BIOLOGIJA. 2006. Nr. 2. P. 68–71

Development of antibodies to Sendai virus in chickens and their isolation from yolk

G. Bižanov, T. Normantienė,

I. Jonauskienė*

Laboratory of Biomodels,
Institute of Immunology,
of Vilnius University,
Molėtų plentas 29,
LT-08409 Vilnius, Lithuania

Egg-laying hens were immunized with the Sendai virus (SV) grown in chicken embryos. The titres of immunospecific anti-SV IgG antibodies varied from $\log_2 12$ to $\log_2 16$ during the 5-month immunization period. Two IgY purification methods based on salt precipitation using lithium sulphate or sodium citrate were developed. These methods were compared with two other purification methods based on polyethylene glycol (PEG) precipitation and chloroform extraction, respectively in terms of yield, total protein content, IgY concentration and immunospecific anti Sendai IgY activity. The total protein and IgY contents when purified by chloroform were 1.4–2.8 times and 1.3–2.3 times higher, respectively, than in corresponding preparations purified by the other methods. However, the proportion of nonsense proteins was approximately 10% higher in the IgY preparation purified by chloroform than in those purified by salt precipitation. The immunospecific IgY activity recorded in the preparations from the new salting out methods was lower compared with the PEG and chloroform purification methods. The results indicate that the purification of IgY by lithium sulfate results in a very pure IgY in high quantities (94% + / – 5% of total egg yolk protein).

Key words: chicken, immunization, immunoglobulin, Sendai virus

INTRODUCTION

There is an increasing interest in the use of chicken egg yolk for polyclonal antibody production for practical and economical reasons [1], and chicken egg yolk antibodies (IgY) have been applied successfully for scientific [2], diagnostic [3], prophylactic [4] and therapeutic purposes [5]. Because of the phylogenetic distance between birds and mammals mammalian proteins are often more immunogenic in birds than in other mammals and antibody synthesis in readily stimulated in hens [6]. In addition, because of the phylogenetical distance, avian antibodies against a mammalian protein may often react with analogous proteins in other mammalian species [7]. An additional advantage of the chicken system for antibody production is that many of the viruses used to induce antibodies can be grown in fertile eggs in order to avoid anti-host antibodies [8]. Egg yolk IgY has a molecular weight of 180 kDa, which is higher than that of mammalian IgG, lower isoelectric points and slightly different physicochemical properties compared with mammalian IgG [9]. 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 and blood sampling obsolete techniques to the benefit of the animals used for this purpose [10]. Hens can be immunized for production of polyclonal antibodies by various antigens such as viral antigens [11, 12]. The large amount of lipid in egg yolk [13], 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 1950 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 yields of the antibody [14]. However, purification methods based on organic solvents like chloroform remain in use [15]. Other methods are based on affinity chromatography [16] or on dilution of the yolk followed by a freezing–thawing process after which the process consists of ion exchange chromatography and/or salt precipitations often combining a number of salts like, e.g., polyethylene glycol (PEG) [14, 17], dextran sulfate [14], dextran blue [18], sodium sulphate [14], ammonium sulphate [14], caprylic acid [19] and sodium citrate [12]. More recently, methods combining chloroform removal of lipids with ammonium sulphate precipitation techniques have been shown to result in a good yield of antibodies of high purity [20].

* Corresponding author. E-mail: Rene@imi.lt

The main aims of the present study were i) to produce immunospecific IgY antibodies to the Sendai virus (SV) and ii) to test the use of lithium sulphate as a new salting out method in the purification process for IgY against three other existing methods: chloroform extraction, PEG-6000 precipitation and sodium citrate precipitation.

MATERIALS AND METHODS

Animals and husbandry. Seven 25-week-old outbred ISA hens were obtained from the breeding house-unit of the laboratory animal resources of the Institute of Immunology Vilnius University (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 × 1 m floor pens equipped with nest boxes in a standard animal room with a 17/7 h light/dark cycle. As bedding, chips of deciduous trees were used, after sterilization at 120 °C, pressure 1.5 kg/cm² during 20 min. The bedding was changed twice weekly. The temperature in the room was 20 ± 2 °C, with a relative humidity within the range of 55–60%, and the noise level was maintained below 50 dB. The chicken feed was based on granulated forage (Biosynthesis, Vilnius, Lithuania). It 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 viral antigen. The Fushimi Sendai virus strain obtained from the Institute of Virology (Moscow, Russia) was used. It was grown, purified by differential centrifugation, and inactivated as described previously [18]. This viral sample was used to immunize the hens.

