light chain variable domains in antibodies: implications for antigen specificity. FEBS J. 2011 Aug;278(16):2858-66. doi: 10.1111/j.1742-4658.2011.08207.x. Epub 2011 Jun 28. PMID: 21651726; PMCID: PMC3562479.

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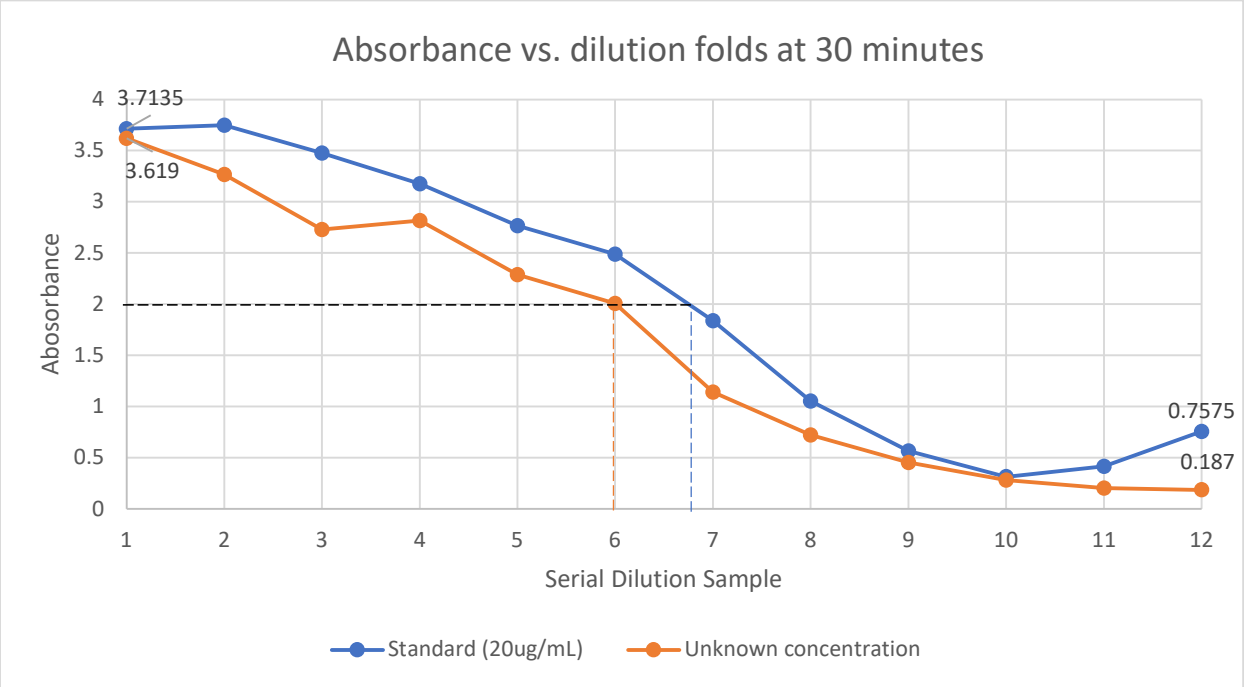

Figure 3 absorbance curve vs. dilution folds taken at 30 minutes after substrate solution was added. The blue curve was used to demonstrate the absorbance rate of the standard sample of IgY sample that has [IgY] = 20 ug/mL. The Unknown concentration curve is drawn in orange. Inflection (50% maX) point of the A405 readings of both the standard and unknown curves are marked by the dashed lines in different colors connecting from the Abs = 2 to the dilution folds number. Data points that represent the maximal signal and the background signal are labeled with the signal value.

The serial dilution was performed by 1:2.5, which means the dilution factor = 2.5.

