Original Article

Development of hen antihepatitis B antigen IgY-based conjugate for ELISA assay

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Abstract

Background: Chicken antibodies have many advantages to the mammalian antibodies and have several important differences against mammalian IgG with regard to their specificity and large-scale production. In this study, the production, purification, and HRP conjugation of polyclonal IgY against hepatitis virus surface antigen (HBsAg) were carried out.

Materials and Methods: Single Comb White Leghorn hens were immunized intramuscularly with hepatitis B vaccine in combination with Freund's adjuvants. Blood and eggs were collected before and during ten weeks after the first immunization.

Results: A highly purified of 180 KDa with specific activity of 200 mIU/ml was obtained by our purification protocol. One milligram of the purified IgY was labeled with horseradish peroxidase (HRP). Sandwich ELISA was used to determine the optimum titer of anti-HbsAg IgY-conjugate which was found to be 1:20.

Conclusions: This study showed that laying hens can be used as an alternative source for production of polyclonal antibodies against HBsAg and anti-HBs IgY could be labeled with HRP enzyme and could subsequently be used successfully as secondary antibody in ELISA for detection of HBsAg in the patients sera.

Key Words: ELISA, hens, hepatitis B, IgY, immunization

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INTRODUCTION

In immunodiagnostic, IgY is suitable to be used in detection systems and immunological assays involving mammalian sera with avoiding interference. This is due to the fact that as compared to mammalian IgG, IgY does bind to Fc receptors, does not activate complement system, nor does react with rheumatoid factors and human antimouse IgG antibodies,[1] and it has shown poor cross reactivity to mammalian IgG.[2] Several types of specific antibodies have been developed in mammals for using in detection of HBsAg in humans.[3] However, producing a large amount of specific- antibodies from the mammals is time consuming, lobar intensive, and requires for immunization of the animals.

Handling live and large amount of HBV may pose a potential risk of infection to laboratory personnel.

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Recent advances in molecular biology together with a newly invented method for producing antigen-specific antibodies in egg yolk (IgY) have created new opportunities to develop a safe, convenient, and inexpensive way for manufacturing various immunodiagnostic.[4] The method of producing specific IgY has some advantages over the production of antibodies from mammals in that the antibodies are produced in a conveniently packaged form and can be collected daily without invasive procedures such as bleeding. The antibodies can be stored in the eggs at 4°C for at least 1 year and for this reason large amount of antibody can be obtained at a relatively low cost. It is feasible to produce specific antibody with small amount of antigen that is poorly immunogenic in mammals.[5]

Several procedures for the isolation and purification of IgY from egg yolk have been described previously.[6,7] As the yolk consists of almost 50% nonaqueous material, the first step involves isolation of IgY in a water-soluble fraction (WSF). Generally the next step involves precipitation of IgY, and the final step involves in chromatographic procedures.[8] However, there are limited reports for using IgY in human immunodiagnostic assays, this study was conducted to develop anti-HBsAg IgY conjugated with HRP and use this conjugate as secondary antibody for detection of HBsAg in sera of patients with HBV infection.

MATERIALS AND METHODS

Immunization of hens

Twenty, 38-weeks-old, Single Comb White Leghorn hens were immunized intramuscularly with the immunizing solutions at two different sites (0.75 ml/site) of breast muscle. Hepatitis B vaccine (Hepavax-Gene, Berna biotech Korea Corp, Gyeonggi-do, Korea) was emulsified with an equal volume of a Freud's complete adjuvant (Biogen, CF112, Mashhad, Iran) for the first immunization, Freud's incomplete adjuvant (Biogen, CF112, Mashhad, Iran) for the first booster immunization at the second week, and administered without adjuvant for the second booster immunization at the fourth week after the initial immunization, as described by Mahdavi *et al*.[9] with minor modification.

Collection of specimens

Specimens (including hens' blood and eggs) were collected weekly from all the hens in both control (unchallenged hens) and experimental groups for 10 weeks starting from the day of initial immunization. Blood samples were collected from the wing vein of each hen. The blood specimens were left at room temperature (20-22°C) for a short time in order to clot formation. Thirty eggs were collected from each groups, marked according to their groups, and stored at 4°C to be processed maximally within 7 days.

