Simple and efficient protocol for immunoglobulin Y purification from chicken egg yolk

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ABSTRACT Besides being a common food component broadly consumed worldwide, egg yolk immunoglobulin Y (**IgY**) has essential therapeutic potentials. In fact, in a time of ever-increasing risk of antibiotic resistance, it is crucial to find new ways to battle infection, and oral administration of preformed specific antibodies represents one of the most attractive approaches against infection. Infectious diseases of bacterial and viral origin in humans and animals can be controlled and passively cured by orally applied IgYs isolated from chicken egg yolks. Despite multiple obvious advantages of oral administration of IgY, harvesting IgY from egg yolk in a pure form is a challenging task.

In this study, we developed a fast, simple, cost-effective, and efficient protocol for IgY isolation from chicken egg yolks. First, egg yolk was collected and diluted with 5 volumes of cold distilled water, homogenized, pH adjusted, and centrifuged. Next, the supernatant was collected, to which caprylic acid at concentration of 2% v/v was added, followed by pH adjustment to pH 5.0, centrifugation at 4°C, and collection of the resulting supernatant. This step was repeated twice, with adding 2% v/v of caprylic acid each time. The final supernatant was concentrated using ultrafiltration, and the IgY purity and activities were checked by SDS-PAGE, western blotting, and ELISA. The sequential (2, 2, 2%) addition of caprylic acid yielded IgY with a purity of 63.5, 90.6, and 95.8%, respectively, and reached 97.9% after ultrafiltration at pH 9.0. The IgY activity increased exponentially to reach 99% after the ultrafiltration step.

The proposed caprylic-acid-based protocol of IgY purification from the yolk of chicken eggs seems to be simple, fast, direct, and very cheap. This indicates that this protocol has great potential for scale-up processing.

Key words: chicken, IgY, purification, structure, benefits

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INTRODUCTION

Immunoglobulin Y (yolk immunoglobulin, **IgY**) is a major low-molecular-weight immunoglobulin produced by oviparous animals, such as chickens (*Gallus gallus domesticus*) and other birds, duck-billed platypus (*Ornithorhynchus anatinus*), reptiles, amphibians, and lungfish (Zhang et al., 2017). Under natural conditions, in order to protect the developing embryo from potential pathogens, large quantities of the serum IgY of laying hens are deposited to the egg yolk (Janson et al., 1995). Researcher interest in IgY is very high, since these avian antibodies have a wide variety of applications within the life sciences, where it can be used as immunodiagnostic and immunotherapeutic biomolecules.

In fact, in addition to its nutritional values and use as an immunological supplement in infant formula, IgY can be bioprogrammed to act against many infectious diseases by immunizing chickens with specific antigens, and these immunized chickens are able to produce eggs with preventative and/or therapeutic potentials (Wang et al., 2011; Spillner et al., 2012). Although the overall structure and functions of IgY are similar to those of mammalian IgG and IgE isotypes (Warr et al., 1995), IgY has many advantages over the other immunoglobulin types, some of which are outlined as follows:

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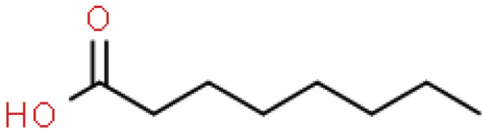
1. Since the concentration of IgY in egg yolk is high (15–25 mg/ml (Akita and Nakai, 1993)), and since it can be recovered from the eggs of laying hens for prolonged periods, IgY can be very easily produced in large amounts and production can be scaled-up, thereby providing a long-term supply of substantial amounts of antibodies (∼40 g/year/hen) (Stadelman and Cotterill, 1973; Hamal et al., 2006),
2. The evolutionary distance between mammals and chicken makes the IgY a unique molecule, which does not interact with the rheumatoid factor, mammalian Fc receptors, and components of the complement system (van de Winkel and Capel, 1993; Spillner et al., 2012),
3. Furthermore, because of the phylogenetic distance between the avian immune system and mammalian proteins, chicken IgY can show an immune response against and/or recognize more mammalian protein epitopes than mammalian IgG (Larsson and Sjoquist, 1990),
4. IgY can be easily tailored against any antigen and produced in the corresponding bioreactor, namely a chicken egg,
5. The production of IgY in eggs helps to avoid the repetitive bleeding of laboratory animals and thereby represents a noninvasive alternative to current methods,
6. Its production is very cheap in comparison to antibodies of blood origin. As a result, the chicken may be considered the IgY factory of choice (Tan et al., 2012),
7. IgY shows particular stability at high temperatures and over a wide pH range (Shimizu et al., 1992).

