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Purification of Avian IgY with Trichloroacetic Acid (TCA)

Hellen Asemota[1], Suzette Curtello[1], Angel Alberto Justiz Vaillant[2]*, Wayne Mohammed[2], Sehlule Vuma[2], Chalapathi Rao AV[2], Arvind Kurhade[2], Shivnarine Kissoon[2], Monica Smikle[3], Brian Wisdom[4] and Geeta Kurhade[5]

[1]*Department of Basic Medical Sciences, The University of the West Indies, Mona, Jamaica*
[2]*Pathology and Microbiology Unit, Department of Para-Clinical Sciences, The University of the West Indies, St. Augustine, Trinidad and Tobago, WI*
[3]*Department of Microbiology, The University Hospital of West Indies Mona, Jamaica*
[4]*School of Biology and Biochemistry, Medical Biology Centre, The Queen's University of Belfast, Belfast BT9 7BL, UK*
[5]*Physiology Section, Department of Pre-clinical Sciences, The University of the West Indies, St Augustine, Trinidad and Tobago, WI*

Abstract

By using trichloroacetic acid (TCA) precipitation to separate egg yolk proteins, mainly IgY was demonstrated that this procedure yield twice more IgY that its counterpart polyethylene glycol (PEG). An ELISA demonstrated that it does not affect the functionality of the IgY molecule. The TCA precipitation procedure provides an efficient and rapid way to purify IgY antibody. It can be used for large scale production of high quality IgY.

Keywords: Trichloroacetic acid (TCA); Polyethylene glycol (PEG); Immunoglobulin Y (IgY); ELISA; Layer hens; Egg yolk

Introduction

Purifying IgY from the yolk of avian egg is of particular interest as a source of specific antibodies for oral immunization to prevent infection [1]. Oral administration of egg antibodies has been successfully used to prevent bacterial infection in animals, including *E. coli* infections in piglets [2] and dental caries caused by *Streptococci* species in rats [3]. IgY antibodies have been used in the immunodiagnosis of several infectious diseases [4].

For the isolation of egg yolk, IgY is compulsory the removal of lipids, which can be found in the egg yolk in high concentrations [5]. Several techniques have been reported for the lipid separation, including use of caprylic acid [6], filtration with hydrophobic membranes [7], hydrophobic interaction chromatography [8], and water dilution method [9]. Livetins are egg yolk proteins that coexist with IgY, which is in extremely high concentrations.

For the isolation of IgY from livetins, several techniques have been improved for its quality and yield. Examples of these methods are alcoholic precipitation [10,11], salt precipitation using ammonium sulphate or sodium sulphate, followed by centrifugation [12] and cation exchange chromatography [13,14]. Among the IgY extraction techniques, the use of chloroform has been used to separate lipids from the watery soluble fraction (WSF) [15].

In general, the action of TCA on all the proteins used can be classified to occurring in three stages. In the first stage, occurring below 5% TCA concentration, most of the protein is found to remain in solution. However, in stage 2, occurring between 5% and 40% TCA concentrations, most of the protein precipitates. When the acid concentration are raised above 50% (stage 3), all the proteins is found to redissolve back into the solution. The results of these experiments show that the presence of three chloro groups (on the alpha-carbon atom) in the acetic molecule is important for the protein-precipitating action, in addition the pH of the solution is not the dictating force in inducing protein precipitation [16], which is more effective than the PEG method, where the PEG-precipitation is highest when the solution pH is near the isoelectric point of the target protein [17].

In this study, we combined the used of chloroform with TCA precipitation to separate IgY from laying hen's egg yolk. We described the superiority of the TCA technique that for its simplicity and high yield can be used by any laboratory working on IgY technology.

Material and Methods

Ostrich and laying hen eggs were purchased in a market and carried to the Molecule Biology Laboratory of the University of the West Indies, Mona Campus, in Jamaica for analysis.

