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Optimized Protocol of Chicken Antibody (IgY) Purification Providing Electrophoretically Homogenous Preparations

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Chicken antibodies isolated from egg yolks (IgY) are a suitable alternative to conventional antibodies from blood of experimental animals. Main advantages of IgY are: (i) larger amounts of IgY could be prepared from repeatedly laid chicken eggs, (ii) collection of eggs is a non-invasive procedure contrary to bleeding of mammals, and (iii) a better response of chicken to mammalian antigens due to the larger evolutionary distance. Here, we report a cost- and labor-effective two-step procedure consisting of yolk extraction by tap water (8-fold dilution, freezing, and filtration) followed by a specific precipitation of IgY at pH 4 with sodium chloride in the final concentration of 8.8%. Using this procedure, the highly purified antibody (97%) was prepared. Resulting IgY preparations are acceptable for a wide range of applications as it was prepared using only bio-compatible chemicals. The purification protocol was developed and optimized in terms of the time, materials and the necessary manipulations.

Keywords: Yolk Immunoglobulin; Purification Protocol; Bio-compatible Chemicals; IgY Precipitation.

Dedication
This paper is dedicated to prof. Gustav Entlicher on occasion of his 70th birthday.

Abbreviations
BSA, bovine serum albumin; HC, heavy chain; IgY, chicken yolk antibody; LC, light chain; PBS, sodium phosphate buffered isotonic saline pH 7.4; PBS-Tw, PBS containing 0.05% Tween 20; PEG, polyethylene glycol 6000; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate; WS, water-soluble fraction.

1. INTRODUCTION

Birds concentrate immunoglobulins in yolks of their eggs to protect the offspring similarly as mammals do *via* milk [1]. The majority of yolk immunoglobulins (~ 100 mg/yolk) belong to a class referred to as IgY; other classes such as IgA and IgM are also present, albeit in lower quantity. Although IgY play the same role for birds as the IgG for mammals, they are structurally rather different, their molecules miss the hinge region, typical for IgG, and in the heavy chain they possess an additional higly glycosylated constant domain. Thus, their molecular mass is higher (~ 170 kDa), compared to IgGs (~150 kDa), and the sugar content is almost doubled. On the contrary to IgG, yolk immunoglobulins do not activate the complement cascade, do not interact with the rheumatoid factor, and the Fc receptors or bacterial proteins A and G. These features make avian IgY advantageous over IgG for various applications.

Even though the transfer of specific antibodies into the egg yolk has already been described in 1893 [2], the practical use of this fact came much later and only within the last two decades IgY are slowly being recognized as a suitable alternative to conventional IgGs prepared from mammalian blood [3]. Because of the IgY productivity (~ 40 g IgY /year/chicken) and in the sense of animal welfare, the laying hens are an excellent source for the large scale antibody production. IgY are therefore suitable not only for research, diagnostics, and therapy, but also for the food industry and cosmetics. Moreover, chicken because of their phylogenetic distance from mammals can produce high-titer antibodies against conserved mammalian antigens compared to other experimental animals commonly used [4].

Several methods have been developed for purification of IgY from egg yolks (for a review see e.g. Kovacs-Nolan and Mine [5] or De Meulenaer and Huyghebaert [6]). Because of significant differences between blood of mammals and egg yolks as sources of antibodies, the IgY require a specific work and handling to be isolated from lipophilic matrix of the yolk. The purification of IgY from separated yolks usually proceeds in two stages: in the first one, the water-soluble fraction (WS) containing IgY is separated from the lipidic fraction composed mainly of lipids and lipoproteins, and in the second stage, IgY are isolated from the other soluble proteins. To remove the lipophilic yolk components from the crude IgY extract, lipid aggregates large enough to be removed by a conventional centrifugation or filtration, are formed using various precipitants (e.g. polyethylene glycol, dextran sulphate, alginate, caprylic acid, organic solvents), by a simple dilution of the yolk, or by a freezing-thawing of the diluted yolk. Alternatively, some methods of a lower throughput, such as ultracentrifugation, hydrophobic or affinity chromatography, are employed for the first purification stage.

In the second stage, the pure IgY fraction should be prepared from the crude immunoglobulin water extract. Frequently, the IgY are salted-out first and further purified using chromatography (gel-filtration-, ion-exchange-, thiophilic-, affinity chromatography), or a repeated precipitation of IgY is employed.

Purity and the overall yield of resulting IgY preparations were reported to be 85-98% and 1.0-9.8 mg/ml of yolk, respectively, depending on the purification protocol used [7].

