Production of Immunoglobulin Y (IgY) against Synthetic Peptide Analogs of the Immunogenic Epitopes of the Hepatitis B Surface Antigen

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ABSTRACT

Introduction. Several studies have been conducted on the use of Immunoglobulin Y (IgY) technology in the fields of diagnostics and therapeutics. IgY is the avian counterpart of the mammalian immunoglobulin G (IgG) which is exclusively transferred from the hen to the yolk thus conferring passive immunization to the growing embryo. However, despite the advantages it offers over the use of mammalian immunoglobulin, IgY technology has remained underutilized.

Objective. The objective of this study is to produce an IgY with activity against synthetic peptide analogs of known immunogenic epitopes of the Hepatitis B Surface Antigen (HBsAg) – a molecular marker of Hepatitis B infection.

Methods. Chickens were immunized with synthetic peptide analogs of previously reported immunogenic epitopes of the S and the pre-S1 regions of the Hepatitis B surface antigen (HBsAg). IgY specific for the synthetic peptides was isolated by delipidation and salt precipitation and was further purified by affinity chromatography. Purity and molecular weights of the whole IgY molecule and its subunits were assessed and determined by SDS-PAGE. Anti-peptide activity and specificity were determined by indirect ELISA. The study was approved by the Ethical Review Board (ERB) and Technical Review Board of the Research Implementation and Development Office (RIDO), University of the Philippines Manila.

Results and Conclusion. The IgY that was purified in this study had an approximate molecular weight of 165 kilodaltons. The heavy and light chains are 60 and 28 kilodaltons, respectively. The affinity purified IgY demonstrated anti-peptide antibody activity against synthetic peptide analogs of known immunogenic epitopes of the HBsAg. Specific binding against a battery of synthetic peptides also revealed that the affinity purified IgY specifically binds to the known immunogenic epitope of the HBsAg.

Key Words: Immunoglobulin Y, Hepatitis B Surface Antigen, Synthetic Peptide

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Introduction

Immunoglobulin Y (IgY), the avian counterpart of the mammalian IgG, is the chicken antibody that is exclusively transferred from the hens to the egg yolks to confer passive immunity to the embryo.[1] Its use in the different fields, which is referred to as IgY technology, has become popular because of the several advantages it offers compared to using mammalian antibody. Among these advantages are: (1) IgY technology is a cost-effective alternative method of antibody production; (2) IgY isolation is non-invasive and more humane; and (3) IgY is non-reactive to rheumatoid factors (RF) and human anti-mammalian antibody (HAMA), which makes it a more efficient tool for diagnostics compared to other mammalian sources.[2,3]

IgY technology has been used in the field of diagnostics. Similar to IgG, it has been used for protein detection assays like enzyme-linked immunoassays (ELISA), radioimmunoassays (RIA) and immunohistochemical analysis of biologic compounds and toxins.[4] Miyamoto et al. reported the use of IgY in the detection of normal and abnormal prion proteins, which is essential in the diagnosis of bovine spongiform encephalopathy in cattle.[5] Ruan et al. showed that IgY can be utilized to determine cell surface molecules such as major histocompatibility complex (MHC).[6] He et al. also reported the potential use of IgY technology to detect the concentration of serum thymidine kinase as a diagnostic tool for the recurrence of breast cancer.[7] In the diagnosis of emerging infections, Lee et al. used IgY to detect the presence of SARS Co-V.[8] Other studies that utilized IgY for diagnosis include bovine leukemia virus,[9] Indian cobra venom in forensic samples,[10] human thalassemia[11] and H. pylori urease B in vitro.[12]

