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GENERATION AND PHYSICOCHEMICAL CHARACTERIZATION OF CHICKEN EGG YOLK ANTIBODIES (IgY) AGAINST *Salmonella typhimurium*

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ABSTRACT

Salmonellosis is one of the most important bacterial diseases with economic concern to all phases of the poultry. The study was aimed to generate chicken IgY against *Salmonella typhimurium* and its physicochemical characterization to unbolt the obstacles for the usage of IgY as therapeutic agent in Poultry. *Salmonella typhimurium* whole cell antigen was prepared and immunized in white leghorn chickens. The immune eggs were collected and IgY was purified from the yolk by PEG extraction method, the purity of the IgY extract was estimated by SDS-PAGE. The peak titer of Anti-*S.tyhpimurium*-IgY in egg yolk was observed after 35th day from the first immunization by ELISA. Total IgY concentration was found to be 30.24 ± 0.25 mg/ml after 25th week of bird's age. The specific reactivity of IgY was relatively stable at 60^{o}C and then significantly decreased at 70^{o}C. The pH stability of IgY was observed between pH4.0 and pH 10. IgY was more resistant to Trypsin compared to pepsin.

KEYWORDS: Chicken IgY, physicochemical property, *Salmonella typhimurium*, Poultry

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INTRODUCTION

World poultry industry is growing, as the population is escalating. In India, poultry is one of the fastest growing subsectors of agriculture. In the last few years the poultry industry has faced a number of challenges because of the disease outbreaks. The diseases in poultry are not only killing the birds but also responsible for poor growth, poor hatchability and poor egg production. At early days of age *Salmonellosis* is one the most important bacterial diseases, even the survivor of *Salmonella* infection becomes the carrier for life and become source of infection for other birds. Hence, the *Salmonella* infection is a problem of economic concern to all phases of the poultry industry and also a source of food borne transmission of disease to humans[1, 2, 3]. Therefore, preventive and curative strategies are of great importance to control the *Salmonella* colonization in chickens at farm level. In a common way the diseases are being controlled by administering the antibiotics. Currently, the antibiotic usage is under inquiry because of the increasing percentage of antibiotic-resistance. Emergence of antibiotic resistance and the need to treat disease caused by pathogens that do not respond to antibiotics have put tremendous pressure to look for viable alternatives[4]. In the recent decades passive immunization by Chicken egg yolk antibodies (IgY) has considerable attention for preventing and controlling diseases in livestocks[5] particularly in poultry. It has been reported by many researchers that oral administration of chicken IgY is effective against variety of intestinal pathogens[6]. However, the results of experimental application of these antibodies to poultry have not always been consistent and still there are many obstacles which make the administration of yolk antibodies to commercial poultry a difficult task to achieve[7]. In particular, there have no studies conducted in India on the physicochemical characterization of IgY. Therefore the present study was aimed to generate chicken IgY against *Salmonella typhimurium* and its physicochemical characterization to unbolt the obstacles for the usage of IgY as therapeutic agent in Poultry.

MATERIALS AND METHODS

(i) Bacterial Strain and Positive serum

Salmonella typhimurium standard strain (MTCC No.98) was procured from Microbial Type of Culture Collection & Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India. The procured strain was characterized morphologically and biochemically in the laboratory and it was found that the standard strain was pure. *Salmonella* positive serums generated in rabbit were purchased from the Division of Biological Products, Indian Veterinary Research Institute (IVRI), Izatnagar, Uttrapradesh, India and used as positive control for the interpretation of test results in this study.

(ii) Salmonella typhimurium whole cell antigen[8]

Salmonella typhimurium was inoculated into brain heart infusion (BHI) broth and incubated at 37°C for 24 hours. After growth in BHI broth, cells were harvested by centrifugation at 10,000 rpm for 15 minutes at 4°C. Cells were washed thrice with 0.1 N PBS buffer (pH 7.4). After harvesting, the pellet was re-suspended in a quantity of PBS to achieve $1.5x10^8$cells/ml (McFarland's No.1) and then, the cells were treated with 3.7% formalin over night. The residual formaldehyde was removed by subjecting the suspension thrice to centrifugation in PBS buffer. Finally, the formalin inactivated cells were re-suspended in PBS and the concentration was adjusted to an optical density equivalent to McFarland's standard number 1 tube (≈$1.5x10^8$cells/ml). The cell suspension was aliquoted and stored under refrigeration for further use.