The desirable potential therapeutic importance of IgY makes harvesting sufficient amounts of this protein in a pure form and at a realistic and reasonable price an important and somewhat challenging task. The main obstacle during the isolation and purification of IgY from the raw egg yolk is the presence of water-insoluble ingredients, such as lipoproteins (Tong et al., 2015). Another issue of working with egg yolk is the high content of the egg yolk phospholipids, which accounts for ∼16% of egg yolk mass, and which can spontaneously form bilayered nanovesicles under the physiological conditions and during IgY isolation (Kondratowicz et al., 2018; Bernardo et al., 2019). These liposome-like structures may contain some egg yolk proteins, including IgY, and subsequently represent one of the main obstacles during IgY purification steps, as well as serve as an important source for impurities in the final isolated IgY samples. To overcome this obstacle during IgY isolation and purification, many approaches were utilized, including the use of water dilution (Akita and Nakai, 1993), chloroform (Polson, 1990), or precipitation with natural polyanionic polysaccharides, such as xanthan (Hatta et al., 1990), dextran sulfate, and pectin (Chang et al., 2000), and κ-carrageenan (Tan et al., 2012), or polyethylene glycol (**PEG**) precipitation method (Marcet et al., 2011; Pauly et al., 2011; Lee et al., 2014), in the preisolation step to exclude the lipid and lipoproteins from the content of the egg yolk.

Using common plant gums pectin and κ-carrageenan in the presence of calcium chloride to delipidate egg yolk mixtures, Tan et al. (2012) got a higher yield (60 mg) of high purity (∼80%) chicken IgY, than any previously reported protocol, including the established PEG extraction method of Pauly et al. (2011). One of the known drawbacks of the (PEG) precipitation method is that it is difficult to remove PEG using dialysis or ultrafiltration (Marcet et al., 2011; Pauly et al., 2011; Lee et al., 2014). Also, the SDS-PAGE showed high levels of impurities in the precipitant (Marcet et al., 2011; Lee et al., 2014). The purity of isolated IgY was significantly improved (∼80%) in comparison with the commercial kits used when delipidation agents were combined with ammonium sulfate precipitation (Tan et al., 2012). However, other researchers reported efficient harvesting of IgY with a comparable purity, yield, and activity simply using ammonium sulfate precipitation without a delipidation agent (Lee et al., 2014).

Other studies used caprylic acid (**CA**) combined with ammonium sulfate for IgY isolation (McLaren et al., 1994) or as a delipidation step during the IgY purification (Araujo et al., 2010; Ren et al., 2016). Although the use of ammonium sulfate precipitation is a very old methodology (Pope, 1939) that represents an attractive protocol for antibody purification, some concerns should be mentioned here. These concerns include the presence of noticeable side effects originating from the use of the therapeutic antibodies purified by this approach (Redwan el, 2006). These include the potential effects of high concentrations of ammonium sulfate on the structure of antibody during purification leading to the (partial) denaturation of immunoglobulins (specifically, when ammonium sulfate is added into the source of antibodies as a solid). As a result, antibodies purified in this way may have altered structures and can cause adverse reactions. Furthermore, this protocol requires a high grade of ammonium sulfate to avoid the companions' nondesirable ions and also needs an excessive dialysis process to eliminate the undesirable materials. Finally, samples produced using this protocol often contain copurified proteins (even if they are minors proteins), which induce nonconvenient immunological reactions as well (Redwan el et al., 2005; Redwan el, 2006). Furthermore, this approach is time-consuming, especially in the scaled-up processing; it is cost-inefficient when analytical-grade ammonium sulfate is used, since cheap lower-grade ammonium sulfate typically includes heavy metal contamination. Wang et al. (2008) enumerated the limitations of the use of ammonium sulfate for antibody purification. For these disadvantages, many companies have moved to other purification protocols instead of using ammonium sulfate precipitation and started to utilize other agents, for example, caprylic acid, for downstream production protocols of their therapeutic antibodies (as discussed further).