The large chicken productivity makes the antigen-specific IgY an excellent tool for prophylactic and therapeutic treatment, where large amounts of antibodies are needed [8]. Given the benefits of IgY production, it is rather surprising that a wider use of IgY for passive immunization and other human related applications is still rare. In part, this may be attributed to a need for economical and easily scaled up methods for IgY purification. Another difficulty is likely associated with the safety reasons arising from the use of chemicals in the purification protocol. Thus, we undertook this work with purpose to overcome these limitations. The aim of our present work was to elaborate a simple and cost-effective procedure for preparation of IgY based on the use of bio-compatible chemicals.

Herein we describe a suitable procedure for preparation of IgY, which is based on freezing-thawing of the water diluted yolks followed by a filtration step to prepare a crude IgY water extract. Then, the specific sodium chloride precipitation of IgY at low pH, resulting in a highly purified fully immunoreactive IgY preparation, was used. Moreover, the purification protocol was optimized as regarded chemicals, instruments and sample handling.

2. MATERIALS AND METHODS

2.1. Chemicals

Anti-chicken IgY rabbit IgG-alkaline phosphatase conjugate, BCIP/NBT tablets, BSA, Freund's adjuvant, and polyethylene glycol 6000 were from Sigma Chemical Co. (St. Louis, MO, USA); p-nitrophenylphosphate, Tween 20 from Serva (Heidelberg, Germany). All other chemicals were of reagent grade and were purchased from Lachema (Brno, CR).

2.2 Immunization

Leghorn hen was immunized weekly by three subcutaneous injections with BSA (0.1 mg/dose/animal). The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic). For the first injection the antigen was emulsified in the complete Freund's adjuvant and for two subsequent boosters in the incomplete adjuvant. Pre-immune IgY sample was purified from 6 eggs laid a week prior to immunization and the antigen-specific IgY sample from 12 eggs collected between 5-6 weeks after immunization. Egg yolks were separated from the whites, washed with tap water and pooled in a calibrated cylinder. The pool was diluted with the same volume of PBS containing 0.1% sodium azide and stored at 4 °C.

2.3. Preparation of water-soluble fraction of yolk

The method for preparation of water-soluble fractions (WS) of yolk introduced by Jensenius et al. [9] and Akita and Nakai [10] was modified for the use of tap water as follows: Aliquots (10 ml) of pooled yolks stored in 1:1 mixture (volume:volume) with PBS (containing 0.1% sodium azide) were further diluted 2-5 times (by volume) with tap water, pH adjusted to 5.0 with 0.5 M HCl, and the mixture frozen in polypropylene centrifugation tubes at -20 °C. After spontaneous thawing the

aggregated egg yolk granules were sedimented by centrifugation at 13,500 g for 15 min at 4 °C and WS collected. The purity and recovery of IgY in WS were monitored by the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Optimization of IgY precipitation

Because of its bio-compatibility, sodium chloride was used for the precipitation in the second isolation stage instead of the other precipitants described in the literature. Conditions for the IgY precipitation with NaCl were optimized in the respect of the salt concentrations and pH. To the aliquots (30 ml) of yolk water fraction, solid NaCl was added to prepare samples containing up to 2 M NaCl. Each sample was divided into two parts and pH was adjusted with 0.5 M HCl to 4.0 or 7.2 to check the behavior at different pH and concentration combinations. Mixtures were stirred for 2 hrs at room temperature and then centrifuged at 3,700 g for 20 min at 4 °C. Supernatants were discarded and the pellets dissolved in PBS (3 ml). Protein concentration was determined in supernatants and dissolved pellets and the aliquots of all samples were analyzed by SDS-PAGE.

For a comparison, IgY of WS were precipitated at 4.0 and 7.2 also with polyethylene glycol 6000 (PEG) (5–12%) or sodium sulphate (6–15%) and the resulting supernatants and dissolved pellets were analyzed as described above.

2.5. PEG purification of IgY

As a reference method for IgY purification, PEG precipitation described by Polson et al. [11,12] was used. Briefly, an aliquot (100 ml) of the pooled yolk was diluted (1:2) in PBS and precipitated with PEG to a final concentration of 3.5%. IgY were precipitated from the resulting supernatant using 12% PEG, then dissolved in PBS and re-precipitated with 12% PEG. Final IgY preparation was stored at 4 °C.