Immunization of hens with Sendai virus. The viral suspension (100 μmg in 500 μml of Tris-buffered saline (TBS) (10 mM Tris, 150 mM NaCl, pH 7.5) was emulsified with an equal volume of incomplete Freund's adjuvant (IFA) (Calbiochem, Corp., La Jolla CA, USA). Seven laying hens were immunized by injection of 1 ml of this emulsion distributed into four sites of the pectoral muscle of each bird. A second immunization was performed after 14 days. Eggs were collected for antibody measurement and also for determination the IgY concentration in egg yolk weekly, beginning 7 days after the first injection, and stored at 4 °C until analysis.

Immunization of rabbits with chicken IgY. Three rabbits were immunized intramuscularly with purified IgY as described previously [18] and then the antibodies were used for radial immunodiffusion assay.

Enzyme-linked immunosorbent assay. SV-specific IgY antibodies in yolk and samples of the IgY isolated from yolk by four methods were measured by enzyme-linked immunosorbent assay (ELISA) as described earlier [19]. The antibody titres were expressed as the reciprocal of the highest dilution of IgY samples (the optical density 492 nm) which was 2-fold higher than that of the negative samples. The titres were converted to a base-2 logarithmic scale.

Isolation of IgY from egg yolk. Seven eggs were collected 4 weeks after the second immunization and egg yolks were separated from egg whites, washed with distilled water to remove as much albumin as possible and rolled on paper towels to remove adhering egg white. The yolks were pooled, mixed, and a mixture of TBS and egg yolk (4:1, v/v) was prepared. From this mixture aliquots were processed according to the four different protocols.

1. Precipitation with PEG-6000 (Fluka) following the procedure as described earlier [17].
2. Chloroform extraction as described previously [15].
3. By the water dilution method as Akita & Nakai [12] have described, but replacing sodium sulphate by sodium citrate.
4. As method #3 but replacing sodium sulphate by lithium sulphate.

Preparation of standard IgY. IgY was extracted from egg yolk with PEG-6000 as described earlier [17] and then purified according to the procedure as by described Hurn & Chantler [21]. This sample of the IgY was used to immunize the rabbits and as a standard IgY in the radial immunodiffusion assay.

Radial immunodiffusion assay. Radial immunodiffusion was used to estimate the concentration of total IgY in egg yolk by the method described earlier [22].

Total protein estimation. The protein content of the product obtained by the different methods was determined as described earlier [23].

Statistical analysis. The mean antibody titres of the yolk were compared using Student's t test. All values were expressed as mean ± standard deviation and differences with p values <0.05 were considered significant.

Ethics Committee approval. We performed the experiment on rodents and birds on receiving permission № 0086 (21.01.2003) from the Ethics Committee on the use of laboratory animals of the State Food and Veterinary Service.

RESULTS AND DISCUSSION

Specific Sendai virus antibodies were detected at week 2 post immunization (Figure). Following reimmunization, the level of specific antibodies continued to increase above $\log_2$ 16 throughout the 20–week period of observation. The titres were below $\log_2$ 3 before immunization of the hens. The IgY preparations purified by use of chloroform contained significantly more total protein as well as IgY than did those purified by the three other methods (Table). The proportion of IgY of the total protein isolated with chloroform was only about 80% as compared with more than 90% in the IgY preparations purified by salt precipitation. The immunospecific anti Sendai virus activity was higher in the IgY preparations obtained by the methods based on PEG precipitation and chloroform extraction as compared with the activity of the IgY preparations purified by the two other methods. The concentrations of total protein and IgY purified by PEG precipitation were significantly lower than those in corresponding preparations

Table. **Characteristics of immunoglobulin Y (IgY) purified from egg yolk by polyethylene glycol (PEG) 6000, chloroform and water dilution methods**[a]

Method of purification	Total protein (mg/ml of egg yolk)	IgY (mg/ml of egg yolk)	Mean geometric anti-Sendai virus IgY antibody titre (log_2)	IgY/protein (%)
PEG-6000	$4.6^{b} \pm 0.4$	$4.4^{b} \pm 0.4$	$15.2^{b} \pm 0.5$	95.6 ± 3.6
Chloroform	$13.2^{c} \pm 0.7$	$10.7^{c} \pm 0.6$	$11.7^{c} \pm 0.5$	$81.0^{b} \pm 1.0$
Lithium sulphate	8.2 ± 0.2	7.7 ± 0.2	8.9 ± 0.5	93.9 ± 4.8
Sodium citrate	9.1 ± 0.7	8.2 ± 0.7	8.6 ± 0.5	90.1 ± 4.0

[a] Egg yolk collected from eggs four weeks after the second immunization.
[b,c] Results with different suffixes differ significantly from results in the same column.