There are also previous studies on the inhibitory effects of IgY on the activity of infectious agents. In 1999, Carlander et al. produced chicken antibodies against *Pseudomonas aeruginosa*, used it in this first oral antibody trial and reported that no positive cultures were observed on patients who received oral treatment of the immunoglobulin.[13] This result was consistent with the findings of Nilsson et al. on the therapeutic and prophylactic use of IgY against *P. aeruginosa*.[14] Sarker et al. reported that IgY from egg yolks of chickens immunized with human rotaviral strain improved the conditions of children with rotaviral diarrhea.[15] Deignan et al. reported that IgY from unfractionated egg yolk reduced the attachment of *Salmonella typhimurium* in the intestinal epithelial cells of murine models in vitro.[16] Nomura et al. and Shimamoto et al. have investigated and suggested the curative effect of IgY-specific for the immunodominant protein of *Helicobacter pylori* against the disease in animals and humans, respectively.[17,18] Sunwoo et al. and Cook et al. also reported the growth inhibiting activity of IgY specific for E. coli 0157:H7.[19,20] The group of Sunwoo attribute the anti-E. coli activity of the IgY to the binding of the avian immunoglobulin to bacterial surface antigen distorting therefore the cell surface structure of the pathogen.[19] In 2000, Carlander et al. also reported the prophylactic effect of IgY from rotavirus infection.[13] The immunosuppressing effect of IgY against certain toxins such as snake, scorpion venom[21,22] and botulinum toxin[23] has already been reported.

The proven applicability of IgY technology and its advantages over mammalian IgG offer an alternative in the detection and prevention of epidemiologically relevant diseases such as Hepatitis B which have infected approximately 360 million people as reported by the World Health Organization.[24] Countries like the Philippines which has intermediate (2-8%) to high (>8%) endemicity of HBV infection[25] must harness its potential and use IgY technology.

The objective of this study was to produce an IgY against known immunogenic epitopes of the Hepatitis B surface antigen using synthetic peptides analogs. The IgY product of this study can potentially be used in Hepatitis B diagnostics and therapeutics.

Method

Chicken Immunization and Care

Sixteen to eighteen week-old white leghorn hens acquired from the National Poultry Research and Development Center, Bureau of Animal Industry, were grouped into three namely: Negative Control Group, Peptide 1 Immunized Group and Peptide 2 Immunized Group. They were separately immunized with synthetic peptide analogs comprising of the known immunogenic epitopes of the S region 39th-147th, herein referred to as Peptide 1, and pre-S1 region (34th-59th), herein referred to as Peptide 2, of the Hepatitis B Surface antigens (HBsAg) synthesized and purchased at Genscript Corporation in New Jersey, USA. The immunogens were prepared by emulsifying the peptide dissolved in PBS with Freund's Complete Adjuvant (Sigma) during the initial immunization and with Freund's Incomplete Adjuvant (Sigma) during booster immunizations. One hundred micrograms of peptide was injected at four different sites in the pectoral muscle of each chicken. An initial and two booster immunizations were administered at two weeks interval.

Chickens were fed with commercially available chicken laying mash and given purified water *ad libitum*. Eggs were collected daily and stored at 4^{o}C until use.

IgY Isolation

The IgY isolation was done as described by Shin et al. with modification.[26] The egg yolk was separated from the egg white, washed with distilled water and poured into a graduated cylinder for measurement.

The egg yolk was diluted with an equal volume of distilled water followed by the addition of 0.15% (w/v) of λ-carageenan (Rico Carrageenan) until a 1:1 diluted egg yolk-carageenan mixture was achieved. The mixture was incubated at room temperature for 30 minutes and then centrifuged at 3,200 x g at 20^{o}C for 30 minutes. The supernatant was collected and was filtered using Whatman filter paper No. 1. Sodium sulfate crystals were added gradually to the water soluble fraction until a total of 19% (w/v) salt concentration was achieved. The solution was then centrifuged for 30 minutes at room temperature and the precipitate was dissolved in 0.01M Phosphate Buffered Saline pH 7.4 (Sigma) and stored at -20^{o}C prior to use. Total protein concentration of the IgY isolates was determined by Bradford method using microtiter plate protocols.

