Production of Antibodies in Egg Whites of Chickens

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Authors’ contributions

This work was carried out in collaboration among all authors. Author AJV designed the study, performed the statistical analysis, wrote the protocol, perform the laboratory investigations, and wrote the first draft of the manuscript. Author BFC managed the analyses of the study and literature review searchers. Author SV managed the literature searches and revised the first draft of the manuscript. All authors read and approved the final manuscript.

ABSTRACT

Background: IgM, which participates in the primary immune response, is the primary antibody in egg whites. There is scant information about the production of antibodies in egg whites. This study describes the preparation of antibodies against a bacterial antigen, staphylococcal protein-A.

Methods: The detection of antibodies against staphylococcal protein-A in egg white was performed by ELISA, and the antibodies were purified by protein-A affinity chromatography. Agglutination inhibition of Staphylococcus aureus Cowan I strains by purified antibodies against protein-A in vitro was investigated.

Results: ELISA showed the production of antibodies against staphylococcal protein-A in the egg whites of layer hens. The antibodies were separated using affinity chromatography. The agglutination of Staphylococcus aureus Cowan I strains occurred when the purified antibodies were incubated with S. aureus.

Conclusion: The results showed that it is possible to produce antibodies against bacterial antigens in egg whites, which can have industrial applications in the preparation of antibodies for immunotherapy of infectious diseases.
Keywords: Chicken; IgM; egg white; antibody; ELISA; affinity chromatography; agglutination inhibition.

1. INTRODUCTION

Eggs are laid by animals of different species, including birds. Avian eggs consist of an eggshell, egg yolk, and egg white, which constitute the embryo from which a chick develops. From day zero, the avian egg contains a powerful immune system comprising antibodies against each foreign agent to which laying hens have been in contact with. Egg yolk possesses a high titer of immunoglobulin Y (IgY), which is equivalent to immunoglobulin G (IgG) in mammalian species, and egg whites contain immunoglobulin M (IgM) and immunoglobulin A (IgA) [1,2].

Purifying immunoglobulin M from the egg white of avian species is of critical interest as an antibody source for preventing enteric infections. The concentration of immunoglobulin M in egg whites was 0.15 mg/ml [2]. Oral administration of hyper-immune eggs has been used to prevent enteric infections in several animal species, such as dental caries in rodents caused by *streptococci* [3] and *E. coli* infections in mammals [4]. Avian antibodies have been used in immunotherapies for infectious diseases [5].

Yao et al. [6] documented that avidin transports drugs, toxins, or therapeutic genes to intraperitoneal tumours. Our objective was to provide information about the production of egg white antibodies against a bacterial antigen (protein-A), which is a surface protein displayed in *Staphylococcus aureus* Cowan I strains [7,8]. Staphylococcal protein A has a molecular weight of 42 kDa. It has the capacity to react to immunoglobulins from many animal species by interacting with the Fc fragment of antibodies from monkeys, dogs, humans, rabbits, cats, hamsters, and other antibodies from laboratory, domestic, and wild animal species [9,10]. Protein-A consists of five domains, four of which show critical structural homology. These bacterial binding sites are located at the interface CH2-CH3 of guinea pigs and human IgGs [9].

The use of birds to produce antibodies decreases the use of laboratory animals for this purpose. Furthermore, immunized hens produce higher amounts of antibodies than rabbits in the laboratory. The hens are farmyard animals and are, therefore, less expensive than laboratory animals, such as rabbits. Antibodies developed in birds recognize more epitopes in mammalian proteins. It is more advantageous to use chicken immunoglobulins Y in immunoassays, which detect mammalian proteins. This is especially true when the antigen is a highly conserved protein such as a hormone. Immunizing chicken with a protein induces the production of idiotypic antibodies that recognize the original protein [11].

2. MATERIALS AND METHODS

All chemical and biological reagents were commercially available (Sigma-Aldrich Co.).

2.1 Production of Antibodies against Protein-A in Egg White from Birds

Six-month-old healthy layer chickens (brown Leghorn) were injected at multiple sites on the breast with one mg of protein-A in 0.5 ml complete Freund’s adjuvant on day zero, and one mg of the same protein in 0.5 ml incomplete Freund’s adjuvant on day 9. Eggs were collected from laying birds before and ten days after the immunization. Egg whites were manually separated [12] and stored at −20°C.

2.2 ELISA for Detection of Antibodies against Protein-A

The 96-well polystyrene microplates were coated with 500 ng of protein-A (Sigma-Aldrich) in coating buffer for four h at 37°C. The microplates were washed four times with buffer (PBS-Tween-20) and blocked with 3% non-fat dry milk in PBS (25 μl/well) for one h at room temperature. The microplates were washed four times. Samples were then added to 50 μl of a 1:50 dilution of egg white. After an incubation period of one h at RT, the microplates were washed four times, and 50 μl of the peroxidase-labelled protein-A conjugate at a dilution of 1:3000 was added. The microplates were then incubated for one h at RT and washed four times. Tetramethylbenzidine solution (50 μl) was added to the plates. After further incubation for 16 min, the reaction was stopped and analyzed using a microplate reader at a wavelength of 450 nm [13,14].