Immunoglobulin Y isolation and determination by Direct ELISA

The IgY fraction was isolated from the egg yolks of laying hens and ostrich eggs. The IgY fraction was isolated by the chloroform-trichloroacetic acid procedure that we reported for effective IgY separation. The eggs were washed with warm water and the egg yolk was separated from the egg white. The membrane was broken and the egg yolk was collected and diluted 1:4 in phosphate buffered saline (PBS), pH 7.4. It was added an equal volume of chloroform, the mixture was then shaken and centrifuged for 10 min (2000×g at 18°C). The supernatant was decanted and treated with cold 25% TCA and stirred and incubated for 10 min at 18°C. The mixture was then centrifuged as previously described. The precipitate containing the IgY was dissolved in PBS (pH 7.4) at a volume equivalent to 1/10 the original volume of the egg yolk and dialyzed against 1L of PBS (pH: 7.4 for 24 h at 4°C). The IgY was removed from the dialysis tubing. IgY concentration was determined by the Bradford method. IgY samples were stored at -20°C.

The chloroform-PEG technique [15] was used to be compared with the chloroform-TCA technique. Briefly, the eggs were washed with warm water and the egg yolk was separated from the egg white. The membrane was broken and the egg yolk collected and diluted 1:3 in Phosphate Buffered Saline (PBS), pH 7.4. To a third part (1/3) of the egg yolk mixture, an equal volume of chloroform was added, the mixture was then shaken and centrifuged for 30 min (1000×g, at room

***Corresponding author:** Angel Alberto Justiz Vaillant, Pathology and Microbiology Unit, Department of Para-Clinical Sciences, The University of the West Indies, St. Augustine, Trinidad and Tobago, WI, E-mail: avail4883@gmail.com

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temperature). The supernatant was decanted and mixed with PEG 6000 (12%, w/v), stirred and incubated for 30 min at room temperature. The mixture was then centrifuged, as previously described [6]. The precipitate containing IgY was dissolved in PBS (pH 7.4) at a volume equivalent to 1/6 of the original volume of the egg yolk and dialyzed against one litre (1L) of PBS (pH 7.4 for 24 h at 4°C). The IgY was removed from the dialysis tubing and its concentration was determined by using the Bradford method. IgY samples were stored at -20°C.

A direct ELISA was used to determine the presence of avian egg yolk IgYs as follows: 96 well microtiter plates were coated overnight with LPS of *S. typhimurium* at 4°C with 2 µg/well in 50 µl of carbonate-bicarbonate buffer pH 9.6. Plates were washed 4X with 150 µl PBS-Teen 20 buffer. Then duplicated serial dilutions of 50 µl of IgY (5 µg/µl) from laying hen and ostrich were added and incubated for 1 hr. The microplates were washed as previously described, and after that, 50 µl of a commercial anti-IgY-HRP diluted 1:30,000 (Sigma-Aldrich Co) in PBS-non-fat milk was added to each well and incubated for 1 h at room temperature (RT). The plates were washed 4X with PBS-Tween. 50 µl of 4 mg/ml o-phenylenediamine solution (OPD) was added and the plates were incubated 15 minutes at RT and recorded the presence of color in positive samples and the lack of colour in negative samples. Appropriate positive and negative controls were included. The positive control was a pooled laying hens sera with a high titer (1:4096) of anti-*Salmonella typhimurium* from clinical cases and the negative controls was a laying hens pooled sera with low triter (1:4).

Results and Discussion

The Bradford method demonstrated that the TCA method yield the double IgY amount as compared with the classic chloroform-Polyethylene-glycol (PEG) method, as seen in Table 1. The chloroform-TCA method produced more IgY from laying hens and ostrich than its counterpart the chloroform-PEG method.

Both methods use chloroform to separate the lipids from the WSF. The TCA method yielded more laying hens IgY: 200-250 mg/egg, as compared with 4200-5000 mg/ml yield from the egg yolk of ostrich. The PEG method yielded lesser IgY: 100-130 mg/egg yolk of laying hens and 2000-2200 mg/egg yolk of ostrich. These differences in the IgY yield are owed to the differences in the egg yolk size of the two bird species, and in addition, in biochemical properties of both PEG and TCA, this last one precipitates larger amount of proteins than PEG. It was demonstrated that the TCA method was more effective for IgY separation.