Purity Assessment and Molecular Weight Determination by SDS-PAGE

Non-denaturing and denaturing Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) by Laemmli were done to assess purity of isolates and determine the molecular weight of purified IgY and its subunits. SDS-PAGE was carried out in the Mini Protean 3Cell (Biorad) using 12% resolving gel and 4% stacking gel for both the non-denaturing and denaturing SDS-PAGE with Kaleidoscope Prestained Molecular Weight Markers (Biorad) and Standard Chicken IgY (Promega) as molecular weight and purity standards. SDS-PAGE of the samples and standards was performed at 180 volts for 45 minutes. Proteins were visualized by staining the gels with Coomassie Brilliant Blue R-250 Staining Solution (Biorad) for 30 minutes, washing it with distilled water, and destaining it with Coomassie R-250 Destaining Solution (Biorad).

Affinity Purification of Peptide Specific IgY

Peptide-specific chicken IgY was purified using the method previously described by Camenisch et al. Two and five tenths (2.5) milligrams of peptide was dissolved in 50ul of distilled water, vortexed, incubated at room temperature for 2 hours, and finally brought to 5 ml with coupling buffer (0.1M $NaHCO_3$ buffer pH 8.3 containing 0.5M NaCl).

Swelling of the Sepharose gel was made according to manufacturer's instructions. One gram of freeze-dried CNBr-activated Sepharose 4B Fast Flow (Sigma) was dissolved in 5 ml of 1mM HCl. The dissolved Sepharose was washed with 200 m1 of 1 mM HCl. Immediately, the swollen Sepharose was combined with the previously prepared peptide solution and incubated with gentle shaking at room temperature for 2 hours. The gel was transferred to Econo-column chromatography column (Biorad) and washed with 15 ml of coupling buffer to remove the unbound ligands. Excess active groups were blocked for 2 hours with 0.1M Tris-HCl buffer pH 8.0. The gel was washed 3 times alternately with 10 ml acetate buffer saline pH 4.0 (0.1M $NaCH_3COO$ containing 0.5M NaCl) and 10ml Tris buffered saline pH 8.0 (0.1M Tris-HCl buffer containing 0.5M NaCl).

Prior to loading into the column, IgY isolates were pooled at 2 weeks interval and dialyzed against the coupling buffer for 1 hour. Dialyzed samples were loaded into the column and fractions were collected at 2ml intervals. The column with IgY isolates was washed with 50 ml 0.1M PBS pH 7.4. Peptide-specific IgY was eluted with 8.5 mL 0.2M glycine pH 2.2 containing 0.15M NaCl at 2ml intervals. To immediately neutralize the IgY fractions, eluents were collected in tubes containing 0.8ml of Tris buffered saline pH 8.0.

After elution, the column was immediately regenerated by washing with 10 ml Tris buffered saline and 10 ml PBS. The column was cleaned by alternately washing with elution buffer and Tris buffered saline three times. The column was stored in PBS containing 0.05% NaN_3 at 4°C.[27]

Antipeptide Activity Determination

Antipeptide activity of purified IgY was done by Indirect ELISA. The wells of a 96-well microtiter plate (Corning) were coated with 50 ul of serially diluted synthetic peptide solutions overnight at 4°C. The wells were then washed with 0.01M PBS containing 0.05% Tween 20, blocked with 100 ul 1% ovalbumin, and incubated for 2 hours at 37°C. Excess blocking solution was removed by washing, and 50 ul of purified IgY was introduced to each well. After incubation for 1 hour at 37°C and washing of the unbound IgY, 50 ul of HRP-conjugated anti-chicken IgY was added to each well. The wells were washed and 100 ul of TMB substrate solution (1 mg TMB dissolved in 1ml DMSO and diluted with 9 ml 0. 01M phosphate citrate buffer pH 5.0 and added with 2ul of 30% H_2O_2) was added to the wells and incubated for 30 minutes at room temperature. The reaction was stopped with 50ul 2M H_2SO_4 and the plate was read at 450 nm in a Model 550 Microplate Reader (Biorad).

