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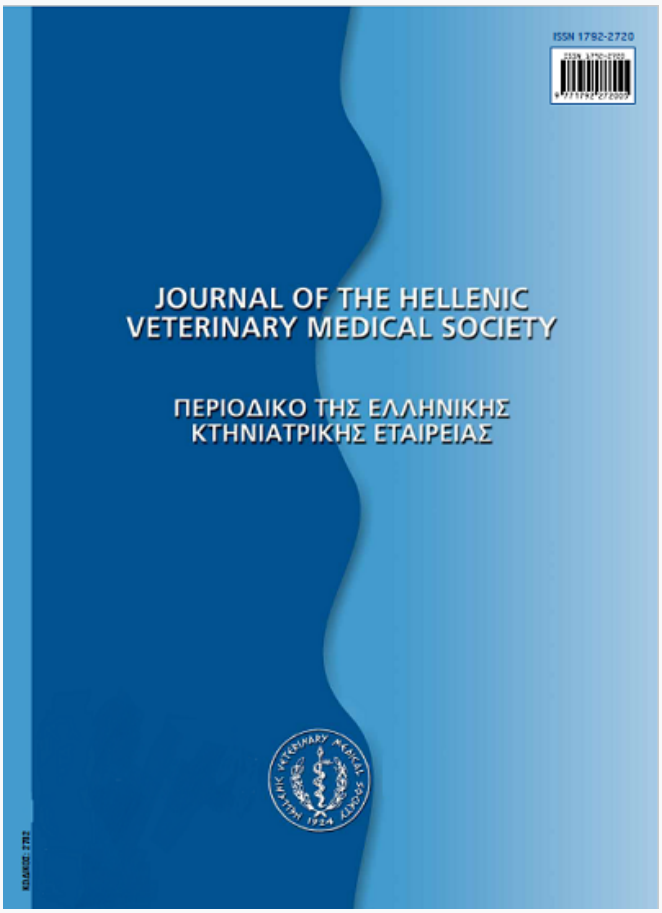

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Review article
Ανασκόπηση

IgY extraction and purification from chicken egg yolk

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ABSTRACT. The interest for immunoglobulin Y (IgY) isolation arises from the possible applications of these immunoglobulins in diagnostics and therapeutics. Powered whole eggs or yolks have been used in veterinary medicine as an inexpensive immunoglobulin Y source for the treatment of enteric diseases. Incorporating feed grade egg yolk antibodies into animal diets has been examined extensively in an attempt to limit pathogenic diarrhea causing by *Escherichia coli* in swine, and limit *Salmonella* establishment in calves and mice, as well as *Campylobacter*, *Clostridium*, and *Salmonella* in poultry. Thus, therapeutic administration of the IgY might reduce the clinical use of antibiotics, and minimize the risk of developing bacterial antibiotic resistance. Methods of immunoglobulins isolation and purification from hen yolk are reviewed. For a large-scale production, one of the problems is separating the water-soluble protein fraction from the lipids and other hydrophobic substances. Precipitation or aggregation of lipid occurs under various conditions: (1) by use of water dilution under acidic conditions; (2) by means of acids; (3) combination of solvents (chloroform, aceton) which selectively solubilize the lipids; (4) using of 3.5% polyethylene glycol and (5) by applying natural gums (polyanionic polysaccharides). Further purification of IgY after crude extraction can be achieved by selective precipitation. IgY precipitation can be carried out by salt precipitation using saturated solutions of sodium or ammonium sulphate, which dehydrate proteins. Furthermore, selective precipitation is achieved by using 8.8% sodium chloride or at 12% of polyethylene glycol. The methods comparison on the basis of IgY yield, showed that the mean IgY yield obtained by water dilution method or precipitation with salts, or by using polyethylene glycol was 5.6 mg/ml of egg yolk, 6.3 mg/ml of egg yolk and 8.7 mg/ml of egg yolk correspondingly and did not significantly differ between them. Water dilution method seemed to offer the best IgY recoveries. Moreover, this method in combination with chromatography and filtration can be applied easily in an industrial environment. Filtration technology offers the best opportunities for industrial applications while precipitation with polyethylene glycol or salts provides a cheap and easy methodology for laboratory use. In the production of safer foods, an important strategy is to exploit natural antimicrobial agents as alternatives to conventional synthetic chemical preservatives. In this regard, the IgY is of much interest for its potential application in fortified foods, such as administration for prevention of enteric diseases. The use of IgY is cost-effective.