2.6. Effect of pH on IgY activity

To test the stability of IgY samples, the aliquots (0.5 mg of protein/ml) prepared in 0.1 M sodium phosphate buffers of pH 2-11 were incubated for 2 hrs at room temperature. After the incubation, the samples were diluted with PBS to a final concentration of 9 μg/ml and their activity examined with ELISA against BSA as antigen. Pre-immune IgY samples treated identically were used as a control.

2.7. ELISA

The antibody immunoreactivity was tested by ELISA. An ELISA plate (Nunc-Polysorp, Denmark) was coated with 100 μl per well of antigen solution (4 μg BSA/ml in 50 mM sodium carbonate buffer, pH 9.6) and incubated at 4 °C overnight. After washing 3 times with PBS containing 0.05% Tween 20 (PBS-Tw), each well was loaded with 150 μl of 2% solution of ovalbumin in PBS-Tw and incubated for 1 h at 37 °C. Wells were washed (3 times) with PBS-Tw and then in doublets loaded with 100 μl of antibody solution in PBS (pre-immune and immune IgY preparations,

concentration series 1, 3, and 9 μg/ml). After washing (3 times) with PBS-Tw, 100 μl of alkaline phosphatase-conjugated rabbit anti-chicken IgG in PBS was added to each well (2000 times diluted commercial preparation, Sigma) and incubated at 37 °C for 1 hr. After washing with PBS-Tw, 100 μl of substrate solution (1 mg/ml p-nitrophenyl phosphate in carbonate buffer) was added and the plate kept for 10 min at room temperature. Reaction was stopped by 50 μl of 3 M NaOH to each well and the color developed was measured at 405 nm with an ELISA reader ELX 800 (Bio-Tek Instruments, Winooski, VT).

2.8. Western blots

The purified IgY were examined by Western blotting on Immobilon-P membrane (Millipore, Bedford, MA). For SDS-electrophoresis (8% polyacrylamide gel), 5 μg of BSA or IgY (positive control) was applied. Blots were developed with the specific chicken anti-BSA- or pre-immune control IgY. Western blotting was carried out as described earlier [13]. Visualization was performed using the alkaline phosphatase-conjugated rabbit anti-chicken IgG (2000 times diluted commercial preparation) and BCIP/NBT tablets containing 10 mg of substrate for alkaline phosphatase.

2.9. Protein concentration determination

Protein concentrations were determined using Lowry method with the Folin-Ciocalteu reagent [14] against a standard curve created using bovine serum albumin. For purified IgY, the concentration (mg/ml) was calculated from the absorbance at 280 nm using the previously determined experimental factor of 1.094.

2.10. Polyacrylamide gel electrophoresis

Protein samples were analyzed on 8% gels by SDS-PAGE under non-reduced or reduced conditions [15]. Gels were run on MiniVE Vertical Electrophoresis System (Amersham Biosciences Corp., USA) and then stained in Coomassie Brilliant Blue R-250 stain (0.25% in 46% ethanol containing 9.2% acetic acid). The gels were destained in 25% ethanol with 10% acetic acid until a clear background was reached.

3. RESULTS

To develop an effective procedure for isolation of chicken IgY using “safe” chemicals only, we used a combination of two stages similar to those described previously [7] with the following modifications. For the first stage, quite inexpensive and efficient precipitation of lipidic granules by water-dilution of yolks and subsequent freezing was adopted. This method was examined for the use of tap water instead of commonly used distilled water.

First, we examined the step for an optimal dilution. Diluted yolk samples (2 to10-fold with tap water) were frozen and the water-soluble fraction (WS) was separated after thawing from aggregated constituents by centrifugation. We have found that the 6- to 8-fold dilutions of yolk resulted in WS having the relative purity and yield of IgY comparable to those obtained by the commonly used 10-

fold dilution [9,10], whereas the 4-fold dilution resulted in a highly opalescent WS not suitable for IgY purification (data not shown). Thus, the 8-fold dilution (as a safer option) was used in further experiments.

In order to simplify the sample handling and to overcome the need of centrifugation, an experimental set-up using the filtration of a gradually melted yolk mixture was tested as an alternative. The yolks were diluted with 7 volumes of tap water and frozen (-20 °C) in a large glass filtration funnel closed with a stopper. The frozen mixture (in a conical shape) was transferred to another filtration funnel (with filtration paper) and allowed to melt freely at the laboratory temperature. Even a conventional filter paper entirely retained the lipid aggregates formed by yolk freezing and provided a clear filtrate – WS of the purity comparable to that resulting from separation by centrifugation. This fraction was further purified as described below.