Peptide Specificity Assay

Peptide specificity of the affinity-purified IgY was tested by Indirect ELISA as described above. Six peptides with sequence shown in Table 1 synthesized by and purchased at Genscript Corporation (New Jersey) were used for peptide specificity test.

Statistical Treatment and Analysis

The total protein determination of samples was done in duplicates. The mean absorbance reading was computed and used for analysis. A standard curve using gamma globulin protein standard set (Biorad) ranging from 0.125 mg/ml – 2.0 mg/ml was generated every time total protein concentration experiment was done. The total protein concentration of the samples was determined by linear regression analysis.

The total protein from the crude IgY isolates of eggs collected weekly was determined. Trends in the protein concentration per week from each group were observed. Differences in the mean protein concentration of the three groups were determined by Analysis of Variance (ANOVA).

For determination of peptide-antipeptide activity and peptide specificity assay, the mean absorbance readings of the samples, done at least in duplicate, were computed. Cut-off values were determined using the formula described by Frey et al. These were used to discriminate negative and positive antibody activity. An absorbance reading greater than the cut off value was interpreted as positive activity while an absorbance reading less than the cut off value was interpreted as negative antibody activity. The cut off values

Table 1. Amino Acid Sequence of Peptides Used for Peptide-Antipeptide Specificity Assay

Peptide	Amino Acid Sequence	N-terminal Modification	C-terminal Modification
Peptide 1	CTKPTDGNC	None	Amidated
Peptide 2	FGANSNNPDWDFNIKDHWPAANQVG	None	Amidated
Peptide 3	ENHSPVNIAHKL	None	None
Peptide 4	CGTIADKDGC	Acetylated	Amidated
Peptide 5	CGDTKEYGC	Acetylated	Amidated
Peptide 6	CGDDTGKIGC	Acetylated	Amidated

were computed from each assay using the method described by Frey et al.[28] The formula used is

$$\textbf{Cut off value} = \textbf{X} + \textbf{SD} * f$$

where X = mean absorbance of the blank,
SD = standard deviation of the absorbance of the blank, and
f = standard deviation multiplier which is dependent on confidence interval and number of replicates

The confidence interval used to compute the cut off value is 99.0%

Results and Discussion

IgY Isolation

Up to 85 milligrams of IgY was isolated in a single egg yolk using the modified Shin et al IgY isolation technique. The mean concentrations from the Negative Control Group, Peptide 1 Immunized Group and Peptide 2 Immunized Group are 4.61 ± 0.65, 4.93 ± 1.10 and 4.87 ± 0.96 mg/ml egg yolk, respectively. Comparison of the mean isolated IgY concentrations from the three groups before immunization and up to ten weeks after the first immunization revealed that there is no statistically significant difference among the three groups.

Akita and Nakai's comparison of four IgY isolation techniques namely PEG precipitation by Polson, dextran sulfate method by Jensenius, Xanthan method by Hatta and the Water Dilution method by Akita and Nakai revealed that the water dilution technique was the most efficient in terms of yield with up to 100 mg/ml of IgY per egg yolk.[29] This was recognized by Shin et al., however, a modification was done which includes the use of λ-carrageenan to efficiently remove lipids in the egg yolk which according to Powrie and Nakai are the most abundant component of the egg yolk.[30]

Antipeptide Activity Assay of IgY Isolates

To determine antipeptide activity of isolated IgY from the three groups, absorbance readings of the IgY isolates were taken and compared against the computed cut off values for each group. Table 2 presents the absorbance readings of the anti-Peptide 1 and anti-Peptide 2 activity assay of IgY isolated from the Negative Control Group, Peptide 1 Immunized Group and Peptide 2 Immunized Group. The cut off values for Anti-Peptide 1 activity assay of IgY isolated from Peptide 1 Immunized Group and Negative Control Group are 0.220 and 0.198, respectively. For Anti-Peptide 2 activity assay of IgY isolated from Peptide 2 Immunized Group and Negative Control Group, the computed cut off values are 0.050 and 0.025, respectively.