(iii) Generation of Anti-S. tyhpimurium-IgY in white leghorn chickens

White leghorn chickens were immunized with prepared *Salmonella* whole cell antigen ($1x10^9$ cells/kg of body weight) in pectoral muscle. Then the chickens received booster

doses with the same concentration at 14 days interval. The pre-immune and post-immune sera were collected at specified time intervals during and after the various immunization schedules in chicken. They were tested for the presence of agglutinating Anti-*S. typhimurium* antibodies. The eggs were collected and stored at 4°C.

(iv) Purification of Egg Yolk anitbodies[9]

The egg shell was carefully cracked and the yolk was separated from white, washed with distilled water to remove as much albumin as possible. The membrane was punctured with a lancet. Then the yolk was poured into a separate centrifuge tube and volume was measured (V_1). Twice the egg yolk volume of PBS was mixed with the yolk ($\sum V_1+V_2$), thereafter 3.5% PEG 6000 (in gram) of the total volume was added, vortexed and stirred for 30 minutes. Then the suspension was centrifuged at 4°C for 20 minutes at 10,000 rpm. The supernatant (V_3) was poured through a folded filter paper and transferred to a new tube. 8.5% PEG 6000 in gram [Calculated according to the new volume (V_3)] was added, vortexed and stirred for 30 minutes. Then the tube was centrifuged at 4°C for 20 minutes at 10,000 rpm. After centrifugation the supernatant was discarded and the pellet was carefully dissolved in 1ml PBS by means of glass stick and the PBS is added to a final volume of 10ml (V_4). Then the 12% PEG 6000 (w/v 1.2 gram) was added and vortexed. Finally, the solution was centrifuged at 4°C for 20 minutes at 10,000rpm and the final pellet was dissolved in 800µl PBS (V_5) (Pauly *et al.,* 2011). Then, the IgY extract was subjected to dialysis for overnight in 0.1% saline and next day morning the saline is replaced by PBS and further dialysed for another three hours. Thereafter the IgY-extract was pulled from the dialysis capsule by a pipette and transferred to storage vials [final volume was around 2 ml (V_6)] and used for further studies. The purity of IgY-extracts was determined by SDS-PAGE technique and the total protein concentration was estimated by Lowry *et al.,* (1951) method. The total IgY concentration in the IgY-extract was estimated photometrically at 280nm (1:50 diluted with PBS) with the extinction coefficient of 1.33 for IgY[9]. Finally, the specificity of Anti-S. typhimurium-IgY in chicken serum and IgY-extract was qualitatively determined by Rapid slide agglutinations test[10].

(v) Determination of IgY titer by Indirect ELISA[8]

Micro titer wells were coated with 150µl/well *Salmonella* whole cell antigen solution at a concentration of 10µg/ml (Reference value - 500µg is corresponding to $1x10^8$ CFU/ml)[11] using coating buffer (0.05M Carbonate bicarbonate buffer pH 9.6) and incubated at 4°C over night for binding. After coating, unbound antigens in the wells were removed by washing with PBS containing 0.05% tween 20 (PBST) for 3 times. The empty sites were blocked by adding 200µl per well of 1% bovine serum albumin in PBS and the Plate was incubated at 37°C for 1 hour. Plate was subsequently washed with PBST and incubated with 100µl IgY-extract at appropriate dilutions. Control wells had PBST and pre-immune sera served as respective controls. Plate was incubated for one hour at 37°C and subsequently washed with PBST. For the chicken antibodies 100µl of diluted (1:1000) rabbit anti-chicken immunoglobulin coupled to horseradish peroxidase (Genei Pvt. Ltd, Bangalore) was added and the plate was incubated for 1 hour at 37°C. After incubation the plate was washed with PBST and enzyme activity determined by adding 100µl of freshly prepared substrate solution (1ml of TMB added with 19ml of sterile $D.H_20$). The plate was allowed to stand at room temperature in the dark for 20 minutes. The reaction was stopped by adding 50µl of 4N H_2SO_4 and plates was read at 450nm in an ELISA reader.