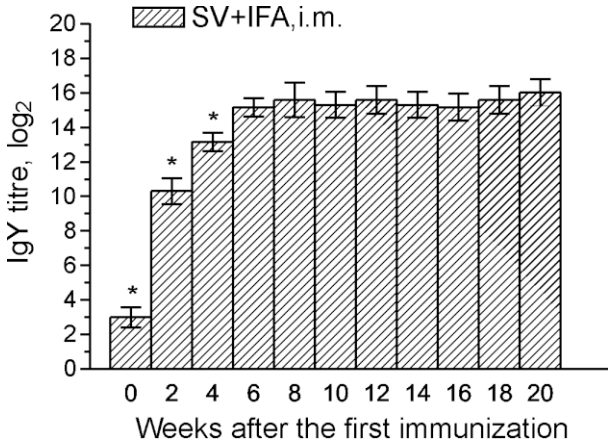

Fig. Development of the anti-Sendai virus IgY antibody titres in chicken egg yolks. Asterics indicate the results differing significantly from results on any week

purified by the three other methods. As early as one week after the initial immunization of laying hens with the Sendai virus, immunospecific antibodies were found present in a high titre in the egg yolk. The titre increased steadily during the following five weeks and remained at a fairly steady high level throughout the 20 weeks of the observation period. This confirms our earlier observations [18], whereas lower titres have been reported in eggs of chickens immunized with the Newcastle Disease Virus [11]. These differences may be due to immunogenicity differences between the viruses as well as to the use of different immunization schemes, different breeds of hens and different assays employed [18, 24]. Other reports have noted high specific immune responses over 3–15 months in the egg yolk from hens inoculated with various antigens [9, 12, 15]. From the productivity point of view, the yield of IgY obtained by the various purification methods is of interest. The literature reports on the amount of IgY obtained by PEG purification range from 40 to 109 mg/egg [25, 26]. These findings are in agreement with the results of the present work.

Other studies have shown that the of use chloroform gave 7–9 mg IgY/ml of egg yolk [18, 27], which is slightly lower than the 10.7 mg IgY/ml of egg yolk recorded in the present study. Using chloroform, the maximal amount of total protein obtained was 264.0 mg/egg [28]. Chloroform extraction is associated with contamination of the IgY preparation with unwanted nonsense proteins to the extent of 20% in the present study. IgY purification by PEG-6000 resulted in a significantly lower yield of total protein as well as IgY compared with the other purification methods. Interestingly, this did not affect the anti-Sendai titre, which was higher than those of the other IgY preparations. The reasons for this difference are not clear, but it is well known that even low levels of PEG significantly improve immune-complex formation [14], and this feature has been utilized in precipitation assays suchas immunoelectrophoresis [29]. It cannot be ruled out that the IgY purified by PEG still contained PEG molecules which might have disturbed the an immunoassay resulting in abnormally high titre estimation.

Both lithium sulphate and sodium citrate precipitation schemes were found useful for IgY purification. A high purity of the IgY preparation is desirable for many immunoassays and for production of labeled 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.

Received 15 March 2006
Accepted 22 May 2006

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ANTIKŪNŲ SENDAI VIRUSUI INDUKCIJA VIŠTŲ ORGANIZME IR JŲ IŠSKYRIMAS IŠ KIAUŠINIŲ TRYNIO

Santrauka

Viščiukų embrionuose išaugintas Sendai virusas panaudotas vištų dedeklių imunizacijai. Penkių mėnesių imunizacijos laikotarpiu bandomųjų vištų kiaušinių trynyje analizuoti specifinių antikūnų titrų ir imunoglobulino Y (IgY) koncentracijos pokyčiai. Specifinių antikūnų titrų reikšmės svyravo nuo $\log_2 12$ iki $\log_2 16$. IgY iš kiaušinių trynio išskirtas išsūdymo ličio sulfato arba natrio citrato druskomis metodu. Šie metodai palyginti su alternatyviais išskyrimo polietilenglikoliu bei ekstrahavimo chloroformu metodais, įvertinant produkto išeigą, bendrą baltymo kiekį, IgY koncentraciją ir specifinio IgY aktyvumą. Ekstrahavimu chloroformu gautas bendras baltymo ir IgY kiekis buvo didesnis atitinkamai 1,4–2,8 ir 1,3–2,3 karto, tačiau naudojant šį metodą pašalinių baltymų kiekis buvo vidutiniškai 10 % didesnis nei naudojant išsūdymą druskomis. Specifinio IgY aktyvumas preparatuose, gautuose pritaikius išsūdymo druskomis metodus, buvo mažesnis lyginant su kitais dviem metodais. Gauti rezultatai leidžia daryti išvadą, kad panaudojus išsūdymą ličio sulfatu gaunamas didelis aukšto grynumo lygmens (94 ± 5 % bendro trynio baltymų kiekio) IgY kiekis.

Raktažodžiai: vištos, imunizacija, imunoglobulinai, Sendai virusas