Keywords: Extraction and purification, immunoglobulin, chicken egg yolk

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INTRODUCTION

During the last decade, antibodies (Abs) have increasingly been used in veterinary medicine as well as in the new context of the so-called functional foods.

The advantage of obtaining Abs *via* laying hens instead of mammals has improved animal welfare. This is in complete accordance with the principle of the 3 R's – reduction, refinement and replacement (Russel and Burch, 1959).

Powered whole eggs or yolks have been used in veterinary medicine as an inexpensive immunoglobulin Y (IgY) source for the treatment of enteric diseases. Incorporating feed grade egg yolk Abs into animal diets has been examined extensively in an attempt to limit pathogenic diarrhea caused by *Escherichia coli* in swine, and limit *Salmonella* establishment in calves and mice, as well as *Campylobacter*, *Clostridium*, and *Salmonella* in poultry (Al-Adwani et al., 2013).

Furthermore, it was shown that anti-bovine rotavirus (BRV) IgY containing yolk provided up to 80% protection in neonatal calves and therefore may be a promising strategy to prevent BRV-related mortality (Vega et al., 2011).

Thus, therapeutic administration of the IgY might reduce the clinical use of antibiotics, and minimize the risk of developing bacterial antibiotic resistance.

The production of safer foods, is now an important strategy. It is necessary to exploit natural antimicrobial agents as alternatives to conventional synthetic chemical preservatives. In this regard, IgY is of much interest for its potential application in fortified foods, such as the IgY administration for prevention of enteric diseases. Moreover, the use of IgY is cost-effective as IgY cost less than $10/gram (Dubie et al., 2015).

The extraction and purification of specific antibodies from chicken from egg yolk are increasingly attracting the interest of the scientific community as demonstrated by the significant growth of interest regarding IgY in literature. However, purity and yield vary greatly from method to method thus making the optimization for each experiment a necessity (Carlander, 2002).

Therefore, in the present paper various IgY extractions from egg yolk and purification methods were described.

COMPOSITION OF THE EGG YOLK

In general, egg yolk contains approximately 50% water. The dry weight of egg yolk is made up of 2/3 of lipids, and 1/3 of proteins (Shenstone, 1969). In fact, egg yolk proteins are distributed in two particular parts: the granules and the plasma in which the former are suspended. Granule proteins are composed of α- and β-lipovitellins (70%), phosvitine (16%) and low-density lipoproteins (12%) (Burley and Cook, 1961). Plasma proteins consist of α-, β- and γ-livetins and low-density proteins (McCully et al., 1962; Williams, 1962; Kovacs-Nolan et al., 2005). The α- and β-livetins were identified as chicken serum albumins and α_2-glycoprotein, respectively, (Hatta et al., 1980), whereas IgY is the predominant fraction of γ-livetin (Kovacs-Nolan et al., 2005). In particular, all livetins are water-soluble and they correspond to serum proteins (Williams, 1962).

For a large-scale production, one of the problems is separating the water-soluble proteins from the lipids and other hydrophobic substances. Therefore, several methods are used for purifying IgY and are based on the strategy of separation of proteins (levitins) from lipoproteins (lipovitellins) and the rest of the yolk lipids using various chemical substances.

Practical applications of hen egg antibody may require removal of lipid, leaving the antibody in the water soluble protein fraction (WSPF). The main components of WSPF are IgY, α-and β-livetin, low density lipoproteins, and albumin (Kim and Nakai, 1998; De Meulenaer al., 2002).

METHODS FOR IgY EXTRACTION

Water dilution method

Precipitation or aggregation of lipid from WSPF by different methods are reviewed**.**

This method is based on the use of water in order for the proteins to be transferred from the emulsion (condition: water in lipid) (Kates, 1972).The use of water dilution method for the extraction of IgY was

Table 1. The yield of IgY after application of the water dilution method

IgY mg/ml of egg yolk	References
5.4	Akita and Nakai, 1992
1.2	Ko and Ahn, 2007
12.6-25.0	Kitaguchi et al., 2008
5.1	Araújo et al., 2010
4.5	Mahdavi et al., 2010
13.9	Sunwoo et al., 2010
54.3	Liou et al., 2011
23.1	Kassim et al., 2012
3.3	Nafea, 2012

introduced by Jensenius et al., 1981. In this study, a property of yolk lipids was used for aggregation under low ionic strength and neutral pH conditions while also it was found that the immunoglobulin was isolated together with the lipid at acidic pH values. In another study, a ten-fold water dilution of the yolk was used in order to obtain the IgY soluble fraction (Jensenius and Koch, 1993).