CA is a naturally occurring eight-carbon fatty acid found in mammalian milk, palm oil, and coconut oil. CA, which is also known as octanoic acid, is a short or medium-chain fatty acid with the structural formula $CH_3(CH_2)_6CO_2H$:



CA has long been used in the pharmaceutical industry as an albumin stabilizer, non-IgG fraction precipitant, and bactericidal agent (Li, 2019). According to the Food and Drug Administration (FDA) classification, CA is listed as a GRAS (Generally Recognized As Safe) substance that can be used in human foods and therapeutics at levels not to exceed good manufacturing practice (that ranges from 0.04% in cheeses to 0.001% in some other food categories) (https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=184.1025). Furthermore, the main players operating in the anti-venom/toxin market, such as BTG plc. Laboratorios Silanes, CSL Ltd., Pfizer, Merck, Bharat Serums and Vaccines Ltd., Haffkine Bio-Pharmaceutical Corporation Ltd., Vins Bioproducts Ltd., Incepta Pharmaceuticals Ltd., VACSERA Inc., and Rare Disease Therapeutics Inc. regularly use CA for their products downstream (Redwan el, Khalil et al., 2005; Redwan el, 2006; Redwan el et al., 2009; Kurtovic et al., 2019).

The goal of this study was to design a simple, efficient, cost-effective, and reproducible protocol for the lab-scale IgY purification from the yolk of chicken eggs utilizing only CA at a certain concentration and pH.

MATERIAL AND METHODS

All aqueous solutions were prepared using deionized water. All chemicals utilized in this study were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Chicken Egg Collection and Yolk Processing

Fresh hen eggs with white shell laid within 72 h were collected from the food-stuff market, kept in the laboratory refrigerator for 1 week, and then used for yolk harvesting. The hen eggs were manually broken, and most albumen (egg white) was eliminated through soaking in cold water, then the resulting sample was carefully rolled on a filter paper to remove chalazion (egg yolk supporting membrane) and albumen adhering to the vitelline membrane. The membrane was then perforated to collect unspoiled egg yolk in a beaker cooled on an iced bath. The yolk was end-to-end mixed for 10 min, then diluted with cold water. The pH of the mixture was adjusted to be close to pH 5.0–4.9, and the resulting sample was left for 2 h at room temperature (RT) with gentle mixing, then centrifuged (3,800 rpm for 30 min at 4°C), and the supernatant was collected, while the pellet was discarded (Figure 1).

Downstream Isolation and Purification of IgY From the Yolk of Chicken Eggs

Downstream IgY isolation and purification were done by drop-wise adding of total 6% CA to the supernatant in 3 steps. At the first step, 2% of CA was added to the supernatant in a drop-wise manner with gentle mixing (350–750 rpm), then the pH was readjusted to pH 5.0 (Figure 1). The mixture was left on the stirrer for 30–35 min at RT, then centrifuged for 30 min at 3,800 rpm and 4°C. The solidified disc was carefully removed from the top of the sample, then the supernatant was collected, and precipitated material was discarded. The resulting supernatant was used for the second round of CA treatment, where an additional 2% of CA was introduced to the sample, which underwent the aforementioned processing (Figure 1). The whole procedure was repeated once again with the addition of another 2% portion of CA. The final supernatant obtained as a result of the divided (2% + 2% + 2%) addition of 6% CA was ultrafiltrated to concentrate the IgY sample, eliminate the low-molecular-weight components dialysis, and change buffer to 50 mmol Tris-HCl, pH 9.0 (Kim and Nakai, 1996) using Amicon Ultra-15 centrifugal filters (Ultracell-100K, Millipore, Darmstadt, Germany). The protein concentration in

