EFFECT OF IMMUNOGLOBULIN Y PURIFIED FROM IMMUNIZED HEN EGGS ON THE GROWTH OF *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

A novel purification method of egg yolk immunoglobulin (IgY) based on precipitation using agar-PEG was developed. This method was compared with chloroform extraction and polyethylene glycol (PEG) precipitation methods. The results showed the protein contents were high with chloroform method followed by agar-PEG then PEG method. The purity of resultant IgY was homogeneous with agar-PEG method followed by PEG method then chloroform extraction method. The IgY purified by agar –PEG method, obtained from hens immunized by formalin-treated *S. aureus*, showed a significant reduction in bacterial growth and the growth inhibition was dependent on specific-IgY concentration.

INTRODUCTION

The immunoglobulin of yolk (IgY) in nature is somewhat different from mammalian IgG in molecular weight (larger), isoelectric point (more acidic) and don't bind with mammalian complement and protein A (1).The animal suffering is reduced, as antibodies are obtained directly from the egg (2). Specific IgY can be produced in egg yolk from immunized hens with specific antigen, (3) noted that, the concentration of IgY in the yolk is ranged from 10-20 mg/ml, however, the amount of antigen specific antibodies of the total pool of antibodies in an egg has been reported to be up to 10% (4).

The major problem in isolation of IgY is removal of lipids which are present in high concentration (5), therefore, IgY normally purified using complex and time consuming procedures (6). There are several procedures used for purifying IgY based on the strategy of separation of proteins from lipoproteins and the rest of the yolk lipids using extraction with organic solvents rather low yields of antibody (7). Other methods are based on dilution of the yolk followed by a freezing-thawing process after which the process consists of ion exchange chromatography (7). Moreover, by using of 3.5% (w/v) of a low molecular weight (PEG) polyethylene glycol (8) or natural gum (9).

Increasing prevalence of antibacterial-resistance in many bacteria has reduced the effectiveness of antibacterial therapy (10), whereas, immunotherapy can be used

against pathogen that are difficult to treat with traditional antibiotics (11). Moreover, IgY as a passive, inexpensive and easy producing antibodies has attracted much attention and been recognized to be efficient in therapy and prevention (11).

IgY have been produced against many bacteria: *Sterptococcus mutans* (12), *Salmonella enteritidis* and *Salmonella typhimurium* (13), *Helicobacter pylori (*14), *Escherichia coli* (15) and *Staphylococcus aureus* (16). Also against viruses including Porcine epidemic diarrhea virus (17) and Rota viruses (18).

The main aims of this study were: 1) test the use of agar-polyethylene glycol in the purification of IgY against other methods including uses of chloroform extraction and uses of PEG 6000 precipitation. 2) Evaluate the purity of these three methods and yield. 3) Study the effect of specific IgY purified with agar-polyethylene glycol on the growth of *Staphylococcus aureus* isolated from milk.

MATERIAL AND METHODS

Bacterial Isolates

Staphylococcus aureus strain was isolated from cow milk; suspected colonies on mannitol salt agar were identified by Gram's staining, colony morphology and hemolysis. The strain was confirmed by the tube coagulase test with rabbit plasma (16).

Laying hens

Four brown laying hens (*Gallus domesticus*) were obtained from a commercial farm. The hens were kept in an environmentally controlled room, and were subjected to regular light cycles. The hens were fed *ad libitum* with commercial diet.

Antigen preparation and immunization of hens

Staphylococcus aureus was cultured in nutrient broth for 18 hrs at 37ºC and cells were harvested by centrifugation (10000 rpm for 10 min) and washed twice with sterile normal saline (0.9% NaCl). Cells were diluted to10^8 cfu/ml in sterile normal saline and inactivated with 0.5% formaldehyde for 18 hrs (14).

The antigen suspension was emulsified with an equal volume of complete Freund's adjuvant and a total volume of 1 ml was injected at four different sites (0.25 ml per site) of breast muscles (two sites per left or right breast muscle). Two booster injections of antigen with equal volume incomplete Freund's adjuvant each 0.5 ml were given on days 10^{th} and 20^{th} after first injection (15).

