PRODUCTION AND PURIFICATION OF IgY FROM EGG YOLK AFTER IMMUNIZATION OF HENS WITH PIG IgG

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The antibody response in chickens immunized with pig immunoglobulin G was studied during a 3.5 months immunization period comparing the efficacy of two doses of the antigen. The titres of immunospecific antibodies to pig IgG in egg yolk varied from log₂ 6 to log₂ 14 during the period of study after immunization, and the immunoglobulin Y (IgY) concentrations varied from 1.0 to 3.8 mg per ml of yolk in two groups of birds. Two IgY purification methods based on salt precipitation using zinc sulphate or cadmium sulphate were developed. These methods were compared with ammonium sulphate precipitation, respectively in terms of yield, total protein content, IgY concentration, and immunospecific anti-pig IgG IgY activity. The specific activity and IgY contents when purified by ammonium sulphate were 1.6-2.0 and 1.6-1.8-fold higher than those purified by the other two methods. However, only the total protein content obtained by the use of zinc sulphate was lower than that of the others.

Key words: hen, immunization, pig IgG, egg yolk, purification.

There is an increasing interest in the use of chicken egg yolk for polyclonal antibody production for practical and economical reasons (6, 28, 30). The chicken egg yolk antibodies (IgY) have been applied successfully for scientific (26), diagnostic (8), prophylactic (2, 24) and therapeutic purposes (18), and veterinarian therapy against bacteria such as enteropathogenic Escherichia coli (3). Because of the phylogenetic distance between birds and mammals, mammalian proteins are often more immunogenic in birds than in mammals (19). In addition, because of the phylogenetical distance, bird antibodies against a mammalian protein may often react with analogous proteins in other mammalian species (12, 13).

Egg yolk IgY has a molecular weight of 180 kDa, which is higher than that of mammalian IgG, lower isolectric points and slightly different physicochemical properties compared with mammalian IgG (6).

From an animal welfare point of view, chickens are an attractive alternative to mammals as antibody producers because large quantities of antibodies can be produced from the egg yolk making restraint from the blood sampling obsolete techniques to the benefit of the animals used for this purpose (9, 25, 28).
Hens can be immunized for the production of polyclonal antibodies against a large variety of antigens as reviewed (26). The large amount of lipids in egg yolk (11), however, renders some purification of IgY necessary for scientific use regardless of the assay in which they are to be used. Several methods were described in the 1950ies for purifying IgY based on the strategy of separation of proteins (levitins) from lipoproteins (lipovitellins) and the rest of the yolk lipids using extraction with organic solvents with rather low yield of antibodies (15). However, purification methods based on organic solvents like chloroform remain in use (22). Other methods are based on dilution of the yolk followed by a freezing-thawing process after which the process consists of ion exchange chromatography or salt precipitations often combining a number of salts like e.g. polyethylene glycol (PEG) (15, 23), dextran sulphate (15), dextran blue (4), sodium sulphate (15), ammonium sulphate (15, 29) caprylic acid (29), and sodium citrate (1).

The main aims of the present study were i) to produce immunospecific IgY antibodies to pig IgG, and ii) to test the use of cadmium sulphate and zinc sulphate in the purification process for IgY against other existing method: ammonium sulphate precipitation.

Material and Methods

Animals and husbandry. Ten 25-week-old outbred Brown Hisex hens were obtained from the breeding unit of Vaisa, the laboratory animal resources of the Institute of Immunology (Vilnius, Lithuania). Three female chinchilla rabbits 3-5 months old and 1.5-2.0 kg body weight were obtained from the same breeder.

The hens and rabbits were kept singly in 1 m x 1 m floor pens equipped with nest boxes in a standard animal room with a 17/7 h light/dark cycle. As bedding, sawdust of deciduous trees were used, after sterilization at 120°C, pressure 1.5 kg/cm² during 20 min. The bedding was changed twice a weeks. The temperature in the room was 20°C ± 2°C, with a relative humidity within the range of 55-60% and the noise level was maintained below 50 dB. The chicken and rabbit feed was based on granulated forage (“Biosynthesis” AB Vilnius, Lithuania). This consisted of dry matter (88%), crude protein (20%), fat (3%) and carbohydrate (4%). The feed was balanced for vitamins and micronutrients, and the moisture content did not exceed 12%. Water was provided ad libitum.