2.3 Purification of Anti-protein-A Antibodies from Eggs Whites of 9 Days Post-Immunized Birds by Affinity Chromatography

Egg white immunoglobulins (300 μl) were purified using a commercially available protein-A
affinity chromatography kit (PURE-1A, Sigma Aldrich Co). The manufacturer’s instructions were followed, and the antibody concentration was set at 0.4 mg/ml. The isolated anti-protein-A antibodies were stored at -20°C [11,12].

2.4 Inhibition of S. aureus Growth by Anti-protein-A Antibodies in Egg Whites of Birds

The effects of purified anti-protein-A antibodies on S. aureus isolates were investigated. The neutralizing ability of purified anti-protein-A antibodies was studied as follows: One ml of brain heart infusion (BHI) broth was placed in 11 sterile test tubes. An equal volume of ten μl of purified anti-protein-A was added to a concentration of 2.5 μg/μl. An inoculum of the ATCC Staphylococcus aureus strain (ATCC #33592) was made to 0.5 commercially prepared McFarland scale standards (1=300 × 10⁶/ml bacteria concentration). Then, ten μl of the inoculum was poured serially from tubes 1 to 10. Tubes 11 and 12 were used as controls [7].

Tube preparations were plated on blood agar and incubated overnight at 35°C. The optical densities of the bacterial growth were assessed and plotted against the bacterial concentrations at various serial dilutions (1:10, 1:100, or 1:1000).

2.5 Agglutination Inhibition of Protein-A-bearing Staphylococcus aureus Cells by Purified Anti-SpA Antibodies in vitro

Serial dilutions of 25 μl at a concentration of 60 μg/μl of purified anti-protein-A were added in duplicate to 96 wells microtiter plates containing 20 μl of S. aureus Cowan I strains and incubated for 1 h at RT. Inhibition of agglutination was observed in positive (+) samples (presenting anti-SpA antibodies), and agglutination was observed at the bottom of the negative samples. Commercially available human serum (Sigma-Aldrich) was used as the positive control.

2.6 Statistical Analysis

Statistically, SPSS version 22 was used. A P<0.05 was considered significant.

3. RESULTS AND DISCUSSION

The authors calculated the cut-off point of the test after several photometric measurements. It was set as the mean absorbance value of three-fold that of the negative control, equal to 0.55 [10]. The sandwich ELISA showed approximately specificity and sensitivity of 98% and 96%, respectively. This ELISA was normalized by determining the optimal reaction conditions and optimal coating antigen, reagent, substrate, and conjugate concentrations using checkerboard titration.

The use of peroxidase-labelled protein-A conjugate and coated microplates ensured that antibodies against the bacterial antigen tested positive in the ELISA, showing a substantial concentration of specific antibodies. One hundred % of the layer hens were positive for the presence of antibodies against protein A, as shown in Table 1.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean OD at 450 nm</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laying hen 1</td>
<td>1.308</td>
<td>0.053</td>
</tr>
<tr>
<td>Laying hen 2</td>
<td>1.455</td>
<td>0.045</td>
</tr>
<tr>
<td>Laying hen 3</td>
<td>1.387</td>
<td>0.050</td>
</tr>
<tr>
<td>Laying hen 4</td>
<td>1.309</td>
<td>0.069</td>
</tr>
<tr>
<td>Laying hen 5</td>
<td>1.415</td>
<td>0.071</td>
</tr>
<tr>
<td>Laying hen 6</td>
<td>1.125</td>
<td>0.058</td>
</tr>
<tr>
<td>Positive control 1</td>
<td>1.650</td>
<td>0.047</td>
</tr>
<tr>
<td>Positive control 2</td>
<td>1.575</td>
<td>0.066</td>
</tr>
<tr>
<td>Negative control 1</td>
<td>0.237</td>
<td>0.006</td>
</tr>
<tr>
<td>Negative control 2</td>
<td>0.195</td>
<td>0.009</td>
</tr>
<tr>
<td>Blank 1</td>
<td>0.088</td>
<td>0.003</td>
</tr>
<tr>
<td>Blank 2</td>
<td>0.073</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Production of antibodies against protein-A antibody production was tested by affinity chromatography from nine days post-immunization egg whites [15]. Table 2 shows the bacterial concentration of serially diluted samples: the mean optical density of the bacterial growth in pooled egg whites of post-immunized birds was 1.475 versus that of pre-immunized birds, which showed a mean OD value of 0.186. There was a notable difference in the absorbance values of pre- and post-immunized animals in the three serial bacterial dilutions. Comparable results have been reported in an earlier study in dogs with specific antibodies against surface antigens [7].