As a second purification stage, we introduced the fractionation of proteins of WS with NaCl. The salting-out procedure was tested for the pH range of 2-11. At pH 4.0, an extensive IgY precipitation was found even at a low NaCl concentration (1 mol/l). Therefore, the salting-out procedure was examined with regard to NaCl concentrations at pH 4.0 in detail. As judged from SDS-PAGE analysis (see Fig. 1), the optimal IgY precipitation (yield, purity) occurred at NaCl concentrations between 1.4-1.6 moles per liter. In addition, to check the effect of pH on IgY stability, the IgY samples were exposed for 2 hrs to pH 2-11 and their reactivity against antigen (BSA) on ELISA was determined. Figure 2 illustrates that IgY samples incubated at the pH range of 3-11 do not show any decrease in their reactivity relative to control IgY samples kept at pH 7. Under the conditions used, only pH 2 was detrimental to IgY, causing ~ 60% drop in their activity.

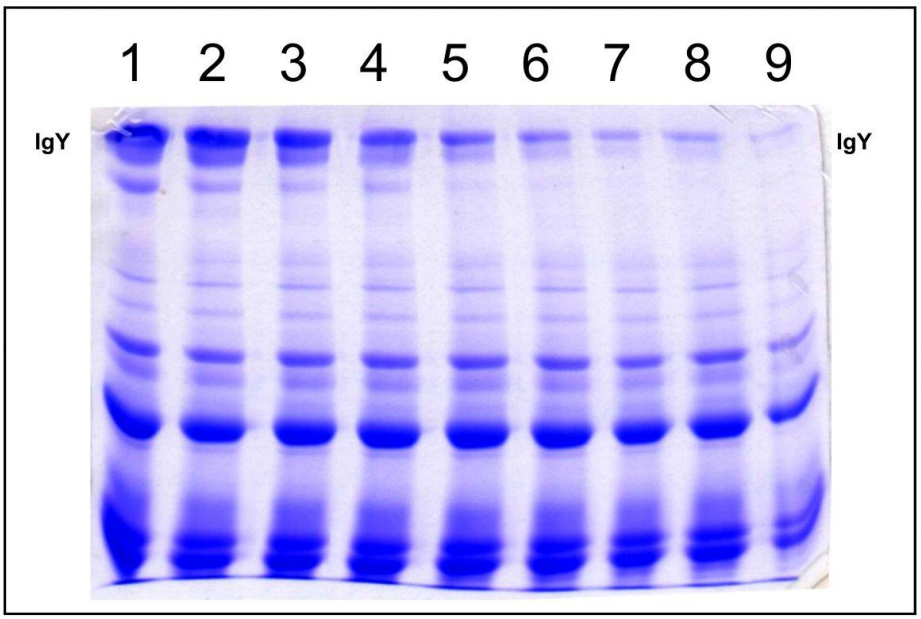

Figure 1. SDS-PAGE of IgY precipitated at increasing NaCl concentrations. IgY samples were precipitated at pH 4.0 with 0 (line 1), 0.6 (line 2), 0.8 (line 3), 1.0 (line 4), 1.2 (line 5), 1.4 (line 6), 1.6 (line 7), 1.8 (line 8), and 2.0 M NaCl (line 9) and after sedimentation equal volumes of supernatants were loaded on non-reduced PAGE. Position of **IgY** label marks protein bands belonging to IgY sub-classes.

To test a possibility of decreasing the NaCl consumption, the IgY precipitation experiments (with 1.5 M NaCl at pH 4.0) were carried out with WS prepared from the lower, suboptimal yolk dilutions, too. These (4- to 6-fold) dilutions resulted in an inferior purity of IgY (see Fig. 3) and also in a decreased IgY yield (by ~ 8%). On the other hand, 10-fold dilution did not represent any improvement in yield and purity of IgY. Thus, the 8-fold yolk dilution seems to be optimal.