Collection of eggs and separation of yolk

The eggs were collected daily after 2 weeks of booster immunization and kept at 4˚C until suitable number was obtained. The yolk of ten eggs were separated according to (19) with minor modification, the egg yolks were separated from egg whites, washed with distilled water to remove as much albumen as possible and

rolled on paper towels to remove adhering egg white. Intact yolks were broken by dropping through a funnel into a graduated cylinder and mixed thoroughly.

Purification of IgY from egg yolk

- **Lipid Removal**

The water soluble protein was prepared from egg yolks by using three main protocols for lipid removal; each protocol was tested three times. These protocols included:

A- Used organic solvent (chloroform) according to (20). Briefly, 15 ml of yolk was brought to 25 ml with sodium phosphate buffer (100 mM, pH 7.6) and mixed vigorously. Subsequently, 20 ml of chloroform was added and the mixture was shaken until a semisolid phase was obtained. Then the mixture was centrifuged at 2000 rpm for 30 min, the supernatant was filtered through filter paper and decanted into another centrifuge tube for further purification of IgY.

B- Used polyethylene glycol in 3.5% according to (8). Briefly, an equal volume of buffer (0.01 M sodium phosphate, 0.1 M NaCl, pH 7.5) was added to yolk and stirred. Solid polyethylene glycol PEG 6000 (Sigma) was added to a concentration of 3.5%, stirred until it all dissolved, and the protein precipitate that formed was pelleted by centrifugation at 10,000 rpm (Hettich,Germany) for 15 minutes. The supernatant was filtered through filter paper and decanted into another centrifuge tube for further purification of IgY.

C- A novel and simple procedure modified from a combination of earlier protocols (8, and 9). Briefly, egg yolk was diluted 1:2 with distilled water, homogenized for 30 seconds and filtered through filter paper. The mixture was mixed with two volumes of D.W. contain **agar** (Oxoid), the final percentage of agar is (0.01%).The resultant mixture was left for 30 min at room temperature, then centrifuged at 12000 rpm for 15 minutes. The supernatant was filtered through filter paper and decanted into another centrifuge tubes. To complete elimination of lipid, 3.5% of solid PEG 6000 (Sigma) was added to the supernatant and stirred until dissolved. The mixture was centrifuged at 12000 rpm for 15 min (to pellet the residual lipoprotein precipitate). The supernatant was filtered through filter paper to remove any floating lipid debris and decanted into another centrifuge tube for further purification of IgY.

Precipitation of IgY

This step was conducted according to (8). Briefly, 12% w/v solid PEG was added to the supernatant and stirred thoroughly, and centrifugation at 10000 rpm for 15min, resulted in the precipitation of IgY. The pellet was redissolved to the original yolk volume in 0.01 M sodium phosphate buffer, 0.1 M NaCI, pH 7.5, and PEG was added to 12% w/v for a second precipitation. The supernatant was decanted and the

pellet centrifuged twice more to remove any residual PEG trapped in the precipitate. This final IgY pellet was then dissolved in a small volume of phosphate buffer (0.01 M, pH 7), and stored at -20˚C.

Total protein estimation

Total protein concentration of product was determined according to (21) with bovine serum albumin (BSA) as standard in the range from 0 to 500 µg/ml.

Protein electrophoresis

To determine the purity of IgY in the egg yolk final product, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was used according to (22). The resultant from the IgY precipitation steps was dissolved in sample buffer with 2% 2-mercaptoethanol and run on a 5% stacking gel and 10% separating gel. The gel was run at 20 mA for 1.5 h and stained with Coomassie Brilliant Blue.

Effects of specific and non-specific IgY on bacterial growth

This assay was conducted to investigate effect of specific IgY or non- specific IgY (from non- immunized hens) on the growth of *S. aureus*. This assay was done according to (14) with modification. Briefly, *S. aureus* was cultured in nutrient broth at 37ºC for 18 hrs, and adjusted to 0.5 McFarland with sterile broth. Specific and non-specific IgY were sterilized with 0.22 µm filter, then diluted with broth to achieve the desired concentration after the addition of bacterial inoculums (5, 10, and 20 mg/ml). Bacteria and IgY was incubated for 6 hrs at 37ºC with shaking at 50 rpm. After incubation *S. aureus* was diluted with nutrient broth via 10 fold series dilution. Each 100µl was inoculated onto nutrient agar with spread plate method and the plates were incubated at 37ºC for overnight (each concentration of IgY and *S. aureus* was cultured three times). The number of colony-forming units (cfu) per plate was counted to determine the total number of bacteria per ml of sample.