Preparation of pig IgG. The isolation and purification of the IgG from pig serum was carried out according to the method described Johnstone and Thorpe (16).

Immunization of hens. The first group of five laying hens were immunized by injection of 1 ml of emulsion containing 1 mg of pig IgG with the complete Freund’s adjuvant (Calbiochem, Corp., La Jolla CA, USA), while the second group of five hens were inoculated with the same emulsion containing 4 mg of pig IgG. This mixture was distributed into four sites of the pectoral muscle of each bird. Boosters were given 4 and 8 weeks after the first inoculation using incomplete Freund’s adjuvant (Calbiochem). Eggs were collected daily, beginning 5 d after the first injection, and stored at 4°C until analysis.

Immunization of rabbits. The immunization of rabbits with IgY were performed as described previously (4).

Purification of IgY from egg yolk. The yolks of five eggs were collected 11 weeks after the second immunization and separated from egg whites, then washed with
distilled water to remove as much albumen as possible, and rolled on paper towels to remove adhering egg white. The yolks were pooled, mixed and then this mixture aliquots were processed according to three different protocols.

1. By the water dilution method as described earlier (1), but with replacing sodium sulphate by cadmium sulphate
2. As method #1 but with replacing sodium sulphate by zinc sulphate and
3. As method #1 but with replacing sodium sulphate by ammonium sulphate.

In methods 1 - 3 the yolk was diluted 1:9 with distilled water, acidified with 100 mM HCl to reach pH 5.0 and incubated at 4°C overnight. After further centrifugation at 7000 x g, 10°C for 20 min, the precipitate containing IgY was resuspended in phosphate buffered saline (PBS), precipitated twice in either cadmium sulphate, zinc sulphate or ammonium sulphate (34% final concentration) and dialyzed against PBS. The procedure was repeated at least three times for each method, with essentially the same results.

Preparation of standard IgY. The standard IgY was prepared as described previously (4).

Enzyme-linked immunosorbent assay (ELISA). The ELISA test was performed as described earlier, except the standard antibody (5). The antibody titres were expressed, as the reciprocal of the highest dilution of IgY at which optical density (492 nm) was 2-fold higher than that of the control values. The titres were converted to a base-2 logarithmic scale.

Radial immunodiffusion assay. Radial immunodiffusion (RID) as described earlier (21) was used to estimate the concentration of total IgY in egg yolk. RID was carried out as described previously (4).

Total protein estimation. The protein content was calculated as described earlier (20).

Statistical analysis. The mean antibody titres of the chicken sera were compared using two-tailed Student’s t-test (31). All values were expressed as mean ± standard deviation and were considered to be statistically significant at P < 0.05.

Results

IgY titres of egg yolk antibodies with specificity against pig IgG. Specific antibodies to pig IgG in egg yolk increased during the first 5 weeks after the initial immunization in two groups (Fig. 1). The egg yolk titre of the second group increased to a maximum at the 11th week after the first immunization, while that of the first group peaked at the 2nd week after the third immunization. The second group demonstrated significantly higher egg yolk antibody titers than that of the first group from week 2 after the second immunization to the end of the study. Finally, at the 14th week after the first immunization, the level of specific antibodies fell slightly to 12 log₂ and 14 log₂, respectively, in the first and second group. Furthermore, antibody response from week 6 after the initial immunization to the end of the study in hens immunized with 1.0 mg of pig IgG was significantly lower than that of birds receiving the same antigen in a dose of 4.0 mg. The titres were below log₂ 3 in the controls.
Fig. 1. Antibody titres in chicken egg yolk purified by ammonium sulphate (full and empty boxes) after immunizations with 1 mg and 4 mg of pig IgG, respectively. The arrows indicate the time of immunization. Bars represent standard deviations.