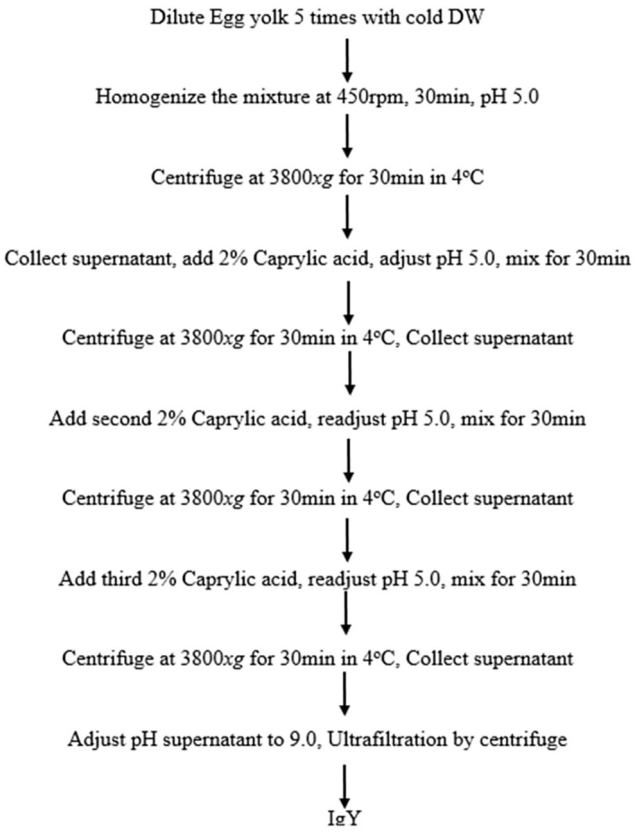


Figure 1. Schematic diagram for the isolation protocol of chicken IgY from egg yolk, DW (distilled water).

Table 1. Extraction of the water-soluble proteins (WSPs).

Extraction methods	Volume (mL)	ELISA OD (405/mL)	Total yield (405 nm)	%
Distilled water (DW) 1:10	90	0.723	65.0	100
Distilled water (DW) 1:5	45	1.05	47.3	72.7
Caprylic acid-B	45	1.113	50.0	76.9
Caprylic acid-A	38	1.510	57.4	88.3

Egg yolk volume used/method: 10 mL, Caprylic acid-A: Caprylic acid was added to the supernatant of centrifuged water dilution (1:5) step. Caprylic acid-B: Caprylic acid (3%) was added to the diluted egg yolk before centrifugation.

the supernatant and precipitate fractions was evaluated using the Pierce Coomassie Plus (Bradford) Protein Assay based on Bradford method (Bradford, 1976). A microplate assay protocol described in the producer manual was utilized, and the standard curve was prepared using BSA.

SDS-PAGE and Immunoblotting

The purified IgY samples were analyzed by 12% SDS-PAGE according to Laemmli (Laemmli et al., 1970) under nonreducing conditions. The gels were stained with Coomassie Blue R-250 (Bio Rad, Hercules, CA) using the standard protocol or transferred into the PVDF membrane, then stained with rabbit anti-IgY alkaline phosphatase conjugate according to the protocol described by Towbin et al. (1979). Briefly, the purified IgY protein was separated by gel electrophoresis then electroblotted onto the PVDF membrane using a Hoffer TE22 Mini Trans-Blotter system (Hoefer, Holliston, MA) for 2 h at 25V. After blotting, membranes were blocked using 2% gelatin, 50 mmol Tris-HCl, 0.1% Tween 20 buffer at 37°C for 60 min, then diluted (1:6000) alkaline phosphatase-conjugated AffiniPure Rabbit anti-chicken IgY (#303-055-003; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was added to the blocking buffer, and the incubation continued for additional 60 min. After thoroughly washing, the membrane was developed for a few minutes with the ready-made BCIP/NBT Liquid (BioShop, Burlington, ON, Canada), and the development was stopped by washing the PVDF membrane thoroughly with water (Redwan, 2002, 2012).