The adjustment of pH to 5.0 combined with ten-fold yolk dilution was found to be crucial for the removal lipids or lipoproteins from the WSPF. In particular, 94.5% of IgY was recovered after WSPF pH adjustment to 5.1, while lower pH values of diluted egg yolk solution resulted in a clear WSPF (Akita and Nakai, 1992). Similar recovery was obtained earlier (Kwan et al., 1991).

Chang et al., (2000) and Ko and Ahn (2007) reported that lipid content at pH 6 was 1.0%, whereas that adjusted to pH 5.0 was 0.08%. In study conducted later, the lipid content at pH 5.0 was 1.75% (Nafea, 2012).

Results of the IgY extraction by water dilution method are listed in Table 1. As seen from Table 1, the yield varied from 1.2 to 25.0 mg/ml of egg yolk.

Thus, water dilution method seems to offer the best IgY recoveries. Moreover this method can be applied easily in an industrial environment.

Extraction of IgY with different chemical substances.

In studies the chemical substances were used to induce removal of the lipoproteins from the egg yolk.

In particular, phosphotungstic acid (Vieira et al., 1986), trichloroacetic acid (Asemota et al., 2013) successfully applied for elimination of lipids. For example, 3.46 mg/ml egg yolk was purified by using of the caprylic acid alone (Araújo et al., 2010).

Bade and Stegemann (1984) used cooled isopropanol to precipitate the water soluble proteins and further removed lipids by consecutive isopropanol and aceton extractions. In other studies, propan-2-ol, acetone and chloroform were used in order to elimination the lipids (Bizhanov and Vyshniauskis, 2000; Deignan et al., 2000; Bizhanov et al., 2004; Al-Edany, 2011). As a result, the yield varied from 7.11 to 12.9 mg of IgY/ml of egg yolk.

In another study, Polson et al., (1980a) were the first

Table 2. The yield of IgY after single or double precipitation by ammonium sulfate

IgY mg/ml of egg yolk	Ammonium sulfate, %		References
3.8	34.0	34.0	Bižanov and Jonauskienė, 2003
13.0	50.0	-	Ruan et al., 2005
4.7-9.2	55.0	31.7	Dai et al., 2012
4.0	19.0	-	Júnior et al., 2012
0.84	24.0	15.2	Parma et al., 2012
9.2	50.0	30.0	Xu et al., 2013

which introduced the use of 3.5% polyethylene glycol (PEG) for extraction of IgY from egg yolk. Moreover, method was improved by increasing the dilution by buffer from a 2:1 to 4:1 ratio (Polson et al., 1985).

In a study conducted by Hatta et al., (1990) was systematically compared the use of twelve natural gums. Xanthan and λ-carragenan seemed to be able to remove most of the lipids. As a result, the yield of IgY was about 6.8 mg/g egg yolk.

These various IgY crude extraction methods give different results considering recovery and purity of levels obtained. The selectivity of precipitation directly depends on the type of precipitating agent applied. So far, for the immunoglobulin extraction the organic solvents (for routine and fast analysis) were applied. However, several precipitation steps using organic solvents there are necessary in order to obtain a purified protein.

Polysaccharides, as safe and nontoxic substance, can be applied for isolation of the IgY in biomedical field.

Purification og IgY

Further purification of IgY after crude extraction can be achieved by selective precipitation.

Salt precipitation is a traditional technique in protein purification. For IgY, mainly, ammonium and sodium sulfate have been applied. The results in Table 2 show the yield of IgY after precipitation with ammonium sulfate at various concentrations. As seen from Table 2 the yield is considerably different.

In other works, double precipitation of IgY by using dextran and sodium sulfate in combination with calcium chloride was applied (Jensenius et al., 1981; Akita and Nakai, 1993). In particular, the yield varied from 7.5 to 15.0 mg of IgY/ml of egg yolk.

