PRODUCTION OF ANTIBODIES FROM POULTRY YOLK (IgY) AND INVESTIGATION OF THEIR IMMUNOCHEMICAL PROPERTIES

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ABSTRACT

A particularly important aspect of immunology is to develop non-invasive methods of obtaining antibodies which could be a great alternative to traditional ones that based on the harmful procedure of isolation of immunoglobulins from animal blood sera. That's why the extraction of antibodies from poultry egg yolks (IgY) is the most promising. Due to the fact of variation of IgY structural features that determine the definite immunochemical properties, yolk antibodies in comparison with mammalian immunoglobulins (IgG) does not interact with rheumatoid factor (Rf), contribute to the activation of the complement system, bind to the Fc-receptor (FcR), and also has weak cross-reactivity, which confirms the possibility of their widespread use in medicine and food. Also the presence of phylogenetic distance between chickens and mammalians guarantees immune response against conservative mammalian protein molecules which is highly important for the creation of new generation test systems.

The aim of this work is to develop a selective method of producing high-purity immunoglobulin Y preparations from the yolk of chicken eggs.

There were adopted selective conditions of isolation of IgY under spontaneous thawing procedure at the room temperature of firstly frozen yolk solution in a sodium-phosphate buffer mixed with water (pH 5.0) in a ratio of 1:6, which leads to receiving a water-soluble fraction further precipitated with the sodium chloride at a concentration of 10% of the solution mass and subsequently concentrated using ultrafiltration with membrane UAM-10, that allows achieving the content of IgY not less than 95% per dry substance in immunoglobulin fraction. It is possible to produce a protein fraction with a protein content of at least 9 g/l.

The purity of the immunoglobulin fraction was verified using polyacrylamide gel electrophoresis. The presence of a light chain in the IgY solution was proved to be a low-molecular compound using the method of gel-filtration-chromatography. The immunological activity of IgY was studied with respect to bovine serum albumin (BSA) as an antigen. The enzymatic resistance of IgY against proteolytic enzymes was tested in area of the gastrointestinal tract.

Keywords: *egg yolk, IgY, immune response, gastrointestinal enzymes, ultrafiltration*

INTRODUCTION

At present time, the development of a new generation of pharmaceuticals based on antibodies' compounds is accelerating. The possibility of their passive administration and the achievement of a rapid therapeutic effect are the main advantages of such drugs.

Egg yolk is a rich source of immunoglobulins(Ig). The total content of gaining Ig is over 100 mg per chicken egg [1], [2]. Most of the yolk immunoglobulins belong to IgY class. Other classes such as IgA and IgM are also antibody components of the yolk but in smaller quantities.

IgY is a systemic immunoglobulin with a molecular weight of approximately 170 kDa. It consists of two heavy (H) and two light (L) chains connected by disulfide bonds and forming a monomeric link (H2L2). The molecular weights of the heavy and light chains are 71 kDa and 26 kDa, respectively [1], [3].

It is known that the variable part of the H-chain encodes a region of the DNA molecule that has variable (V), connective (J), and diversity (D) segments. The rearrangement of these segments doesn't lead to the phenomenon of gene hyperconversion, because of the absence of any development of IgY immunogenetic diversity during the process.

IgY is currently being considered as a promising substitution for mammalian immunoglobulins (IgG). The greatest interest is focused on the variation of IgY structural features which determine the characteristic immunochemical properties. Due to the absence of the hinge region of IgG, chicken antibodies (IgY) are less exposed to proteolytic degradation and fragmentation. There's a limited flexibility zone based on proline and glycine amino acid residues instead of the flexibility zone of the hinge region in IgY [4]. Moreover, IgY does not interact with the Fc-receptor, which is responsible for the implementation of numerous effector functions; they are not able to activate the human complement cascades. Also IgY do not have cross-reactivity and does not bind to the rheumatoid factor (Rf-factor) [5].

Purified yolk antibodies (IgY) are able to conserve their activity for six months at room temperature. In addition, affine-purified and biotinylated IgY retains high activity after five years of storage at 4°C [6].

The technology of production of IgY from the egg yolk requires a specific treatment that provides the isolation of immunoglobulins from the lipophilic matrix of the yolk. This is achievable by a two-step purification procedure of IgY from the yolks. The first stage is the separation of the water- soluble fraction (WF) containing IgY from lipids and lipoproteins. The second stage is based on the segregation of IgY out of the water-soluble protein fraction [7]. To remove the lipophilic components of the yolk, the crude extract of immunoglobulins is precipitated under the action of various reagents (polyethylene glycol, dextran sulfate, alginate, caprylic acid, organic solvents). After that the lipid aggregates could be removed by centrifugation, filtration, diluting of the yolk solution or even by freezing-thawing procedure of the diluted yolk solution. In the second stage, the pure IgY fraction should be prepared from the crude aqueous extract of immunoglobulin. Yolk antibodies are firstly salted out and then purified by chromatography

(exclusive, ion exchange, thiophilic, affinity chromatography). In some cases it's necessary to carry out repeated precipitation of immunoglobulins. Depending on the using purification method the degree of purity and the total yield of the final immunoglobulin preparation are in rang of 85-98% and 1,0-9,8 mg/ml, respectively [7]. These methods for the isolation of IgY are promising for their implementation in technologies for the development of medicines, food additives, functional nutrition, as well as the creation of modern test systems for the early diagnosis and prevention of many diseases, which will reduce the usage of non-invasive therapies on animals aimed to obtain mammalian antibodies (IgG) [8], [9]. That's why the creation of high-quality medicines is required to carry out high degree purification, which guarantees the exact composition of the immunoglobulin fraction.