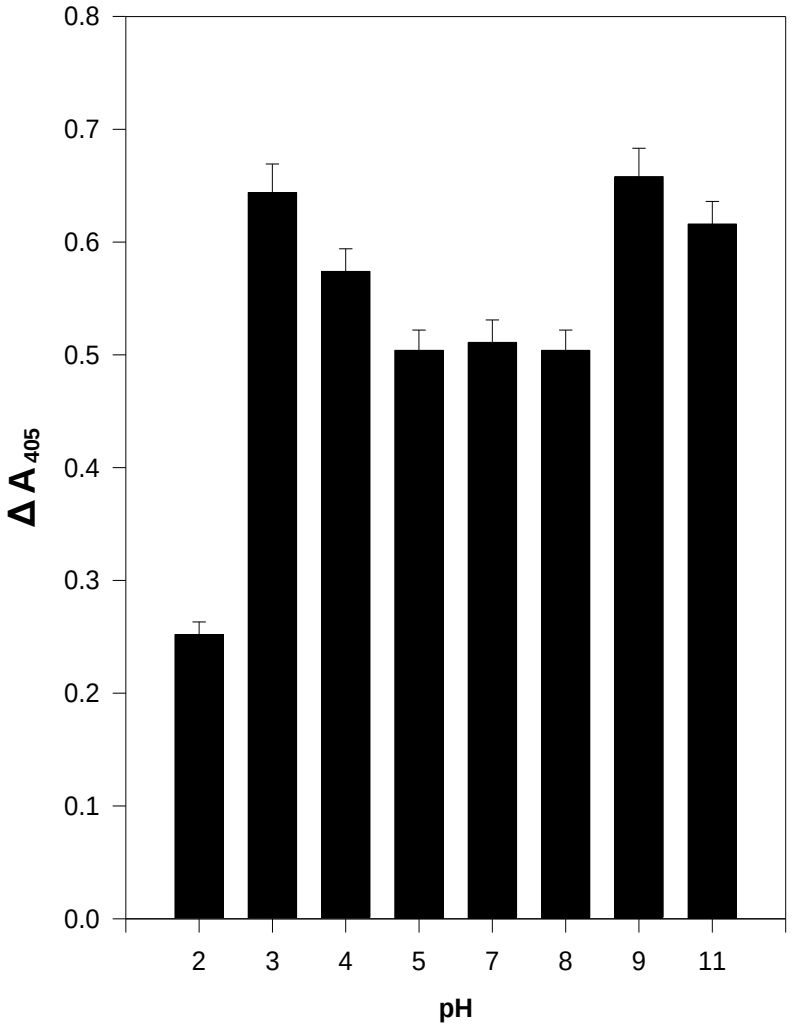

Figure 2. Effect of pH on stability of IgY. IgY samples (0.5 mg/ml) were exposed to various pH values for 2 hrs and then examined on ELISA with BSA as antigen. Immunoreactivity of IgY samples (loaded at 9 μg/ml concentration) is expressed in the plot as a difference in absorbances at 405 nm of anti-BSA IgY samples minus pre-immune IgY controls treated identically. Data are means of triplicates with standard errors plotted.

Based on the above-mentioned results, we developed a new purification protocol consisting of the 8-fold yolk dilution, freeze-thawing followed by a filtration, and IgY precipitation with 1.5 M NaCl at pH 4 (for its scheme, see Fig. 4). The effectiveness (IgY yield and purity of the final IgY preparation) of the developed method was compared with the reference PEG precipitation method. The new protocol results in a higher IgY yield: 8.9 mg of IgY per ml of yolk as compared to 7.2 mg

IgY/ml. The purity of both preparations was tested by SDS-PAGE (see Fig. 5). On non-reduced gels the purity of both samples was comparable, however, on reduced gels the final IgY obtained by the newly developed method contains only the heavy and light chains, whereas two additional protein bands with higher molecular weight are seen in the IgY sample obtained by the PEG method (Fig. 5).

The activities of final IgY samples prepared by both methods were examined by ELISA and Western blotting. Using ELISA and Western blots, specific anti-BSA IgY proved to be equally active in the recognition of BSA antigen regardless the method of IgY preparation (A_{405} = 1.256 ± 0.116 for the new method as compared to A_{405} = 1.306 ± 0.178 for PEG precipitation method).