Because of its bio-compatibility, sodium chloride was used for the precipitation instead of the other precipitants described in the literature. Hernández-Campos et al., 2010 found that sodium chloride at 0.88% and 8.7% decreased the selectivity and purity factor. In another study, it was observed that the optimal IgY precipitation occurred at NaCl concentration between 0.3%-9.36% (Ko and Ahn, 2007; Hodek et al., 2013). As a result, the yield varied from 2.7 to 8.9 mg of IgY/ml of egg yolk.

The precipitation with sodium and ammonium sulfate, sodium chloride as well as polyethylene glycol offers a cheap and easy methodology and can be used in laboratory practice. Furthermore, the purity of IgY, obtained after precipitation with sodium sulfate was 94% (Akita and Nakai, 1993).

Table 3. The yield of IgY after precipitation by polyethylene glycol

IgY mg/ml of egg yolk	References
4.4	Bizhanov and Vyshniauskis, 2000
4.4	Bizhanov et al., 2004
9.7	Shahbazi et al., 2009
4.6	Al-Edany, 2011
4.8	Marcet et al., 2011
5.43	Sudjarwo et al., 2012
8.6	Wen et al., 2012
2.87	Sampaio et al., 2014

The most successful precipitation method with respect to IgY recovery and purity consists of adding PEG at a 12% level as a the method of Polson et al., 1985. For the most part, the PEG may be removed by precipitation of the IgY either with 40% precooled ethanol at -20°C or by 2 M ammonium sulfate (Polson et al., 1980b).

The results of the IgY yield after application the PEG precipitation are summarized in Table 3. As seen from the data, the yield peaked at 9.7 mg/ml of egg yolk. As follows from data represented in three tables, the mean yield of IgY obtained by three methods did not significantly differ between them.

The precipitation with polyethylene glycol or salts offers a cheap and easy methodology for the laboratory use.

Various chromatographic techniques can be used to further purify the IgY. These techniques include the following methods, such as anion exchange chromatography (Bade and Stegemann, 1984), affinity chromatography (Ntakarutimana et al., 1992), thiophilic chromatography (Hansen et al., 1998), or ion exchange chromatography (Ko and Ahn, 2007). As a result, the yield of IgY was not more than 1 mg/ml of egg yolk (Hassl and Aspöck, 1988; McCannel and Nakai, 1990; Verdoliva et al., 2000). Furthermore, it was reported that purity of IgY obtained by cation exchange chromatography was not high and contained too many contaminating proteins.

Thus, column chromatography is an expensive and impractical for large-scale production of antibodies (Ko and Ahn, 2007).

Filtration methods by using traditional funnel filtration or otherwise column- and ultra- filtration offer another possibility for IgY purification. These methods for separation are based on using different membranes (Kim and Nakai, 1996; 1998; Hernández-Campos et al., 2010) and columns (Kim and Nakai, 1996). However, by using synthetic membranes it is necessary considered that only under certain conditions (pH and ionic strength solution) can be obtained good results of purification of IgY.

It was noted that ultrafiltration seems more suitable method for the large industrial applications because of lower operation costs and direct scale up. (Akita and Nakai, 1992). These methods also allowed

the isolation of immunologically active IgY which can be used for further downstream immunotechnological processes.

CONCLUSION

IgY extraction methods require an initial delipidation step prior to extraction of insoluble lipids and lipoproteins.

The water dilution method was found to be most efficient for the extraction of IgY from the WSPF. This technique is a simple and rapid and also it is available for large scale production of IgY production. Moreover this method can be applied easily in an industrial environment.

Various IgY crude extraction methods give different results considering recovery and purity of levels obtained. The selectivity of precipitation directly depends on type of precipitating agent applied.

Further purification of IgY after crude extraction can be achieved by selective precipitation.

The precipitation with sodium or ammonium sulfate, sodium chloride as well as polyethylene glycol offer a cheap and easy methodology and can be used in laboratory practice.

Column chromatography and ultrafiltration are expensive and impractical for large-scale production of antibodies.

The knowledge of this method can be used for the development and refinement of a new methodology approach. The extraction of IgY from aqueous solution can be achieved using several methods, resulting in variations in the yield of the extract. However, each method has a specific purpose and can be improved.

The choice of a suitable method depends on the yield and purity desired, final application of the IgY as well as material cost, technology, labor skills and scale of extraction.

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