(vi) Physicochemical properties and stability of IgY[12]

- ***Heat stability***
  IgY solution was incubated at 4ºC, 10ºC, 25ºC, 37ºC, 60ºC, 70ºC, 80ºC and 90ºC for 30 minutes. The heat treated IgY was cooled in a water-ice bath. The remaining antibody activity was measured by ELISA.

Antibody activity was represented as a percentage of the untreated control.

- ***pH stability***
  The pH of IgY was modulated to the desired pH 2, 4, 6, 8, 10 and 12 with NaOH or HCl. Then the solutions were incubated at 37°C for 2 hours. After incubation each IgY solutions was neutralized. The remaining antibody activity was measured by ELISA. Antibody activity was represented as a percentage of the untreated control.

- ***Stability of IgY against Pepsin and Trypsin***
  For the pepsin stability test, the pH of IgY solution was adjusted to 2, 4, 6 respectively. IgY solution of each pH was mixed with pepsin at a rate of 20:1 (m/m). The mixtures were incubated at 37°C for 1, 2, 3, and 4 hours. After the incubation, each IgY solution was neutralized to inactivate the pepsin. For trypsin stability test, the pH of IgY solution was adjusted to pH 7.5 and all other procedure were same like pepsin digestion. Residual IgY activity was estimated by ELISA.

RESULTS AND DISCUSSION

1. Total protein and IgY concentration in IgY-extract

The IgY-extract purified from the immune egg yolk by Polyethylene glycol (PEG) extraction method was subjected for the estimation of total protein and IgY concentration. The concentration of protein was 18.94 ± 0.24 mg/ml in the IgY-extract obtained from the egg yolk at birds age was 19 weeks. Then the total protein concentration was gradually increased and reached to 40.07 ± 0.57 mg/ml after 24th weeks of chicken age after that it was found to be relatively constant during the study period of 20 weeks. As like total protein concentration, the total IgY was also low during the 19th week of chicken age (12.57 ± 0.57mg/ml) then it was increased proportionally to the birds age and attained the concentration of 30.24 ± 0.25mg/ml after 25th week of chicken age (Fig 1&2). The results revealed that the concentration of IgY was dependent on age of the bird. A clear 180 kDa protein band was observed in the SDS-PAGE and some minor impurities protein bands were also observed but it was negotiable; the result was compared with standard IgY (Fig.3).

Total Protein Concentration in the IgY-extract

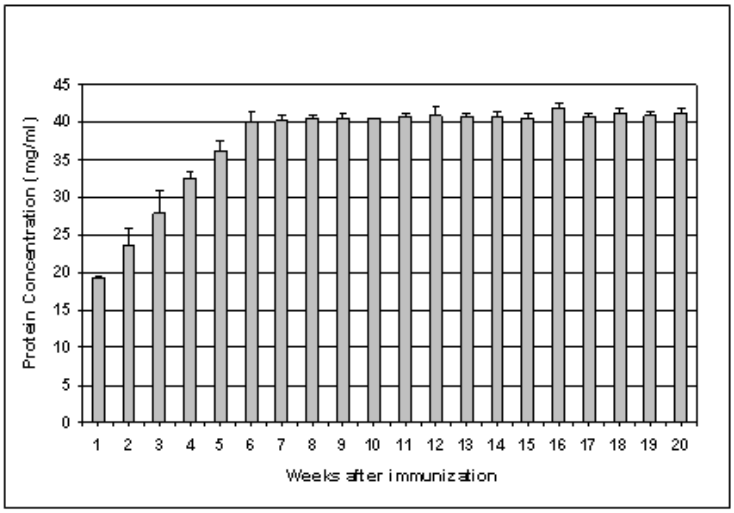

Figure 1
Concentration of total protein in the IgY-extract obtained from chicken eggs mmunized with Salmonella typhimurium. Values are the mean of triplicate samples.Vertical bars indicate the standard deviation.