Isolation of water-soluble fraction from egg yolk

For extraction of IgY, the WSF containing IgY was prepared from egg yolk using water dilution method as described by Akita and Nakai[10] with minor modification. Total protein concentration was determined in all WSF by the Bradford method[11] in which was using bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) as the reference protein. Additionally, total lipid concentration was determined in all WSF by sulfo-phospho-vanillin reaction as described by Frings *et al*.[12]

Measurement of specific anti-HBsAg IgY in Sera and WSF with ELISA

Specific anti-HBs Ag IgY in both laying hens sera and WSFs of experimental and control groups were quantitatively measured weekly by ELISA, kit (Delware biotech, USA). The principle of the assay depends on sandwich ELISA and the trail of procedure were carried out according to the manufactured instruction.

Purification of IgY

The WSF with high titer of anti-HBsAg IgY were selected for IgY purification. They were re-centrifuged at 12,000 × g for 20 min at 4°C to pellet the trace amount of lipid and lipoproteins. The supernatant was collected and filtered through Whatmann paper no.1 and ultrafiltration was carried out using Millipore (Amincon ultra-4 centrifugal filter device) with the molecular weight cut off 100 KDa, in order to have further purification.

Sephacryl S-200 column was used in ÄKTA FPLC system with flow rate of 0.2 ml/min to equilibrate the column. The pressure in both pumps of the system for column in use was (0.45 MPa). The sample loading loop was washed with 1× PBS (0.15 M, pH 7.4). SDS-PAGE (under nonreducing condition) 7% polyacrylamide gel, was carried out on the fractions obtained from ultrafiltration and gel filtration-FPLC to monitor the efficiency of each method in purifying of IgY, as mentioned by Laemmli.[13]

Identification of pure IgY with western blot

For western blot, IgY was electrophoresed in 7% polyacrylamide gel, then the blotting method was done by transferring IgY band to nitrocellulose membrane as described by Towbin *et al*.[14] Enhanced chemiluminescence (ECL) western blotting detection reagents and analysis system was supplied from Amersham/Sweden.

Conjugation of IgY with HRP enzyme

For conjugation, the periodate method was used according to the findings of Wilson and Nakane.[15] First, 4 mg of HRP was dissolved in 1 ml of distillated water. Then 0.2 ml freshly prepared sodium periodate solution (0.1 M) was added to the enzymatic solution and incubated on shaker for, 20 min at room temperature. The solution was dialyzed against acetate buffer (pH 4.4) at 4°C, overnight. About 8 mg of the purified monoclonal antibody was dissolved in 1 ml sodium carbonate (10 mM, pH 9.5). The pH of the dialyzed enzyme was reached to 9 and immediately the solution containing IgY was added and shacked for 2 h at room temperature. Then, 0.1 ml of the freshly prepared sodium brohydrate was added to the solution and was incubated for 30 min at room temperature. The final solution was precipitated with ammonium sulfate ($(NH_4)_2SO_4$) and then was dialyzed against PBS buffer. The IgY- HRP conjugate was aliquoted in several cryo-tubes and stored at –20°C until further usage. For determination of conjugation, HRP-IgY conjugate was electrophoresed under nonreducing condition and 10% polyacrylamide gel was used.

Optimization of anti-HBs Ag IgY-HRP conjugate

The optimum titer of anti-HBs Ag IgY-HRP conjugate was determined by preparing several dilutions (1:5 to 1:200) from it with 0.15 M PBS (pH 7.4). ELISA kit for detection of HBsAg in human serum and plasma, working in principle of sandwich ELISA, was used for testing these dilutions. Each dilution of conjugate was tested with the control positive and control negative of the commercial kit, instead of commercial kit conjugate. After determination of the optimum dilution, it was tested with sera of patients with HBV infection who were previously checked to be positive.

Statistical analysis

Statistical analyses of measured traits were done using the General Linear Models (GLM) procedure of statistical analyses systems (SAS, 2001). A probability level $P < 0.05$ and $P < 0.01$ were considered statistically significant and highly significant, respectively.