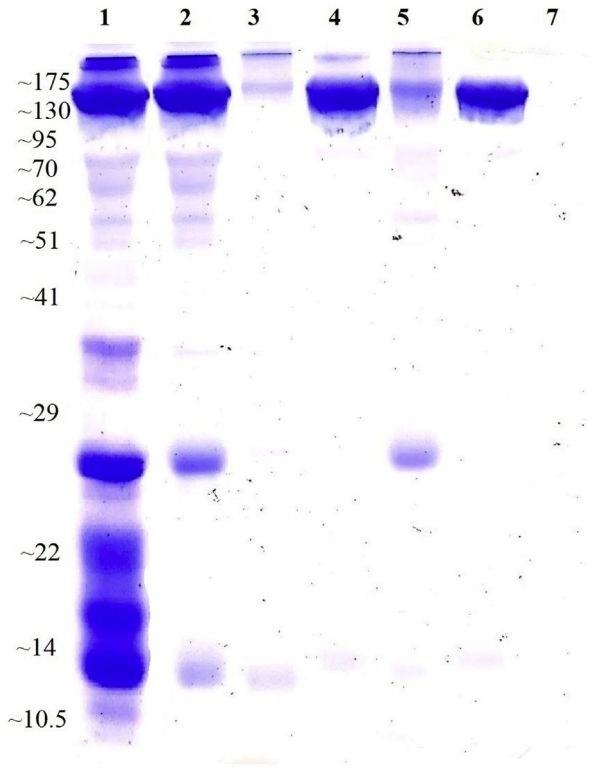


Figure 2. Twelve percent nonreducing SDS-PAGE profile of sequential caprylic acid addition. Lane 1 is a crude sample of WSF, lanes 2, 4, and 6 are supernatant, whereas lanes 3, 5, and 7 are precipitates of the sequential 2 + 2 + 2% addition of caprylic acid, respectively.

ELISA Assays and Purity of IgY

Both SDS-PAGE and ELISA were used to estimate the purity of the isolated IgY. The purity of IgY protein in SDS-PAGE was calculated by converting the density of protein bands in the gel picture using the ImageJ software (National Institutes of Health, Bethesda, MD) as the percentage of the total gel density (Lee et al., 2014). On the other hand, ELISA assays were carried out to quantify and check the IgY purity at each stage of the purification procedure. Standard curves were prepared using the IgY (Sigma-Aldrich, Taufkirchen, Germany) with serial dilutions. For each standard, the content of IgY in protein fractions (precipitates and supernatants) collected at each purification step was estimated by coating the 96-well microplate (Coster, South Elgin, IL) with 50 µL/well of 10 µg/mL of target fraction in 50 mmol Tris-HCl, pH 8.8 (**TBS**) followed by incubation for 2 h at room temperature, then overnight at 4°C. After washing the wells 4 times with a TBS solution containing 0.05% Tween 20 (**TBST**), 50 µL/well of 2% gelatin in TBST was added as a blocking buffer. The plate was incubated for 1 h at 37°C, then washed 4 times with TBST. Three dilutions of samples were added to duplicate wells. Water-Soluble Fraction (**WSF**) of egg yolk prepared according to Akita and Nakai (Akita and Nakai, 1992) represents the positive samples during the estimation of IgY concentration and comparison. Plates were incubated at 37°C for 1 h and washed again. Diluted (1:50.000) alkaline phosphatase-conjugated AffiniPure Rabbit anti-chicken IgY (Jackson ImmunoResearch Laboratories, West Grove, PA) in TBST was added into triplicate wells and incubated for 1 h at 37°C. After washing 4 times with TBST, 50 µL/well ready-made PNPP

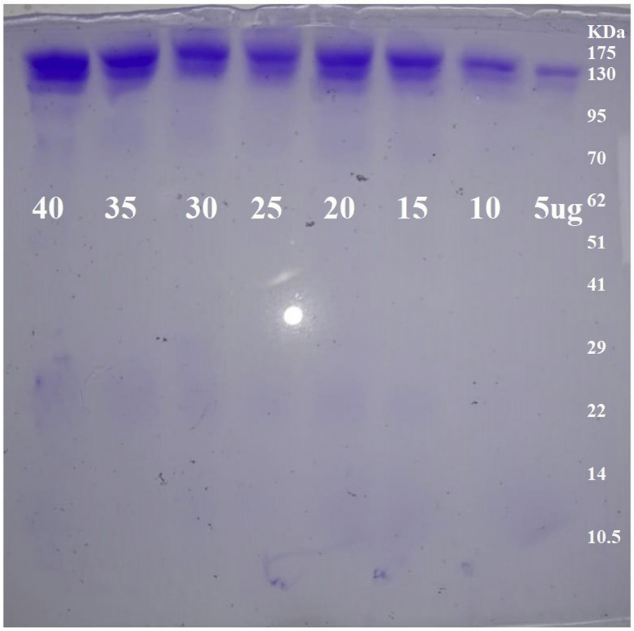


Figure 3. Twelve percent nonreducing SDS-PAGE profile of serial dilution (40-4 μg/well) of IgY after ultrafiltration.

substrate solution (*p*-Nitrophenyl Phosphate, Disodium Salt (Sigma-Aldrich, Taufkirchen, Germany) was added, and the plates were incubated at room temperature for 30 min. The absorbance at 405 nm was read in an ELISA reader (Multiskan EIA, Thermo Fisher Scientific, Waltham, MA).