RESULTS

Purification of IgY

The IgY was purified by two steps including lipid removal and precipitation of IgY.

- **Lipid Removal**

In this study a novel procedure was used to purify the IgY from egg yolks. Lipid removal from yolk was done with salt precipitation including agar-PEG. This method was compared with 2 traditional methods include precipitation of lipid by using PEG 3.5% alone (8) and lipid extraction with chloroform (20).

Table (1) and Figure (1) show the protein concentration of water soluble protein after lipid removal. Chloroform extraction method was gave the highest protein

concentration (mg/ml) followed by agar-PEG, then PEG with mean ± SD, 21.4± 0.62, 19.7± 0.3, and 16.8± 0.2 respectively.

- **IgY Precipitation**

The IgY was precipitated from water soluble protein resulted from different lipid removal methods. Table (1) and Figure (1) show the protein content of IgY precipitation by different methods. Chloroform extraction method was gave the highest protein concentration (mg/ml) followed by agar-PEG, then PEG method with mean ± SD, 12.9 ± 0.15, 10.8 ± 0.42, and 4.6 ± 0.15 respectively. The yield of protein obtained by various methods was high with chloroform method (60%) followed by agar-PEG, then PEG method, 54.8% and 27% respectively.

Table (1): Protein concentrations of resultant solutions after lipid removal and after IgY precipitation.

Methods of IgY purification	**Protein concentration mean ± SD (mg/ml) After lipid removal**	**Protein concentration mean ± SD (mg/ml) After IgY precipitation with 12% PEG-6000**	**Protein yield %**
Chloroform extraction	21.4± 0.62	12.9 ± 0.15	60
PEG precipitation	16.8± 0.2	4.6 ± 0.15	27
Agar – PEG precipitation	19.7± 0.3	10.8 ± 0.42	54.8

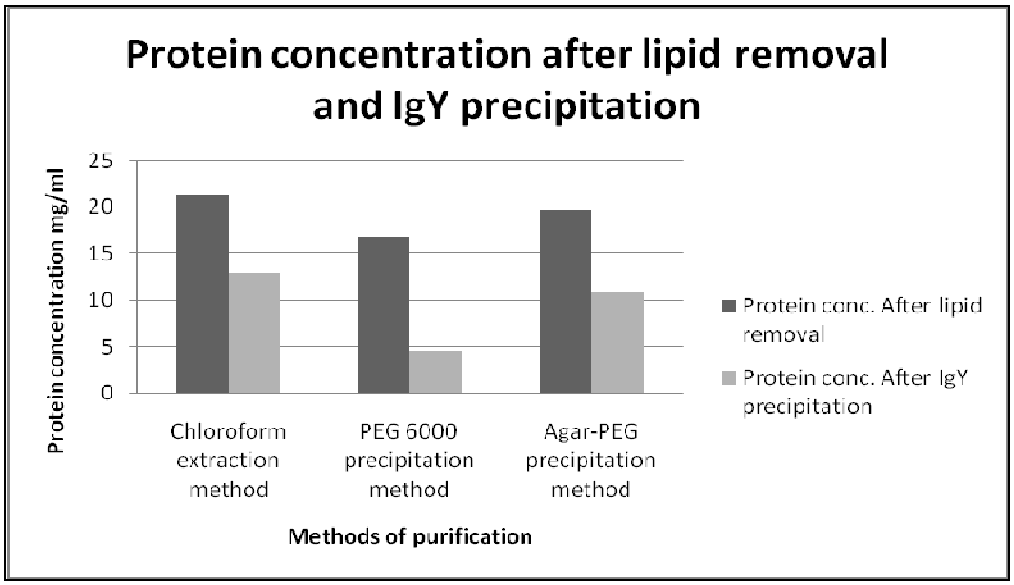

Figure (1): Comparison between protein concentrations of resultant solutions after lipid removal and IgY precipitation.