RESULTS

Purification of IgY

After preparing of WSF, the sulfo-phospho-vanillin reaction indicated availability of trace amount of lipid in WSF. Therefore, to improve the ultrafiltration efficiency and to prevent clogging of the filtration membrane, the remaining lipids and lipoproteins in WSF were minimized (delipidation) by re-centrifuging of WSF under the similar conditions were used to isolate the WSF. Then the supernatant was filtrated through Whatmann paper no.1 and after that by 0.20 μm disposal filter. The delipidation of WSF, which obtained after using the Akita and Nakai method,[10] was confirmed by measuring the turbidity at 600 nm. The average turbidity (±SE) of WSF before and after delipidation was 0.584 ± 0.024 and 0.005 ± 0.002, respectively. The low turbidity of WSF was attributed to better delipidation. SDS-PAGE gel showed that there is no substantial difference among WSF, and concentrate fraction in their purity, as shown in Figure 1.

According to the SDS-PAGE analysis, ultrafiltration was found to be inefficient in removing other soluble proteins that present with IgY in WSF. The average concentration (±SE) of total IgY content in WSF was 3.30 ± 0.27 mg/ml. After two times concentrating of WSF by ultrafiltration, the total IgY content increased up to 12.35 ± 0.94 mg/l; however, this improvement reached to 19 ± 0.83 mg/mL after eight times concentration. The concentrated fractions containing IgY were purified by gel filtration-FPLC using Sephacryl-S200 column. This chromatographic process was fully automated by FPLC system (AKTA FPLC, Upsala Sweden) and elution of protein was monitored by its optical absorbance at 280 nm. Then the fractions were collected by the fraction collector in average of 1 ml/fraction. The relationship between the absorbance and the fractions was plotted; there were three peaks consisted of one major and two minor peaks, as shown in Figure 2.

The fractions of each peak were scrutinized by SDS-PAGE (under nonreducing condition) to determine

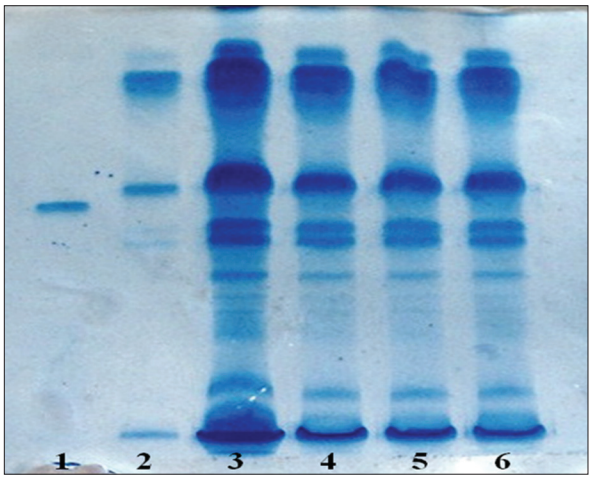

Figure 1: SDS- PAGE (non-reducing) on 7% gel (Mini-protein II cell) of WSF after ultrafiltration. Lanes 1: marker (66K Da), 2: filtrate fraction, 3: concentrate fraction, 4- 6: WSFs

the peak of IgY and also checking the degree of its purity. Our result showed that the first peak with the sharp end belongs to IgY. On the other hand, the fractions of other peaks belong to other water-soluble proteins of egg yolk. The fractions that were suggested as IgY, first fractions, were appeared on SDS-PAGE gel as a single band with 180 KDa molecular weight. The last fractions had bands of other water soluble proteins, as shown in Figure 3. This is because of the fact that IgY has a molecular mass of 180 kDa which is heavier than that of mammalian IgG (150 kDa).[16] Gel filtration separates molecules according to difference in size as they pass through a gel filtration media packed in column. It might be occurred when the last IgY fractions were reached to the end of the column and during their dropping, the other water-soluble proteins were started to drop too. The few number (2 to 4) of IgY fractions, that contain other water soluble proteins bands, and the appearance of both IgY and other water-soluble proteins bands in light form on the SDS-PAGE gel confirm this finding.