Exosome Extraction

Exosome or liposome-like structures were isolated from the purified egg yolk IgY before the ultrafiltration step and from the WSFs (Akita and Nakai, 1992) using the InVitrogen (Carlsbad, CA) extraction kit according to the company instructions. The precipitated material was checked by SDS-PAGE.

Statistical Analysis

The data were analyzed using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA). Differences in mean values were compared by student's *t*-test. All values were expressed as the mean ± SD and were considered statistically significant when $P < 0.05$. The entire purification process was repeated 3 times.

Table 2. Estimation of IgY distributed in supernatant and precipitate after caprylic acid (CA) purification.

	Protein concentration				Anti-IgY reactivity in sup		
CA (v/v)	mL	mg	%	IgY purity	mL	A405	%
WSF	35	53	100	49.6%	35	0.611	100
2%	35	51	96	63.5%	35	0.571	93.5
2%	33	50	94	90.6%	33	0.585	95.7
2%	31	48	90.6	95.8%	31	0.600	98.2
aUF	30	46	86.8	97.9%	30	0.605	99.0

Abbreviations: A405, absorbance at 405OD; aUf, after Ultrafiltration; CA, Caprylic acid; ppt, precipitate after centrifugation; sup, Supernatant after centrifugation; WSF, water-soluble fraction.

The soluble protein concentration sample was 1.51 mg/mL.

RESULTS AND DISCUSSION

After egg yolk was diluted (1:5) with cold water (Figure 1), a set of preliminary experiments were conducted to select the appropriate CA percentage and appropriate addition time. These experiments were needed to check if the addition of CA to diluted egg yolk before centrifugation would be more effective than after the centrifugation. Results presented in Supplementary Figure 1 (panel A and B) clearly indicate that the addition of CA after centrifugation is more effective, since the PAGE profile showed lesser load and lower intensity of different protein bands. Tabulated data on the total yield (Table 1) supported this conclusion as well. Based on the previous reports (McLaren et al., 1994; Araujo et al., 2010; Ren et al., 2016), it was clear that 6% CA should work for IgY isolation. Therefore, we decided to use the same CA concentration but utilize the divided addition approach. Supplementary Figure 2 shows the results of such sequential addition of CA (3% + 3%) at pH 5.0. The addition of the first 3% of CA did not cause much change in comparison with the crude extract (lanes 1, 2, and 3), since only a single band (∼23 KDa) had been removed. On the other hand, the addition of the second 3% of CA (lane 4 and 5) resulted in the decrease in the number of contamination bands (from 10 to 4 bands) and their intensities in the supernatant, while the precipitate contained ∼20% of IgY accompanied by 3 contamination bands. The final results seem to be similar to those reported earlier (McLaren et al., 1994; Ren et al., 2016), although previous studies used the addition of 6% CA at once. Since it seems logical to remove contaminants in steps, we decide to use sequential or divided addition of CA as 2 + 2 + 2%.

To select the optimal pH for CA, a range of pH was scanned. After the diluted egg yolk was centrifuged, the supernatant collected and 2% CA were added, the pH was adjusted to a range from pH 4.0 to pH 6.6. The results obtained for the pH 4.0–5.0 range and 2% CA are depicted in Supplementary Figure 3, which shows that the number and intensity of the non-IgY bands were the lowest at pH 4.9–5.0, but increased exponentially outside this pH range to reach the highest values at pH 4.0–4.3. A nearly similar profile was obtained by using the pH range of pH 5.10–5.90 (Supplementary Figure 4), within the narrow pH range of pH 5.1–5.4 showing an IgY purity comparable to that seen at pH 4.9–5.0 (Supplementary Figure 3), then the number and intensity of the non-IgY proteins bands increased exponentially from pH 5.5 to pH 5.9 (Supplementary Figure 4A). On the other hand, the use of the pH 6.0–6.6 range did not show any significant decrease in non-IgY proteins (Supplementary Figure 4B).

Based on these observations, it seems that the pH range 4.90–5.10 is the best pH interval, at which CA

Table 3. Turbidity and total yield of IgY distribution in different purification steps.