S1 = 20 ug/mL, n = number in the serial dilution

Equation 1 Calculation of unknown concentration using standard concentration

$$S_{n=}\frac{S_1}{(Dilution\ factor)^{n-1}}$$

$$S_{\frac{1}{2}max} = U_{\frac{1}{2}max}$$

$$S_{6.7} = U_6$$

$$U_{n=}\frac{U_1}{(Dilution\ factor)^{n-1}}$$

$$\frac{20\ ug/mL}{(2.5)^{6.7-1}} = \frac{U_1}{(2.5)^{6-1}}$$

$$U_1 = 10.53\ ug/mL$$

Around dilution number 6 to 8, the curve appeared to be close to linear.

The maximal signal of the highest concentration of biotinylated IgY = 3.714, the background signal = 0.758

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$$ratio = \frac{3.714}{0.758} = 4.90$$

For the unknown, the signal of the highest concentration of biotinylated IgY = 3.619, the background signal = 0.187

$$ratio = \frac{3.619}{0.187} = 19.4$$

Both ratios seemed not low (~1). A low value means that the production of p-nitrophenolate is not high after the reaction of p-nitrophenyl phosphate was not high, indicating a low concentration of biotinylated IgY in the well.

Since from 6 to 8, there seemed to have a linear range of the curve, there is a proportional increase in signal produced by the increase in concentration of biotinylated IgY. The concentration range could be calculated using the serial number in dilution.

$$\frac{S_1}{(Dilution\ factor)^{n-1}} = \frac{200\ ug/mL}{2.5^{6-1}} = 2.048 \text{ ug/mL}$$

$$\frac{200\ ug/mL}{2.5^{8-1}} = 0.328\ ug/mL$$

The concentration range is between 0.328 ug/mL to 2.048 ug/mL

The increase in concentration could give a very big change in the signal. This tells that the method is strong in determining the concentration of biotinylated IgY.

Discussion

In the experiment, the Coomassie Blue staining served to check the purity of each sample of IgY. The crude IgY was presented on Lane 14 of Figure 1. When comparing this lane with other lanes that contain the biotinylated and the non-biotinylated IgY, a big difference can be noticed. The crude IgY lane contains numerous more bands than the purified samples (non-biotinylated and biotinylated IgY). Thus, the isolated IgY does not have a high purity.

The Coomassie Blue staining requires 0.3 to 1 ug per band. Ponceau staining requires at least 0.2 ug per band. Thus, only the crude IgY was stained because of its high concentrations of protein in each band.

The ELISA and Western blot both have very high sensitivity to the biotinylated IgY in different aspects. The ELISA mechanism is especially sensitive to the concentration of the Biotinylated IgY. The Western blot is highly sensitive to the presence of the targeting protein in the samples. The Western blot mainly serves to visualize the difference in the chains after the binding of the biotin to the IgY when compared to the Coomassie Blue Staining gel. ELISA is especially useful when it comes to figuring out the concentration of the unknown sample. In this case, the unknown sample can be calculated based on the sample with the known concentration. Since ELISA requires time for the color to appear intensely, Western blot could be said to have a higher sensitivity. However, both mechanisms serve to analyze different aspects of the samples. A higher sensitivity does not tell which mechanisms is the better option for the experiment.

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On the Western blot, there could be some contamination of the non-biotinylated antibodies with the biotins. In Figure 2, Lane 3 had a very vague color on the position of the light chain band. This could be resulted in the overflowing of the samples when they were loaded into the wells during the setup of SDS-PAGE. Since both lanes loaded with the biotinylated IgY were stained after S-AP was added, there was not any IgY degradation. Some potential reasons for protein degradation could be not keeping the samples on ice and overheating the protein samples when preparing for the SDS-PAGE.

If this experiment were to be repeated, the staining step during the Coomassie Blue staining could be done differently with less Coomassie Blue stain to make the bands more visible. In Figure 1, all the bands except for the heavy chain bands were very difficult to see.

New questions such as how dilutions of S-AP would affect the binding between S-AP and the biotin in this experiment. S-AP with different dilution factors could be used to compare the difference in the resulted binding. Since the experiment used an antibody instead of an antigen to perform ELISA, another experiment using an antigen to perform ELISA can be performed to compare the difference in results when ELISA is done using antibody or antigen.

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