For further confirmation, western blot analysis was carried out for the same band, that was previously suggested as IgY. As shown in Figure 4, the present findings indicated that the electrophoretically separated IgY was strongly reacted with antichicken IgY-HRP conjugate, as secondary antibody.

After confirming of IgY purity by SDS-PAGE and Western blot, the purification of IgY was repeated 11 times by gel filtration-FPLC to obtain a sufficient amount of pure IgY. Consequently, the purity of IgY fractions were checked by SDS-PAGE (which was under nonreducing condition) after each run. The result showed that concentrating of WSF by ultrafiltration improved the efficiency of purification with gel filtration. In this regard, when the loaded sample was concentrated two times, the average (±SE) IgY concentration, after gel filtration, was 0.17 ± 0.04 mg/ml. Likewise, the loaded sample was concentrated eight times, the IgY concentration enhanced up to 0.69 ± 0.07 mg/ml.

Anti-HBs Ag IgY-HRP conjugate

The HRP enzyme was used to conjugate the pure IgY against HBs Ag by the periodate method. The final concentration of anti-HBs IgY-HRP conjugate was 1 mg/ml. Sandwich ELISA was used to determine the optimum titer of conjugated IgY against HBsAg. Several dilutions from anti-HBsAg IgY-HRP conjugate (1:2 to 1:500) were tested. The principle of the test was based on using the microwells of the kit that coated with a murine monoclonal antibody (mAb) against HBsAg. The result showed that the optimum dilution of HRP-conjugated IgY was 1:20. This dilution gave an optical density above 1 (positive) with the control positive serum that supplied with the kit.

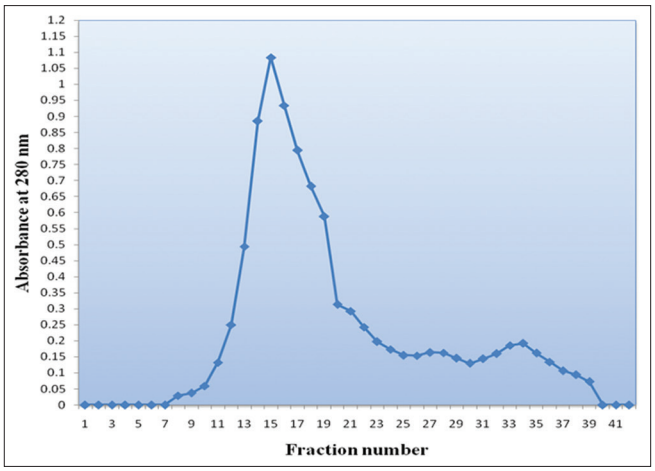

Figure 2: Purification of concentrated WSF by gel filtration- FPLC with HiPerp sephacryl S-200 high resolution column (length 600 mm, i.d. 16 mm). Elusion was done with BPS (0.15 M, pH 7.4) at flow rate of 0.17 ml/min, 1 ml for each fraction

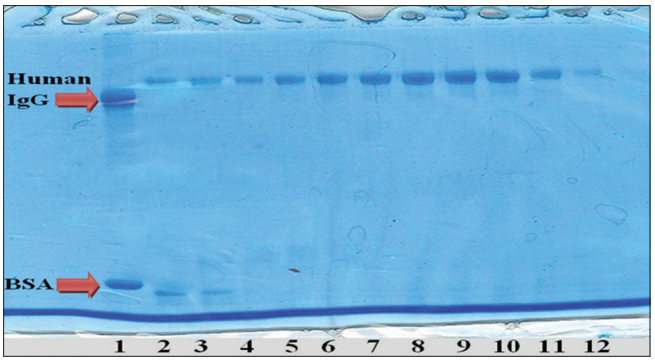

Figure 3: SDS- PAGE (non-reducing) on 7% gel (Mini-protein II cell) of first peak fractions that obtained from purification of concentrated WSF with gel filtration- FPLC. Lanes 1: marker (150 KDa+66K Da), 2-3: partially Pure IgY bands (last fractions), 4-12: Highly pure IgY bands (first fractions)