Absorbance readings higher than the computed cut off values were only observed in Anti-Peptide 2 activity assay of IgY isolated from the Peptide 2 Immunized Group. This means that IgY isolated from the Peptide 2 Immunized Group has an anti-Peptide activity against Peptide 2. However, this was not observed with anti-Peptide 1 activity assay of IgY isolated from Peptide 1 Immunized Group, as well as with the anti-Peptide 1 and anti-Peptide 2 activity assay of IgY isolated from the Negative Control Group.

A concentration-dependent increase in the absorbance readings was also noted in the anti-Peptide 2 activity assay of IgY isolated from chickens immunized with Peptide 2. This was not observed with the IgY isolated from chickens from Negative Control Group (Figure 1). The same was not detected in the anti-Peptide 1 assay of IgY isolated from Peptide 1 Immunized Group as well as the IgY isolated from the Negative Control Group when anti-Peptide 1 activity assay was done.

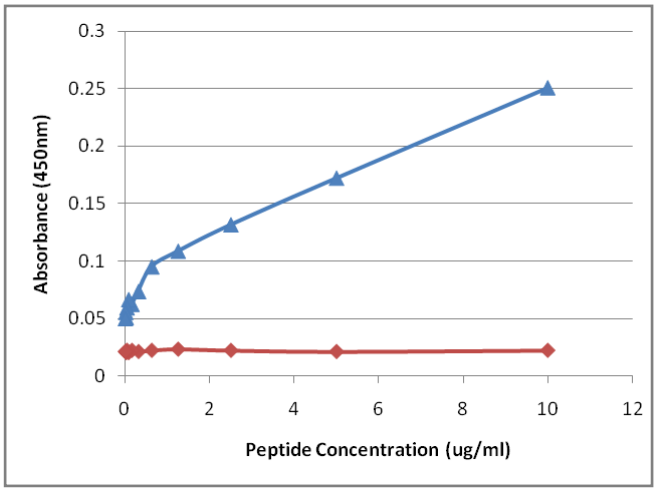

Figure 1. Preliminary Anti-peptide Activity Assay of IgY Isolates from Group P2 and Negative Control Group against Peptide 2. ▲ – IgY from Egg Yolks from Peptide 2 Immunized Chickens, ◆ - IgY from Egg Yolks of Negative Control Group. Peptide 2 concentration-dependent increase in the absorbance was observed with IgY isolated from egg yolks of chickens immunized with Peptide 2 – an indication that the absorbance reading is due to Peptide 2-Antipeptide 2 IgY binding.

Peptides have already been used to elicit immune response. Use of peptides as immunogen is well described by the role of Cytotoxic T Lymphocytes (CTL) in presenting the peptide antigen through the Class I MHC proteins. However, questions of efficiency of using peptide antigens, especially synthetic peptide antigens, have been reported and needs experimental confirmation because of several factors that must be considered in the immune response eliciting activity of synthetic peptides, as well as their clinical applicability. Coligan et al. have discussed several parameters that must be considered in using synthetic peptides for the production of antibodies which have

Table 2. Summary of Results for Anti-Peptide 1 and Anti-Peptide 2 Activity of IgY Isolated from Egg Yolks of Chickens from Negative Control Group, Peptide 1 Immunized Group and Peptide 2 Immunized Group