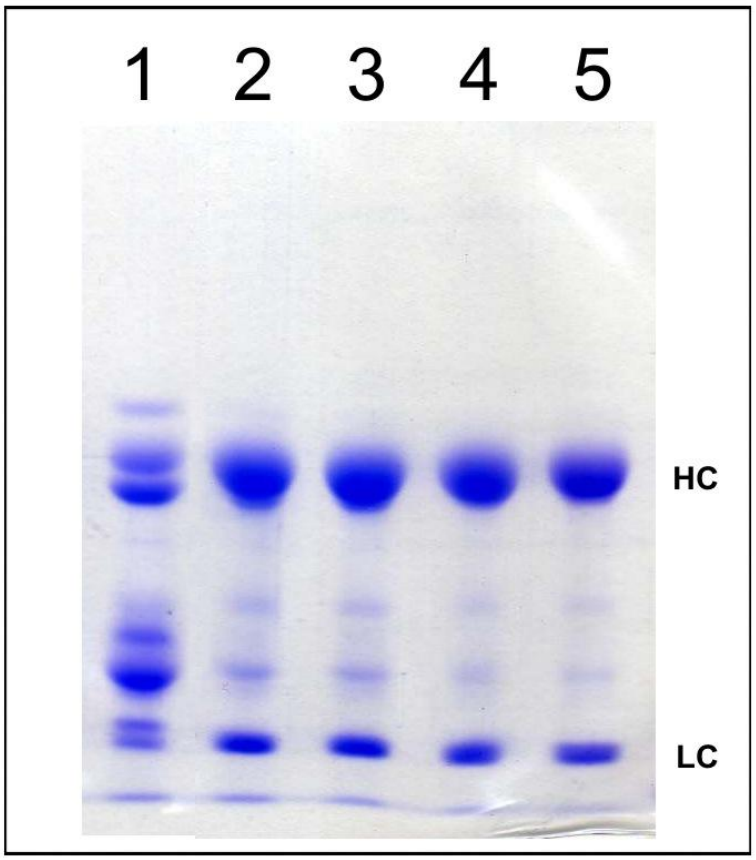

Figure 3. SDS-PAGE of IgY salted-out from 4 to10-folds diluted yolks. WS of yolks (representative WS sample - line 1) diluted 4- (line 2), 6- (line 3), 8- (line 4), and 10-folds (line 5) were precipitated with 8.8% NaCl at pH 4 and dissolved IgY sediments (5 μg per line) were loaded on PAGE carried out under the reduction conditions. Labels **HC** and **LC** show the position of heavy and light chains, respectively.

4. DISCUSSION

The aim of this study was to develop a procedure suitable for IgY purification from chicken egg yolks using bio-compatible chemicals, a procedure as simple and cost-effective as possible in the

terms of required equipment and sample handling. The method we present here fulfills these criteria, offering the following main advantages:

(**i**) in the first stage of the procedure, only tap water and HCl are added to the yolks,

(**ii**) the handling of diluted yolks is very simple, consisting of freezing, transfer of the frozen cone to the filtration assembly and filtration,

(**iii**) for the second stage of the procedure, only NaCl and HCl are added, and the specific IgY precipitation occurs in one step, providing a preparation with sufficient yield and purity.