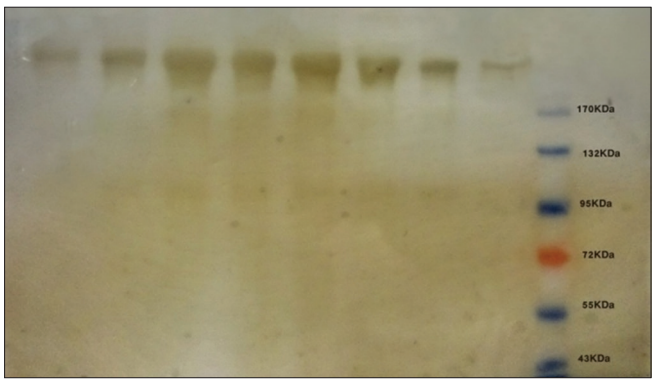

Figure 4: Western blot analysis for the highly pure IgY band. The first well on the right is protein ladder. In another well strong reaction observed between IgY and anti-chicken IgY-HRP conjugate

DISCUSSION

Development of suitable IgY based conjugate for ELISA assay require IgY with high purity. The recent study concluded that the concentrated WSF by ultrafiltration followed by gel filtration-FPLC was suitable and beneficial for obtaining highly pure IgY. Obtaining of IgY with a suitable purity against HBsAg might be due to a combination of several reasons. First, since it allowed elution of IgY in the void volume, the use of sephacryl-S200 as a purified column; thereby, improving the efficiency of purification in terms of speed and resolution.[10] Second, the low flow rate (0.18 ml/min), because decreasing the flow rate usually improve the resolution of gel filtration.[17] Finally, the small particle size of stationary phase that especially designed for the column that working with FPLC system gives the advantage of increasing resolution power of chromatography column.[18] Ko and Ahn,[19] reported that ammonium sulfate precipitation method produce IgY with higher purity than the HPLC, but our data indicated that gel filtration-FPLC could be used for obtaining of IgY with higher purity. This result is consistent with the findings of Akita and Nakai.[10] They recommended that an efficient purification procedure should employ salt precipitation, alcohol precipitation, ultrafiltration, or a combination of these in initial steps; whereas, gel filtration or ion-exchange chromatography should be used as the final steps.

For the following using of IgY in preparation of HRP-conjugate, pure IgY fractions were pooled together. Then, IgY solution was subjected to ultrafiltration by using Millipore with molecular weight of 10 KDa, in order to reduce the excess volume of buffer containing IgY and increase the IgY concentration. After ultrafiltration, the final volume of IgY solution was reached to 4 ml, while its IgY concentration was 1 mg/ml with the specific activity of 200 mIU/ml. As a result, the maintenance of IgY-specific activity in high level indicated that the implemented purification scheme was efficient and it had no adverse effect on the activity of antibody (IgY).

Furthermore, as reported by Majidi *et al.*,[20] the same results were obtained when it was tested with the positive patient sera, which previously had been proven by the same kit. But they had used rabbit antibovine IgG with the final concentration of 8 mg/ml and optimum titer of 1:12,800. The variation in optimum titer between the report of Majidi *et al.*[20] and recent study was attributed to the differences in the main concentration of antibody in the conjugation solution. Whenever the concentration of conjugated antibody is high, it needs to be diluted more until it gives reliable result. The successful preparation of anti-HBs IgY-HRP conjugate depends on the efficiency of conjugation process. It indicated that the enzyme was completely linked to the antibody without deactivation of both antibody and enzyme.[21] The preparation of anti-HBs IgY-HRP conjugate is considered as a step forward to support and develop a detection tool for HBV infection. Thus, it has great advantages over polyclonal antibodies produced in mammals such as goat and rabbit as mentioned by Makvandi and Fiuzi.[22]

CONCLUSION

Anti-HBsAg IgY could be labeled with HRP enzyme and could subsequently be used successfully as secondary antibody in ELISA for detection of HBsAg in the sera of patients with HBV infection.

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