Anti-peptide Assay		Peptide Concentration (ug/ml)											Cut-off Values
		10.00	5.00	2.50	1.25	0.63	0.31	0.16	0.08	0.04	0.02	0.01	
Anti-Peptide 1 Activity of IgY Isolated from Group P1	Mean Absorbance	0.197	0.188	0.195	0.196	0.198	0.199	0.200	0.202	0.208	0.202	0.218	0.220
	Interpretation	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	
Anti-Peptide 1 Activity of IgY Isolated from Negative Control Group	Mean Absorbance	0.169	0.165	0.171	0.170	0.171	0.174	0.178	0.180	0.178	0.184	0.184	0.198
	Interpretation	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	
Anti-Peptide 2 Activity of IgY Isolated from Group P2	Mean Absorbance	0.251	0.172	0.132	0.109	0.095	0.074	0.063	0.067	0.06	0.051	0.056	0.050
	Interpretation	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	
Anti-Peptide 2 Activity of IgY Isolated from Negative Control Group	Mean Absorbance	0.022	0.021	0.022	0.023	0.022	0.021	0.022	0.02	0.022	0.020	0.021	0.025
	Interpretation	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	

reactivity against the native protein. Among them are (1) the correct choice of peptide sequence to be mimicked by the synthetic peptide; (2) length of the synthetic peptide that may confer immunogenicity to the model animal; and (3) conformational limitations of synthetic peptide.[31]

The peptides that were used in this study have already been identified to be immunogenic in mammalian models. Of the two peptides used, Peptide 2 is significantly longer, with 24 amino acid residues, compared to Peptide 1 with only 9 amino acid residues. This may explain why, in this study, the IgY isolated from Peptide 2 Immunized Group had an antipeptide activity against Peptide 2, while no anti-Peptide 1 activity was observed from IgY isolated from Peptide 1 Immunized Group. In practice, the usual number of amino acid residues used as synthetic peptide immunogen is 10 to 15. However, Coligan et al., also noted that synthetic peptides ranging from a minimum of 6 amino acid residues up to 35 amino acid residues have been successful in eliciting immune response and have produced anti-peptide antibody.[31] The limitation on the number of amino acid in a synthetic peptide to elicit detectable immune response might not have been overcome by Peptide 1. Even conjugating it to a carrier protein, which is the most common way to resolve the problem of having a shorter peptide as immunogen, has not been successful in making the peptide immunogenic in this study.

Amino acid sequence of Peptide 1 and its position in the tertiary structure may have contributed to non-immunogenic nature of the synthetic peptide analog of Peptide 1. The two cysteine residues in the N-terminus and C-terminus of the synthetic peptide may have a role in its immunogenicity. Vyas et al. reported that reduction of the disulfide bonds at this position largely destroys the activity of HBsAg.[32] Further, Prince et al. inferred that the cysteine residues are vital for the formation of the loop which is critical to the antigenic activity of HBsAg.[33]

Aside from this, there is a possibility that the amino group of lysine found in Peptide 1, might have also been conjugated to glutaraldehyde during peptide-BSA conjugation thus affecting its immunogenicity.

Inter-species variation might also explain the absence of an immune response of Peptide 1 immunized chickens. Chickens, the animal which was used in this experiment, might have a different immune response to the peptide immunogen. This claim can be supported by studies concerning variation in the immune response of genetically different organisms. Though no previous studies have been cited comparing immune response of chickens and mammals, Gengozian et al. have already reported variation in the immune responses of two marmoset species both *in vitro* and *in vivo* as early as 1978.[34] Gengozian et al. observed that the marmoset species *Saguinus fuscicollis* had a significant immune response to *Escherichia coli* lipopolysaccharide and *Salmonella typhi* flagella compared to *Saguinus oedipus*.[34] Immunogenetics and immunogenomics may also explain the heterogeneity of the immune responses of organisms belonging to different species.[35]

Affinity Purification of IgY with Anti-peptide Activity

Affinity purification was done to capture the peptide specific IgY from isolates of peptide 2 immunized chickens. Total IgY content from the eluates pooled at two weeks interval were determined by Bradford Total Protein Analysis Microplate Technique. Figure 2 shows the concentrations of the putative Peptide 2-specific IgY. As expected, no putative Peptide 2-specific IgY was observed in the IgY isolated prior

to peptide immunization. A gradual increase in the total IgY concentration of the pooled samples was observed from Weeks 1 and 2 up to Weeks 9 and 10. The IgY concentration peaked at Weeks 5 and 6 at 0.409 mg/ml. However, a decrease in the total IgY concentration was observed from Weeks 7 and 8 and Weeks 9 and 10.