The plates containing antibodies against protein-A indicated the growth inhibition of S. aureus. However, when the antibodies were not
present as specimens from pre-immunized birds, bacterial growth was observed. Table 3 shows the percentage inhibition of 94%, which was statistically significant.

Another outcome was the inhibition of agglutination of protein-A-bearing Staphylococcus aureus cells by purified anti-protein-A antibodies in vitro, which confirmed the prior findings. This could be explained by the coupling of anti-protein-A immunoglobulins to SpA-bearing S. aureus cells, blocking those from agglutination at the base of the wells. A pooled positive specimen for anti-protein-A antibodies in egg white samples hindered the agglutination of protein-A-bearing Staphylococcus aureus cells at dilutions of 1:4096, in contrast to a pooled negative specimen that agglutinated protein-A-bearing Staphylococcus aureus from 1:16 to 1:8192 dilutions.

We are unaware of any similar studies like this one. However, the production of antibodies in egg whites of birds immunized with a protein has been reported by other authors, such as Kowalczyk et al. (2019), who reported that IgM was found only in egg white extracts. In comparison to IgY, IgM antibodies were not transferred to the serum of turkey poults [2]. Hamal et al. (2006) documented that chicks first synthesized IgM, followed by IgA and IgY. Anti-Newcastle disease virus (NDV) and anti-infectious bronchitis (IBV) antibody levels were detected in the plasma, egg yolks, and plasma of chicks on days 3 and 7 [16].

ELISA is highly specific for antibodies against protein-A. This is also extremely sensitive. A similar ELISA was previously used to measure the protein-A binding capacity to immunoglobulins in many zoo animal species and it was shown to be overly sensitive and specific as well [10]. In future studies, we recommend comparing the discriminatory capacities of the antibodies against protein-A by western blotting and ELISA to determine whether the sensitivity and specificity of protein-antibody interactions could be stimulated even more. The fact that the purified antibodies interacted with the Staphylococcus aureus Cowan I strain inhibiting their growth is proof of their nature in the recognition of protein-A expressed in the cell wall of Staphylococci. In a previous study, it was shown that the in vitro inhibition of Staphylococcus aureus Cowan I strains by anti-anti-protein-A idiotypic antibodies in chicks fed anti-protein-A hyper-immune egg yolks, supporting the hypothesis that hyper-immune eggs can be considered a type of oral anti-idiotypic vaccine [17].

The mechanism by which protein-A elicits an immune response after intramuscular vaccination includes the recognition of this bacterial antigen by antigen-presenting cells, including B cells; after processing the antigen, it proliferates and differentiates into plasma cells that produce large quantities of antibodies of IgM isotype, memory cells are formed that will react quickly if the antigen is presented in another opportunity to the immune system. In addition, the initiation of the acquired immune response involves the travel or movement of antigen-presenting cells to secondary lymphoid organs, where presentation of protein-A to CD4+ T lymphocytes occurs after antigen processing, which involves MHC class II molecules. In addition, protein-A is a superantigen and, in contrast to conventional

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**Table 2. Show the laboratory results of bacteria growth observed in immunized and pre-immunized birds 9 days post-immunization**

<table>
<thead>
<tr>
<th>Bacterial concentration &lt;sup&gt;a&lt;/sup&gt;</th>
<th>Immunized/pre-immunized hens &lt;sup&gt;b&lt;/sup&gt;</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>0.186/1.475</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>1:100</td>
<td>0.175/1.490</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>1:1000</td>
<td>0.155/1.338</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

<sup>a</sup>Bacteria concentration in ml after serial dilutions; <sup>b</sup>Optical density values of pooled egg white from post-immunized and pre-immunized birds; P values <0.05 are statistically significant.

**Table 3. Percentage inhibition of the growth of S. aureus in blood agar plates streaked out with egg white preparations. The presence of antibodies against protein-A in the egg white was responsible for the inhibition of the growth of the bacterium S. aureus**

<table>
<thead>
<tr>
<th>Birds</th>
<th>N. of Inhibited/Total</th>
<th>% Inhibition</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunized laying hens</td>
<td>47/50</td>
<td>94</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Pre-immunized laying hens</td>
<td>4/50</td>
<td>7</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

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antigens, where epitopes are processed intracellularly, staphylococcal protein-A can simultaneously activate large numbers of T cells carrying a particular T-cell receptor Vβ gene by binding directly to MHC class II molecules at a site distinct from the antigen-binding groove. IL-2 is involved in this process, which activates T cells, which signal B cell differentiation into plasma cells and antibody production [18].

4. CONCLUSION

The results showed that it is possible to produce antibodies against bacterial antigens in the egg white, which can have industrial applications in the preparation of antibodies for immunotherapy of infectious diseases.

ETHICAL APPROVAL

This study was approved by the ethical research committee of the University of West Indies. Mona Campus. Jamaica.

DISCLAIMER

The products used in this study are commonly used in our research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for litigation but for the advancement of knowledge. Moreover, the research was not funded by the producing company; rather, it was funded by the authors' personal efforts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES


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