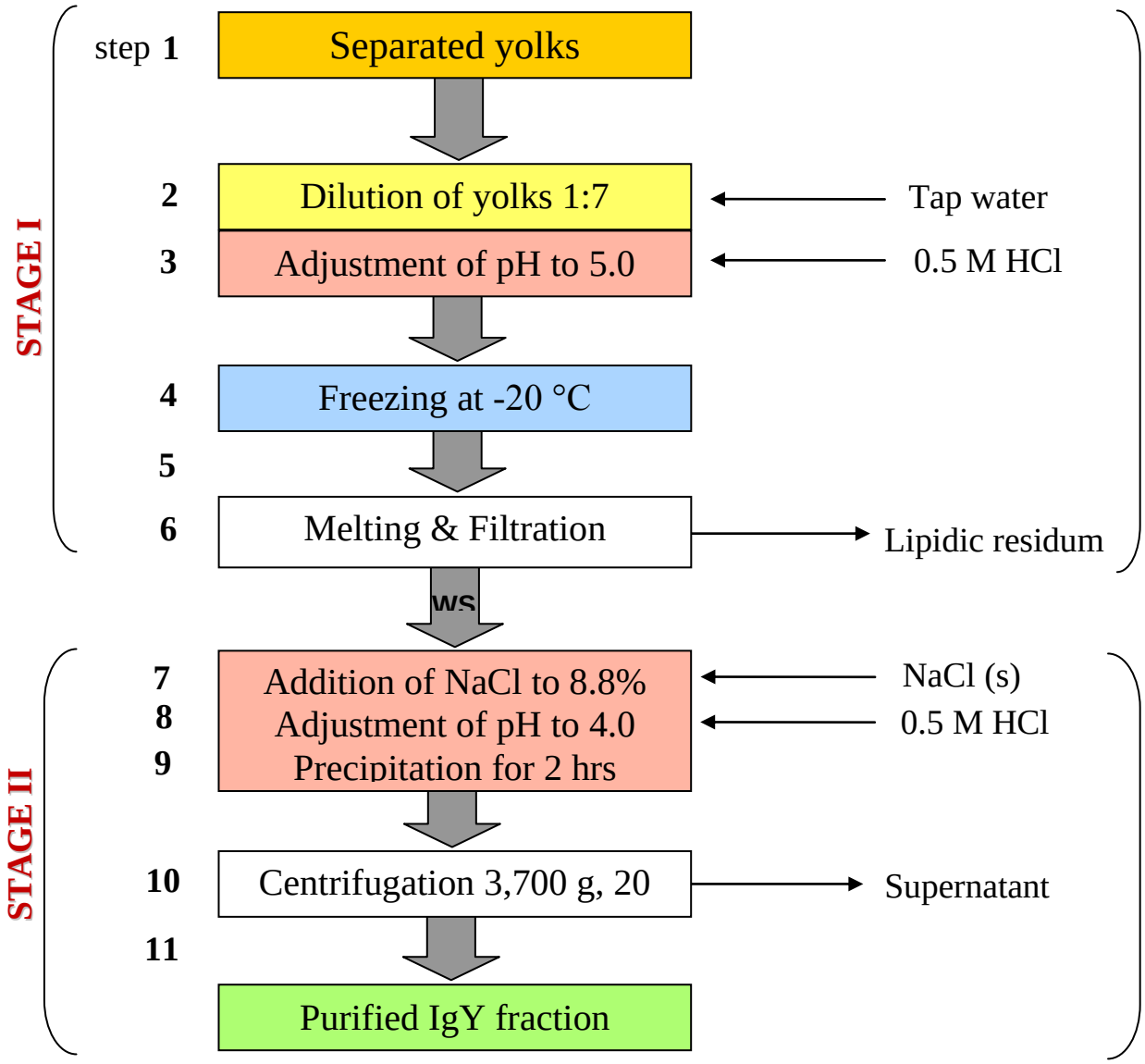

Figure 4. Scheme of the IgY purification protocol. Water-soluble fraction (WS) resulting from the Stage I (steps 1-6) was fractionated with NaCl in the Stage II (steps 7-10). Individual steps: **1**. Separation of yolks (volume measured). **2**. Dilution with 7 volumes of tap water. **3**. Adjustment of pH value (5.0) with HCl. **4**. Freezing of the mixture in a stoppered funnel (-20 °C). **5**. Transfer of the frozen cone to the second funnel with a filtration paper. **6**. Melting at laboratory temperature, collection of filtrate. **7**. Addition of NaCl (to 1.5 M concetration). **8**. Adjustment of pH (4.0) with HCl. **9**. Precipitation & 2 hrs standing at laboratory temperature. **10**. Centrifugation (3,700 g for 20 min at 4 °C). **11**. Dissolving of the sediment in PBS.