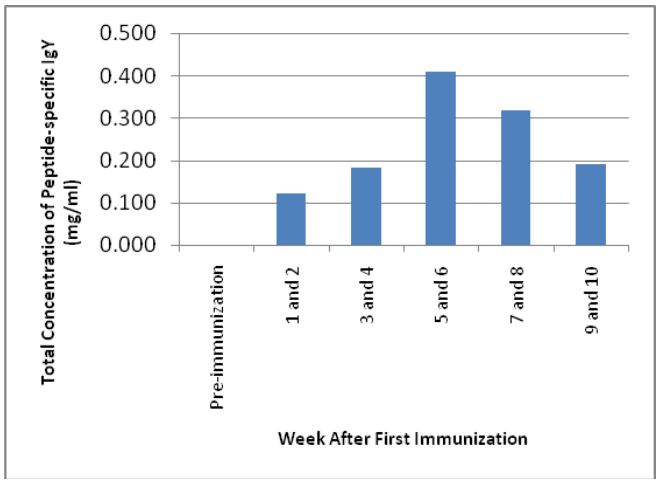

Figure 2. Total Protein Concentration of Putative Peptide 2-specific IgY from Pooled IgY Isolates from Pre-immunization up to Week 10 after the First Immunization Dose.

Consistent with previous studies done by Chui et al. and Rahimi et al., IgY with activity against the target antigen was produced and was detected starting on the first two weeks after the first immunization. Chui et al. noted the presence of IgY acting on formalin-inactivated and heat-inactivated *Mycobacterium avium* starting Week 2.[36] Rahimi et al. also reported that IgY with specific activity on *Salmonella* species was observed starting on the first two weeks.[37] Rahimi further reported that titer increased in both the chicken sera and egg yolk 1 week after administration of *Salmonella enteriditis* whole cell antigens.[37]

In this study, the peak IgY concentration or optimum density was observed after the second booster at weeks 5 and 6. This is inconsistent with the results of Lee et al., Chui et al. and Rahimi et al., wherein optimum IgY densities were obtained at weeks 7, 8 and 9, respectively. This might be attributed to differences in immunization schedules, laboratory conditions and the composition of feeds given to the chickens.

SDS-PAGE of Affinity Purified IgY

To assess purity and confirm the presence of the IgY molecule in affinity purification products, SDS-PAGE was done to the protein-containing eluates collected after addition of elution buffer. Denaturing SDS-PAGE revealed the presence of two distinct bands located at approximately 60 and 28 kilodaltons from Weeks 1 and 2 up to Weeks 9 and 10 (Figure 3). These bands, which have consistent electrophoretic mobility with the bands observed in IgY standard, can be inferred as the heavy and light chains of the IgY. The molecular weights of the whole IgY, as well as the molecular weights of its heavy chains and light chains, are consistent with the approximate molecular weights reported by Leslie and Clem, Akita and Nakai and Schade et al.[4,29,38]