-Purity of IgY

Purity of IgY (the final step of purification) was detected with using SDS-PAGE under reducing conditions. Figure (2) demonstrate that IgY purified with agar-PEG contained two distinctive protein bands. However, IgY extracted with chloroform method contained 4 major protein bands and 5 minor bands, also IgY purified by PEG method contained 4 major protein bands and 3 minor bands.

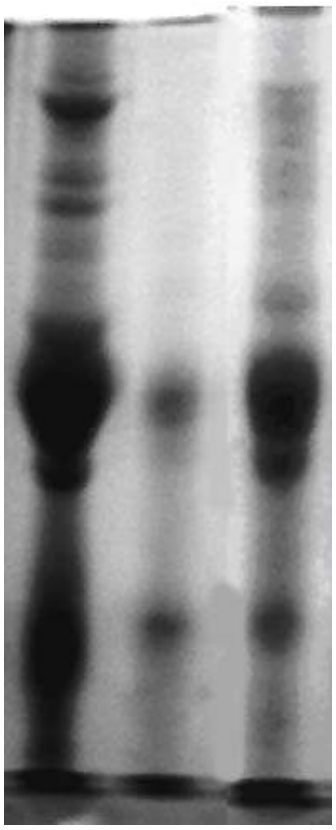

Figure (2): SDS-PAGE of IgY precipitate of various purification methods under reducing conditions, from left to right chloroform extraction methods, agar-PEG precipitation method and PEG 6000 precipitation method.

Effects of specific and non-specific IgY on bacterial growth

When the specific IgY was added to *S. aureus* in broth, the growth inhibition was dose dependent as noted by number of cfu/ml after 6 hrs incubation at 37°C. Figure (3) showed decrease the growth rates of *S. aureus* with increment of specific IgY concentration. On the other hand, non-specific IgY didn't have pronounced effect on the growth of *S. aureus* even with increasing of IgY concentration.

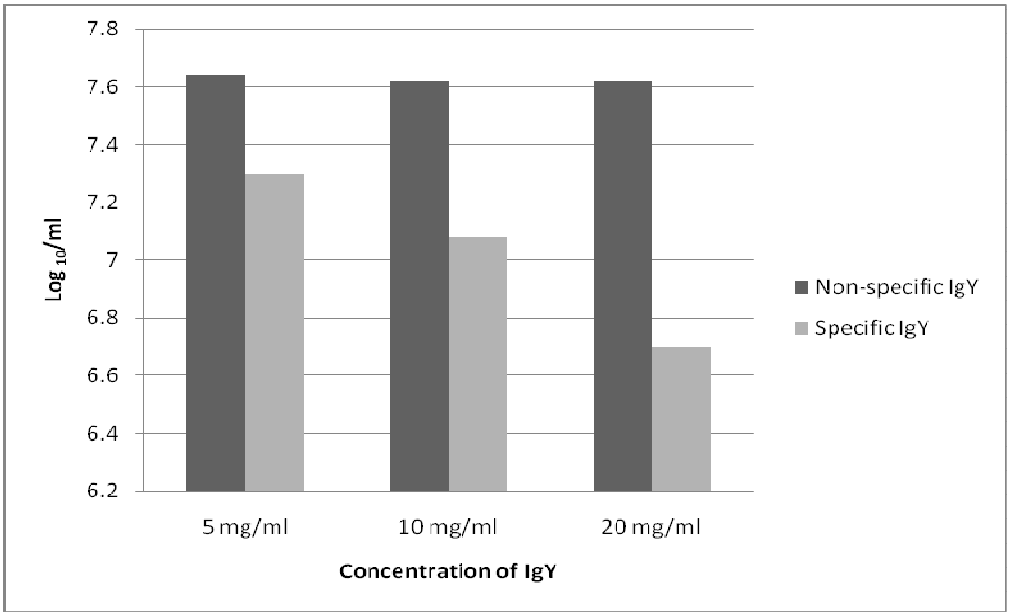

Figure (3): comparison between the effects of different concentrations of specific IgY and non-specific IgY on growth of *S. aureus*.