Fig. 2. Time-dependent immunoglobulin concentration in IgY preparations purified by ammonium sulphate (full and empty boxes) after immunizations with 1 mg and 4 mg of pig IgG, respectively. The arrows indicate the time of immunization. Bars represent standard deviations.
Concentration of total IgY in egg yolk. The IgY concentration in egg yolk determined by RID increased during the immunization period until week 11 where it reached 3.0 mg/ml, 3.8 mg/ml, respectively, in the first and second group (Fig. 2). After week 11 the levels decreased gradually to reach a value of 2.7 mg/ml, 3.2 mg/ml, respectively, in the first and second group. Whereas the amount of IgY/ml of egg yolk in the group of hens that received 4.0 mg of pig IgG was significantly higher than that of birds immunized with the same antigen in a concentration of 1.0 mg from week 3 after the first immunization to the end of the observation period.

Quantitative characteristics of the IgY preparations purified by three methods. The IgY preparations purified by use of ammonium sulphate contained significantly more total protein than that purified by the other two methods (Table 1). The IgY preparations purified by use of zinc sulphate or cadmium sulphate contained the IgY concentration in egg yolk, anti-IgG activity as well as the proportion of IgY of the total protein a significantly lower than did those purified by ammonium sulphate. The IgY concentration in yolk from eggs of unimmunized birds varied in the range 0.4 – 0.9 mg/ml of egg yolk.

Table 1
The characteristics of IgY purified by different methods from 20 ml of egg yolk*

<table>
<thead>
<tr>
<th>Methods of purification</th>
<th>Total protein (mg/ml of egg yolk)</th>
<th>IgY (mg/ml of egg yolk)</th>
<th>Mean geometric anti-pig IgG titre (log₂)</th>
<th>IgY/protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulphate</td>
<td>6.1 ± 0.07</td>
<td>3.8 ± 0.04a</td>
<td>15.6 ± 0.5a</td>
<td>62.1±1.0a</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>5.8 ± 0.04a</td>
<td>2.4 ± 0.05b</td>
<td>9.6 ± 0.5b</td>
<td>41.2 ± 1.2b</td>
</tr>
<tr>
<td>Cadmium sulphate 1.2c</td>
<td>6.2 ± 0.08</td>
<td>1.9 ± 0.08c</td>
<td>8.4 ± 0.5c</td>
<td>31.8 ±</td>
</tr>
</tbody>
</table>

* Egg yolk collected from eggs 11 weeks after the second immunization
a,b,c Results with differing suffixes differ significantly from results in the same column
Discussion

As early as two weeks after the initial immunization of laying hens with pig IgG, the immunospecific antibodies were present in high titre in the egg yolk from hens of two groups. The titre increased during the following seven weeks, and remained at a high level throughout the seven weeks observation period in both groups. This appears to confirm previous observations where high specific immune responses in the egg yolk from hens inoculated with human IgG were noted over 15 months (5, 7).

In the present study, the IgY concentration in both groups varied in the range 1.0-3.2 mg/ml of egg yolk throughout the immunization period. Similar levels have been reported by other authors (27).

From a productivity point of view, the yield of IgY obtained by various purification methods is of interest. Literature report on the amount of IgY obtained by ammonium sulphate (60% v/v) precipitation was 0.6 mg/ml of egg yolk (10). This finding indicates lower efficacy than that of the present study.

According to earlier publications (14, 17) the neutral inorganic salts, such as zinc sulphate and cadmium sulphate induce the precipitation of the proteins. This is similar to our results, where zinc sulphate and cadmium sulphate precipitation schemes were found useful for IgY purification.

A high purity of the IgY preparation is desirable for many immunoassays and for the production of labelled second antibodies. It should be noted, however, that for other assay types like many of the immunoelectrophoretic assays high purity is often less important than monospecificity.

Importantly, the IgY technology seems to be well suited to meet many of the diagnostic requirements in European and developing countries. Specific pathogen free birds are not required in this context since affinity chromatography with appropriate ligands remit the isolation of specific antibodies out of a pool of specific and non-specific antibodies.

References