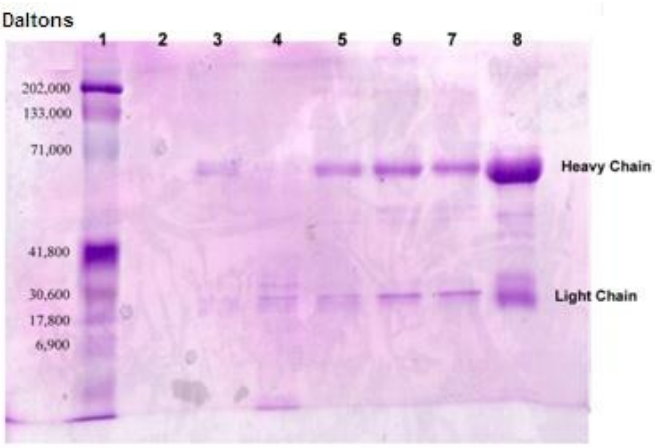

Figure 3. Lane 1: Kaleidoscope Molecular Weight Marker, Broad Range; Lane 2: Pre-immunization; Lane 3: Weeks 1 and 2; Lane 4: Weeks 3 and 4; Lane 5: Weeks 5 and 6; Lane 6: Weeks 7 and 8; Lane 7: Weeks 9 and 10; and Lane 8: Standard IgY.

Determination of Peptide Specificity of Affinity Purified IgY

Among the peptides tested, only Peptide 2 gave an absorbance reading that is higher than the cut off value which is 0.185 (Table 3). This means that the IgY that was purified through affinity chromatography is specific for Peptide 2. This result is expected since the affinity chromatography procedure was done to purify the Peptide 2-specific IgY.

Table 3. Summary of Peptide-Antipeptide Binding Specificity Assay of Affinity Purified IgY from Egg Yolks of Chickens Immunized with Peptide 2

	Peptide						Cut-off Value
	Peptide 1	**Peptide 2**	**Peptide 3**	**Peptide 4**	**Peptide 5**	**Peptide 6**	
Mean Absorbance Reading	0.181	0.216	0.174	0.170	0.170	0.168	0.185
Anti-peptide binding	(-)	(+)	(-)	(-)	(-)	(-)	

Frey et al defined cut off as the upper prediction limit derived from the negative controls.[28] This cut off value is the absorbance due to background, which is defined as the reading obtained from the detection system alone exclusive of any test sera, or in this case, the peptides coated in the wells. Absorbance readings higher than the cut off value mean positive peptide-antipeptide binding activity.

The major contributing factor in the specificity of the affinity purified IgY is the amino acid sequence of the peptides. Peptide 2, which was used to immunize the chickens, is intended to generate an antibody which reacts with it. This is also the reason why the affinity-purified IgY, which was also purified using Peptide 2 as the capture molecule in affinity chromatography, was specific to Peptide 2.

Summary and Conclusion

Consistent with the objectives of the study, Immunoglobulin Y (IgY) with activity against synthetic peptide analogs of the Hepatitis B Surface Antigen (HBsAg) was isolated and purified from egg yolks of chickens immunized with peptide antigens. Based on the indirect ELISA, only the IgY isolated from Group P2 chickens, immunized with synthetic peptide analog corresponding to the 34^{th} – 59^{th} amino acid residue of the pre-S1 region of the HBsAg, exhibited antipeptide activity. The IgY isolated from chickens immunized with Peptide 1, the synthetic peptide analog of the 139^{th} – 147^{th} amino acid residue of the S region of HBsAg, did not exhibit antipeptide antibody activity.

The putative Peptide 2 specific IgY was obtained from affinity chromatography of IgY isolates from Peptide 2 Immunized Group. The affinity-purified IgY, which demonstrated peak concentration during the 5^{th} and 6^{th} week after immunization, showed specificity to Peptide 2 when tested against a battery of synthetic peptides. SDS-PAGE of the affinity-purified products showed two bands with the molecular weights of approximately 60 kilodaltons and 28 kilodaltons. The electrophoretic profiles of the affinity-purified samples were consistent with that of the IgY standard. Moreover, the molecular weights of the two bands as shown in the SDS-PAGE were consistent with the reported molecular weights of the heavy and light chains of the IgY molecule.

In conclusion, IgY with activity and specificity against synthetic peptide analog of the immunogenic epitope of the pre-S1 region was produced using a synthetic polypeptide whose sequence is similar to the 34^{th} – 59^{th} residue of the pre-S1 region. However, further investigations must be done to improve efficiency of the immune induction and IgY isolation technique to harness optimum amount of specific IgY in chicken egg yolks.

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