A limitation of this study was that we could not immunize with a specific antigen to an ostrich, we assessed the IgY quality (functionality of both Fc and Fab regions of IgY), by measuring the capacity of an ELISA to detect natural antibodies against *Salmonella typhimurium*. Since much farmed and wild birds developed during their life anti-*Salmonella* spp, which demonstrate that birds at least once has been in contact with the microorganism, may be by picking food from the soil, bird droppings, ecetera. A titer of 1:256 and 1:128 was recorded, respectively, for laying hens and ostrich using the TCA method, and 1:64 and 1:32 titer was recorded for laying hens and ostrich, respectively, using the PEG method. Once again, the TCA was more effective, displaying a higher titer of antibody than the PEG procedure, as shown in Table 1.

The TCA purification method yields the double of IgY than the classic PEG method [15]. The TCA method proved that it did not have adverse effect on the antibody activity, since titers of *anti-Salmonella* spp were within the normal limits. It was found that the chloroform-polyethylene glycol method yielded 2.57 times more IgY than the conventional polyethylene glycol method [15]. The ratio of titres of IgY anti-Jasus lalandii haemocyanin antibody purified by the two procedures was very nearly 2.57, indicating that the chloroform had no adverse effect on the antibody activity [11]. This study demonstrates that the chloroform-TCA procedure is the one that more IgY yields among the other precipitation methods. Polyclonal IgY antibodies from chicken egg yolk constitute an alternative to the production of mammalian IgG type antibodies in mammals [18,19]. Chicken egg yolk antibodies have been used for prophylaxis and therapy of infectious intestinal diseases [2].

Even macroscopically, we visualized that the TCA-precipitation in the eppendorf microtubes were twice the amount in comparison with the PEG-microtubes. This was also confirmed by the Bradford method and the ELISA for anti-*Salmonella* antibody detection, as shown in Table 1. Furthermore, we assessed IgY protein in an ELISA for proving whether or not the chloroform had no adverse effect on the detection of natural *anti-salmonella* antibodies. The objective of this study was to demonstrate a technique to purified IgY from the WSF, using trichlroacetic acid that for its simplicity and high IgY yield could be successful in IgY technology.

The water dilution method (WD) was compared with three other methods, namely polyethylene glycol (PEG), dextran sulphate (DS) and xanthan gum (Xan), in terms of yield, purity, ease of use, potential scaling up and immunoactivity of IgY. The WD method gave the highest yield, followed by DS, Xan and PEG methods in that order. 9.8 mg IgY/ml egg yolk was routinely obtained from the WD method compared to 4.9 mg IgY/ml egg yolk with the popular PEG method [6]. Our method is superior to the WD, in which is simpler, reproducible, functional, cost-effective and practical. I was demonstrated again the higher antibody titer as compared with the PEG technique.

These antibodies can be used for the immunodiagnosis of viral [20] and bacterial infections [21]. As some of the IgY, including the ostrich one, can interact with immunoglobulin-binding protein [22], they can become in a suitable immunomarker for the detection of other types of infections using procedures, such as immunoblotting or ELISA [23]. The application of purified laying hen's antibodies to treat gastrointestinal infection and respiratory infections and in the near future would be able to be able to carry out with purified IgY. Larsson and Callander [24] reported that they have treated one patient with yolk antibodies against antibiotic-resistant strain of *P. aeruginosa* daily for more than eight years in a patient with cystic fibrosis, which shows that it is possible to use yolk immunotherapy over long treatment periods [25].

Conclusion

The TCA precipitation technique provides an efficient and rapid way to purify IgY antibody, yielding approximately 2 times more IgY than that of the PEG method. It can be used for large scale production of IgY with high quality.

Method of IgY Separation	IgY Protein concentration		Titer of anti-salmonella Ab	
	Laying hen	Ostrich	Laying he	Ostrich
TCA method	200-250 mg/egg yolk	4200-5000 mg/egg yolk	1: 256	1: 128
PEG method	100-130 mg/egg yolk	2000-2200 mg/egg yolk	1: 64	1: 32

Table 1: IgY concentration and Anti-salmonella typhimurium titer of Laying hen and Ostrich in different Separation techniques.

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