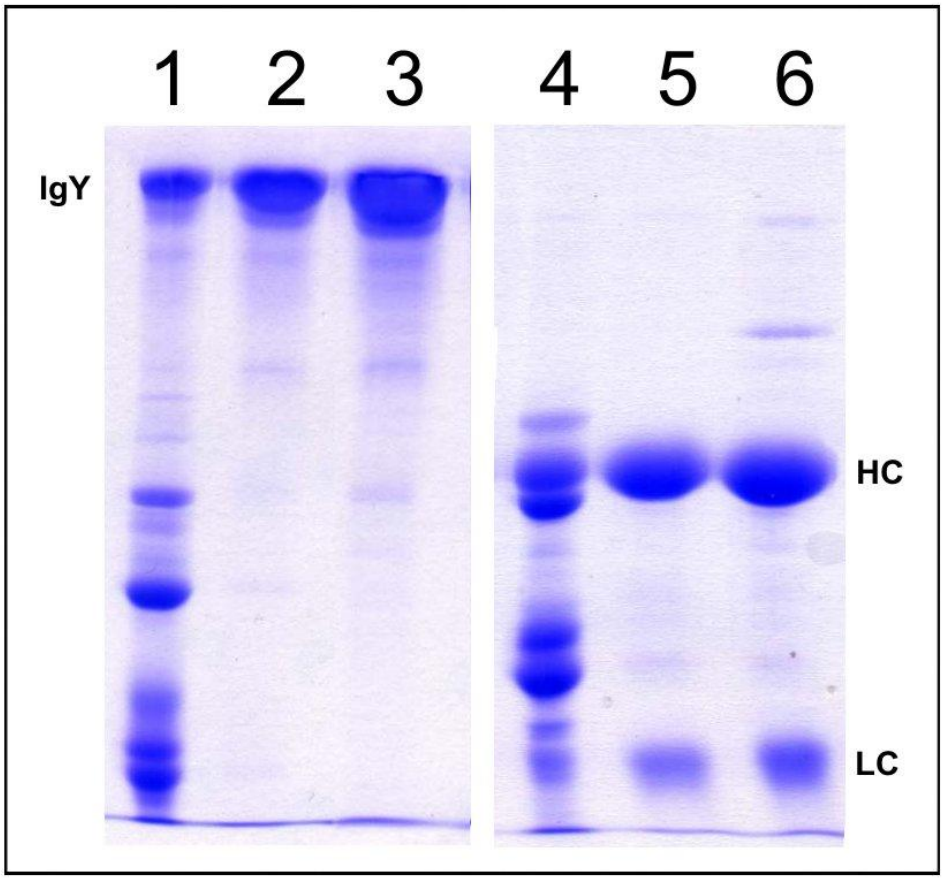

Figure 5. Comparison of final IgY preparations on SDS-PAGE. IgY samples (10 μg per line) resulting from a novel method (lines 2,5) and a PEG method (lines 3,6) were separated on non-reduced PAGE (left panel) and reduced PAGE (right panel). Lines 1 and 4 were loaded with WS of yolks. Labels **IgY, HC** and **LC** show the position of IgY immunoglobulin, their heavy and light chains, respectively.

The major improvement of the isolation procedure, in comparison to the methods described previously, was achieved by the use of acidic pH (4.0) instead of the commonly used values (e.g., 7.2). This allowed for a single step specific precipitation of IgY instead of the usual two-step precipitation. Moreover, the use of acidic conditions resulted in more than 30% reduction of the amount of NaCl consumed. The specific salting-out of IgY at pH 4.0 used for IgY purification is, to our best knowledge, reported for the first time.

The use of pH 4.0 may raise a concern of IgY deactivation, similar to the effects reported by Shimizu et al. [16] and Lee et al. [17]. Our experimental data, however, show that under conditions used for precipitation (2 hr incubation at pH 4.0), no decrease in IgY activity was observed (see Fig. 2). On the contrary, about 25% increase in IgY activity in comparison with a control sample kept at pH 7 was detected under the acidic or alkaline pH 3-4 or 9-11, respectively. This enhancement in antibody binding is associated with the specific IgY only. No analogous increase was detected with the pre-immune IgY treated identically as specific ones. We are not able to provide a full explanation of this fact, but it might be attributed to the release of specific IgY from some complexes as a consequence of

the reversible denaturation of IgY. Such complexes may result from interaction of IgY with some cross/reacting molecule(s) present already in the yolk, or from the purification procedure (e.g., introduction of small amounts of egg-white proteins).

It is worth-mentioning that the change to acidic pH might be helpful also for other IgY purification protocols. In this respect, we tested other precipitants, PEG and sodium sulphate, for salting-out of IgY from WS at pH 4.0. To achieve an optimal IgY precipitation, concentrations lower by at least 35% (7.5% PEG or 8.0% sodium sulphate final concentrations) than commonly used at pH 7.2 were sufficient, and – similarly to the situation with the precipitation using NaCl – one step is sufficient. The purity of the resulting IgY samples corresponded to that of IgY prepared either by NaCl precipitation at pH 4.0 or by repeated fractionation with PEG or sodium sulphate at pH 7.2.

Taking together, the novel protocol reported herein makes the IgY purification of egg yolks as easy and cost-effective as possible. For the first operations (dilution with tap water, pH adjustment, freezing) no costly equipment is required. The filtration set-up reduces sample handling and might be conveniently carried out over night. The precipitation of IgY at pH 4.0 allows the specific IgY salting-out in a single step with the reduced precipitant concentration (by 33%). Although purities of IgY obtained with the novel method and the reference PEG method were similar, the recovery of IgY obtained was higher by at least 24% by the method described here. As the specific antigen binding activity (expressed per mg of protein) of both final IgY samples were comparable, the reactivity of IgY were not affected by low pH during the purification. Moreover, the most common and safe materials (tap water, NaCl) were used within the whole procedure providing the final IgY preparations fully acceptable for various human applications, such as food additives, *peroral* medications or cosmetics.

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