Total IgY Concentration in the IgY-extract

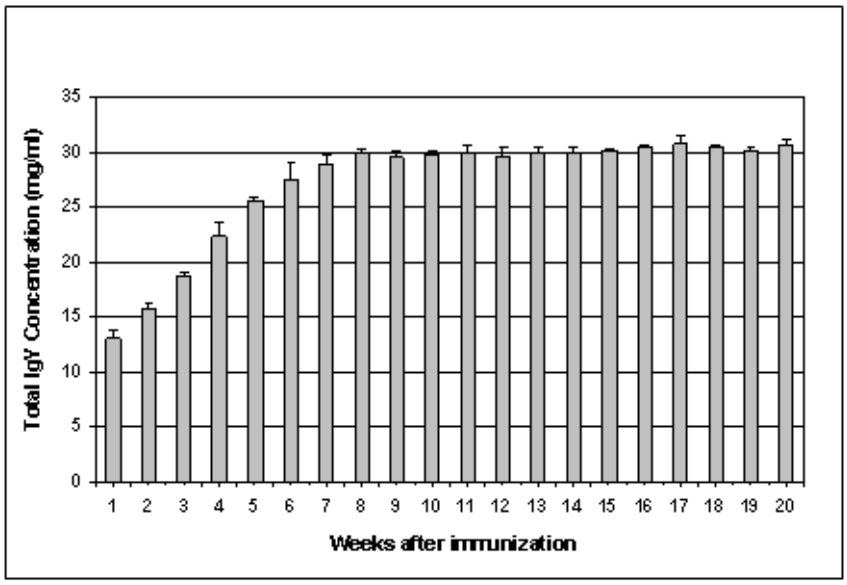

Figure 2
Concentration of total IgY in the IgY-extract obtained from chicken eggs immunized with Salmonella typhimurium. Values are the mean of triplicate samples. Vertical bars indicate the standard deviation

Figure 3
SDS-PAGE profile Lane 1: Standard IgY (Genei, Bangalore); Lane 2: Anti-S.typhimurium-IgY Extract

2. Titer of Anti-S. typhimurium-IgY in Serum and Egg Yolk

Specific reactivity of Anti-*Salmonella*-IgY in serum and IgY-extracts with their corresponding antigens was determined by Rapid slide agglutination test. Agglutination reaction was observed within 2minutes after mixing the serum and antibody solutions with respective antigens separately. The observation was compared with the agglutination reaction of standard anti-serum (IVRI, Izatnagar) with the *S. typhimurium* antigens, which was considered as positive control for interpretation of test results.

Titer of Anti-S. typhimurium-IgY in Serum and Egg Yolk

Figure 4

Level of Anti-S. typhimurium-IgY in serum and egg yolk of chicken immunized with S. typhimurium antigen during the study. Values are the mean of triplicate samples. Vertical bars indicate the standard deviation

The result indicated the presence of Anti-*S. tyhpimurium*-IgY in the serum samples and egg yolks. With this qualitative determination further titration of specific IgY was carried out by indirect ELISA. The level of specific antibodies against the *S. typhimurium* antigens in serum was increased after 1 week and slowly it reached the maximum titer on day 21st from the date of initial immunization. However, the specific antibody level in the egg yolk was very weak on 21st day and gradually increased and reached the peak between 35th and 49th day. The titer of specific antibody was found to be 1:100000 on 35th Day and the titer were maintained with booster doses (Fig 4). Results indicated that there was a delay in the appearance of Anti-*S. tyhpimurium*-IgY in yolk when compared to serum after the first immunization. It was possibly due to the gradual accumulation of IgY during the yolk formation period by selective active transport. Similar finding was reported by Kitaguchi *et al.,* (2008)[13].

3. Physicochemical properties and stability of IgY

In order to evaluate the efficacy of IgY in the prevention and treatment of *Salmonella* infection in chickens, the stability of IgY was investigated at different physicochemical conditions. The results showed that the purified IgY was stable between 4^{o}C and 37^{o}C. Approximately 25% of its activity was lost at 60^{o}C and then significantly decreased at 70^{o}C. It was almost completely lost at 80^{o}C (Fig 5). The purified IgY was stable between pH 4.0 and pH 10.0, it has retained only 20% of its activity at pH 2 and completely lost its activity at pH 12.0 (Fig 6). The activity of purified IgY after pepsin treatment was almost completely lost at pH2.0, but almost 70% and 80% of activity was retained at pH 4.0 and pH 6.0 respectively. In contrast to pepsin treatment, purified IgY showed broad stability to trypsin, approximately 80% of the antibody activity was retained even after 4hours. These results revealed that IgY was relatively stable to high temperature and broad pH range and IgY was more resistant to the effects of trypsin compared to pepsin.