Item	Turbidity (600 nm)	Volume	IgY mg/mL	Total IgY (volume X IgY)
Diluted yolk (1:5)[1]	20.268	45	1.45	65.25 (97.6%)[4]
CA 2%	7.560[3]	35	1.39	48.65 (72.8%)[4]
CA 2 + 2%	0.200[3]	33	1.33	43.89 (65.6%)[4]
CA2+2 + 2%	0.060[3]	31	1.25	38.75 (57.9%)[4]
aUF	0.013[3]	30	1.76	52.80 (78.9%)[4]
WSF (1:10)[2]	0.011[3]	90	0.743	66.87 (100%)

aUF: after Ultrafiltration; CA, caprylic acid.
[1]diluted egg yolk (1:5).
[2]WSF: Water-Soluble Fraction (1:10) (Akita and Nakai, 1992).
[3]significance $P \leq 0.05$ in comparison to diluted yolk (1:5) value.
[4]significance $P \leq 0.05$ in comparison to diluted yolk (1:10) value.

efficiently precipitates the most of non-IgY egg yolk proteins. These results seem logical taking into account that CA pKa is 4.89, which likely plays a role in determining the pH range for the selective precipitation (Li, 2019). When CA is at pH above its pKa, the ionized form is dominant, whereas, at pH below pKa, the nonionized form is dominant. With a pH around its pKa, the soluble nonionized form may have the maximal population (Li, 2019). The capability of CA to bind and precipitate proteins depends on its ionization state (Bull and Breese, 1967). During the purification of the monoclonal antibodies from the human culture supernatants, the best pH ranges for the precipitation of non-IgG proteins were 5.0–5.4 and 5.0–6.0 (Zheng et al., 2015) depending mainly on the isoelectric points (*pI*) for both the monoclonal antibodies and the host cell proteins.

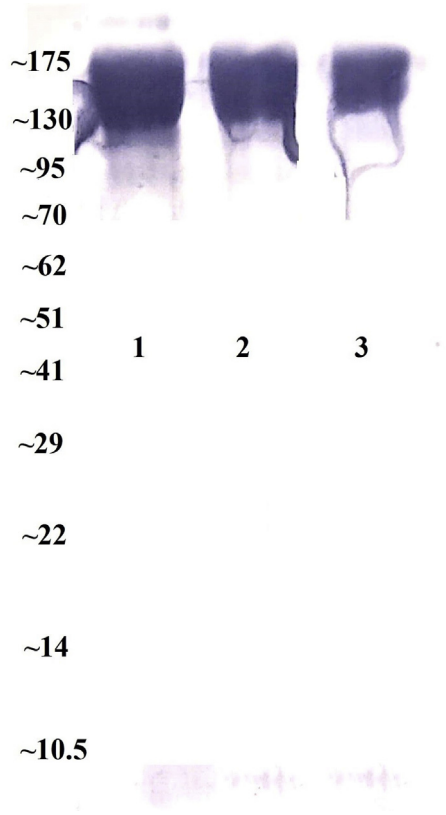


Figure 4. Twelve percent nonreducing SDS-PAGE-western blot of purified IgY. Lanes 1–3 are IgY concentrations 20, 15, 10 μg/well.

Therefore, our results clearly showed that pH 4.9–5.10 represents the optimal pH range, where CA exists in a soluble nonionized form (Li, 2019), which seems to be most suitable for precipitation of non-IgY proteins from chicken egg yolk. Figure 2 shows the results of the sequential addition of CA (2 + 2 + 2%) at pH 5.0. The non-IgY bands gradually disappeared from the supernatants (Figure 2, lanes 2, 4, 6) to appear in the corresponding precipitates (lanes 3, 5, 7). On the other hand, Figure 3 shows the serial dilution of the pure IgY after the ultrafiltration step. The IgY purity (Table 2) improved from the first to third addition of 2% CA. At the same time the turbidity, which coincides with lipid content (Ko and Ahn, 2007), of the supernatant was significantly decreased (Table 3). It efficiently decreased while moving from the diluted yolk (1:5) to the first, second, and third additions of 2% CA (pH 5.0). These results indicate a more efficient delipidation by using CA in comparison with previously used protocols (Ren et al., 2016) and also show that the CA-driven delipidation at pH 5.0 removes lipids and lipoproteins more efficiently than at any other pH (Akita and Nakai, 1992; Ko and Ahn, 2007). The presence and content of the liposome-like structures (exosomes) were analyzed for the IgY samples at different purification stages before ultrafiltration. Supplementary Figure 5 represents the corresponding results and shows that this plot is generally similar to the profile in Figure 3 except for the presence of minor bands at low molecular weight. This subject needs further analysis to clarify the nature of these newly appeared bands.