DISCUSSION

The main components of yolk are lipids (about 65% of the dry matter) and the lipid to protein ratio is about 2:1, lipids of yolk exclusively associated with lipoprotein assemblies (23), the major problem in isolation of IgY is removal of lipids (5). Therefore, the first step of isolation of IgY is to separate the water soluble protein from lipids and lipoproteins.

From a yield point of view the content of protein obtained by various methods was high with chloroform extracted method followed by agar-PEG and at the last PEG method.

In present study the reported concentration of IgY extracted with chloroform was 12.8 mg/ml, this result is slightly higher than that reported by (24). SDS-PAGE analysis of IgY purified with chloroform extracted method appears to confirm previous observation by (24) whom reported that the IgY extracted with chloroform is contaminated with 20% unwanted non-sense proteins.

IgY purified with PEG method resulted in a significantly low total protein content compared with other purification methods, this result is in accordance with (24, 5, 25 and 26). Result of SDS-PAGE analysis of IgY purified with PEG-6000 procedure are in agreement with (19 and 27).

Results of SDS-PAGE analysis of IgY purified with agar-PEG showed very few contaminant proteins in comparison with IgY purified with other methods of purification. However, the purity of IgY purified with agar-PEG appeared more homogeneous in comparison with that purified by other methods.

The growth of *S. aureus* incubated with specific IgY showed a significant reduction in bacterial growth after 6 hrs incubation, however, non-specific IgY had no effect on bacterial growth. This result is in accordance with (13), whom noted that the bacterial growth in presence of specific IgY against *Salmonella enteritidis* and *Salmonella typhimurium* proliferated 16 times less than the control group (non-specific IgY). Also, (28) reported that IgY, obtained from hens immunized with a mixture of formalin-treated pathogenic bacteria, inhibited the growth of *Pseudomonas aeruginosa*. Moreover, (15) noted that, the growth of *E. coli* in the presence of non-specific IgY was similar to the blank control (no IgY).

The growth inhibition is dose dependent; this result is in agreement with (15) whom noted that the growth decreased with increased specific IgY concentration.

The mechanism by which antibodies can suppress bacterial growth is not clearly understood (13). However, Particular components expressed on the bacterial surface, which are crucial factors for the bacterial growth, may be recognized and bound by related polyclonal antibody (13).

In conclusion the results of this study indicate that the IgY purified by agar – PEG method, obtained from hens immunized by formalin-treated *S. aureus* may provide a novel approach to the management of *S. aureus* infections.

تأثير الجلوبيولين المناعي Y المنقى من بيض الدجاج الممنع على نمو المكورات العنقودية

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الخلاصة

تم تطوير طريقة لتنقية الجلوبيولين المناعي Y من صفار البيض وهي مبنية على أساس الترسيب باستخدام الاجار - متعدد الاثيلين جلايكول. تم مقارنة هذه الطريقة بطريقة الاستخلاص بالكلوروفورم وطريقة الترسيب بمتعدد الاثيلين جلايكول. المحتوى البروتيني الناتج كان مرتفع باستخدام طريقة الاستخلاص بالكلوروفورم يليه الاجار- متعدد الاثيلين جلايكول ثم طريقة متعدد الاثيلين جلايكول. نقاوة الجلوبيولين المناعي Y (IgY) الناتج من طريقة الاجار- متعدد الاثيلين جلايكول كان الأكثر تجانس يليه ناتج طريقة متعدد الاثيلين جلايكول واخيرا" طريقة الاستخلاص بالكلوروفورم. الجلوبيولين المناعي Y المنقى باستخدام طريقة الاجار - متعدد الاثيلين جلايكول والمأخوذ من دجاج ممنع بالمكورات العنقودية *S. aureus* المعالجة باستخدام الفورمالين ابدى تأثير واضح على نمو المكورات العنقودية والتأثير كان يعتمد على تركيز IgY الخاص.

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