Thermal stability of IgY

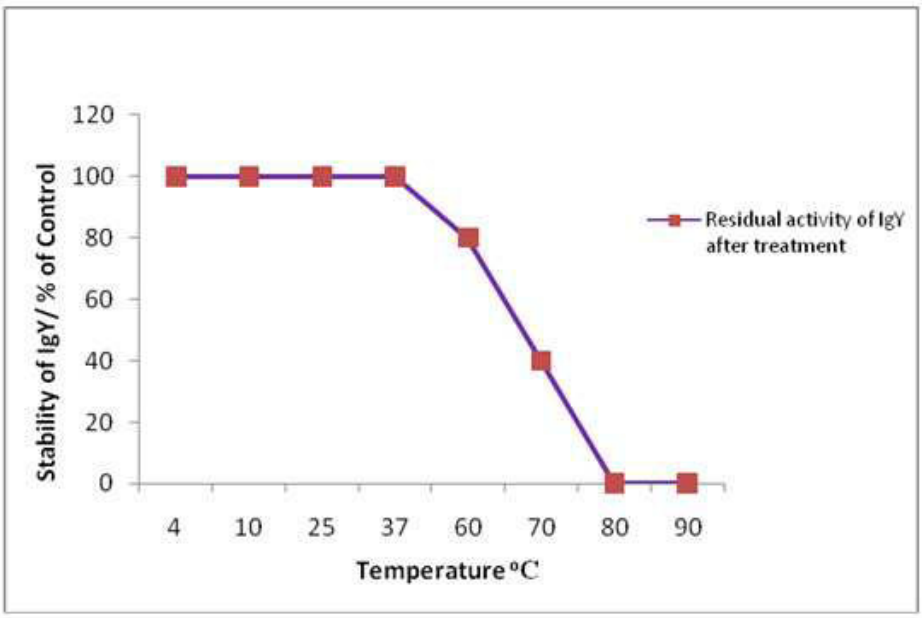

Figure 5
Remaining activities of IgY after treated at various temperature were measured by ELISA and expressed as percentage of untreated control

Stability of IgY at various pH

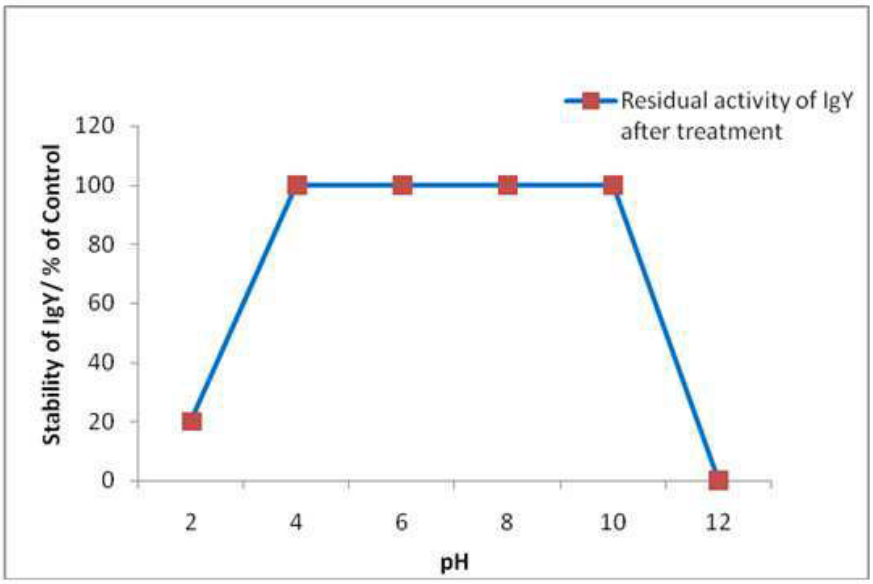

Figure 6
Remaining activities of IgY after treated at various pH were measured by ELISA and expressed as percentage of untreated control

CONCLUSION

The present study indicated that chickens could be used as best choice for antibody generation due its higher productivity and the physicochemical characterization of IgY revealed that once the IgY get ahead of the stomach acidic condition, it could keep hold of its maximum activity and

therefore, can combat or minimize the effect of intestinal pathogens such as *Salmonella* in poultry and other livestock. The study could form a platform for further research on egg yolk antibodies and its commercial application in India since, it might be the first report on physicochemical characterization of chicken IgY from India.

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