One should keep in mind that egg yolk is rich in different phospholipids, which can be involved in liposome formation (please refer to (Kondratowicz et al., 2018; Ahmed et al., 2019; Bernardo et al., 2019) for some illustrative examples). Therefore, any IgY isolation protocol that involves dilution, mixing, and vortexing steps generates an appropriate set of conditions for the liposome formulation and subsequent entrapment of some of the surrounding proteins. We explored this point by extracting and analyzing exosomes (which is a type of natural phospholipid vesicles with a diameter of 100–200 nm). Our analysis showed that exosomes extracted from the WSF of egg yolk contained some co-purified minor proteins, whereas exosomes extracted from the purified egg yolk IgY before the ultrafiltration step

did not contain copurified minor proteins (see Supplementary Figure 5). This also gives us a clear indication that the utilization of CA does not only allow efficient isolation of the IgY, but also removes lipid as also shown in previous studies.

Using our protocol, the reactive IgY recovery was 99.0% with purity reaching 97.9% (Table 2), and the final yield being 79.9% (Table 3). These results were confirmed by western blot analysis (Figure 4). Many studies reported various protocols for IgY purification showing similar or lower recovery and purity percentage (Schade et al., 2005; Ko and Ahn, 2007; Araujo et al., 2010; Pauly et al., 2011; Lee et al., 2014; Ren et al., 2016). Most of these studies used ammonium sulfate or PEG precipitation, sometimes combined with the CA treatment. The IgY yield reported in Table 3 seems to be comparable with the recently reported yield of IgY purification by ammonium sulfate precipitation (Lee et al., 2014) and noticeably exceed IgY yields reported in any published work using CA as a first step (Ren et al., 2016), or combining it with ammonium sulfate (McLaren et al., 1994). The brilliant advantage of our protocol, in addition to its simplicity, high efficiency, fast speed, and low cost, is its suitability for all applications, ranging from various laboratory purposes to analytical uses and to therapeutic applications. Furthermore, our data are in sharp contrast to a previous study by Svendsen et al. (1995), who concluded that CA was not able to precipitate egg yolk proteins and therefore considered it useless for IgY isolation (Svendsen et al., 1995). Instead, these authors used dilution and freeze-and-thaw cycling as a first step of IgY separation from egg yolk followed by the precipitation with 25–45% of ammonium sulfate, which resulted in 58% yield of IgY with high purity (Svendsen et al., 1995). It is likely that the inability of (Svendsen et al., 1995). to precipitate egg yolk proteins with CA could be attributed to the failure of these authors to find appropriate conditions for successful precipitation.

Curiously, most, if not all, previous studies dedicated to the development of protocols for IgY purification used sample treatment with CA as a preliminary delipidation step. Furthermore, some of those studies compared the efficiency of this CA-based step with the effects of polysaccharides on lipid removal and subsequent IgY purity and yield (McLaren et al., 1994; Ren et al., 2016). However, none of these studies utilized CA as an essential, or the only, reagent to purify the IgY. In contrast, our approach is based on a simple idea of using CA as a single reagent to achieve our goal. As far as we know, there is no study where CA would be used as a single reagent to isolate IgY from the egg yolk.

CONCLUSIONS

The report presents a simple, quick, safe, and cheap protocol for IgY purification from the yolk of chicken eggs using a single and environmentally friendly chemical agent, CA. We show here that a sequential addition of CA yields pure and active IgY, avoiding the use of any delipidating and/or harmful agents. CA acts as a double-acting agent, ensuring delipidation and purification of IgY. Current results combined with previous observations suggest a potential scalability of our protocol. Therefore, the proposed protocol makes chicken egg IgY production a promising approach for unlimited purposes and applications.

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Availability of data and materials: The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

DISCLOSURES

The authors declare that they have no competing interests.

SUPPLEMENTARY DATA

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