The aim of this work is directed to the development of a method for obtaining high-purity immunoglobulin Y preparations from the poultry yolk.

MATERIALS AND METHODS

Poultry eggs of Public Joint Stock Company «Snezhka» plant production were used as the object of the study. The eggs have next characteristics: humidity is about 74%; the fat content of the yolk is 32.6%; the content of phospholipids in egg yolk is 29.6%; the crude protein contents in egg protein fraction and in the yolk are 10.6% and 16.6%, respectively.

Determination of crude protein content was performed by the Kjeldahl method, the concentration of protein content in the solution was measured using the biuret method. The determination of the molecular weight of the protein and the degree of its purification was evaluated by gel-filtration-chromatography and polyacrylamide gel electrophoresis in denaturing conditions.

The process of concentration of IgY solutions was performed using ultrafiltration with UAM-type membranes (ultrafiltration acetate cellulose membranes) with molecular weights cut-off of 100 and 10 kDa.

The immunological activity of IgY was investigated using the method of precipitation antibodies with antigen. Bovine serum albumin (BSA) was chosen as an antigen. BSA was prepared in aqueous solution of distilled water with a concentration of 1 mg/ml. The turbidity of the solution after adding equal aliquots of antibodies and antigen preparations was checked against the control sample at a wavelength of 440 nm in cuvettes with a light-absorbing layer thickness of 10 mm.

The enzymatic resistance of IgY against proteolytic enzymes of the gastrointestinal tract was studied using the parallel profile technique.

To build the parallel profile of the gastrointestinal tract area, aliquots of IgY solution and definite enzyme solution were added to preliminarily prepared sodium-phosphate buffer salt (PBS) solutions and hydrochloric acid (HCl) solution with required value of pH the way shown on Figure 1.

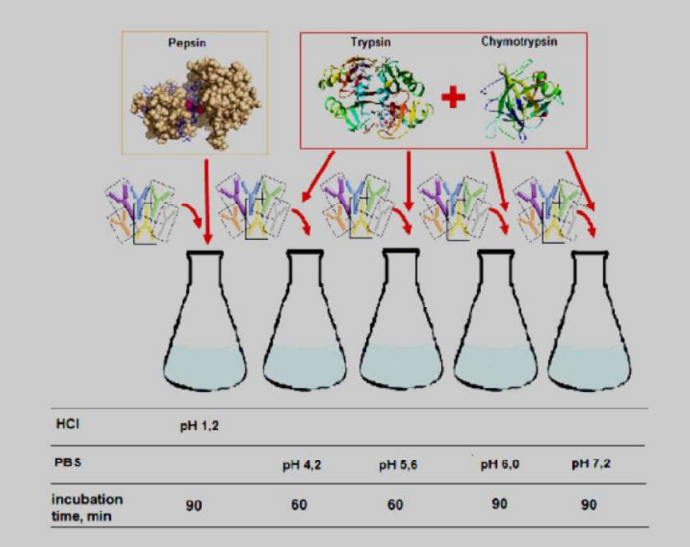

Fig. 1. *Parallel profile of gastrointestinal tract area.*

Consequently, prepared solutions were incubated at 37-40^{0}C being periodically stirred during 60 or 90 minutes depending on gastrointestinal tract section. The dynamics of IgY destruction were constructed by taking samples every 15 minutes from the solutions. All of the above pH values correspond to the acidity values of the human small intestine.

Pepsin was transformed in solution with concentration of 0.1 mass %. According to the literature data average activity of trypsin and chymotrypsin in the human gastrointestinal tract is 15 U/l [10].

Activities of enzymes were defined using the modified Anson method. Degree of IgY destruction was mesuared using the modified Lowry method.

RESULTS AND DISCUSSION

During the previously conducted methods of isolation of IgY, based on precipitation process to segregate proteins and residual fats from the native and ethanol-treated yolk. However, these methods did not allow to obtain a final product with a protein content of more than 54%.

At the next stage of work, after separating the eggs into white and yolk, the yolk was transferred to filter paper for thorough removal of the protein trace. The yolk-shell was additionally decanted.

Then the yolk mass was mixed with an equivalent volume of sodium-phosphate buffer salt (PBS) solution with a pH of 7.4 using a magnetic stirrer to achieve homogeneity. Aliquots were taken from the prepared solution for subsequent dilutions in tap water adjusted to pH 5.0 using 0.2 N HCl in 6 and 8 times [7]. The mixtures were frozen at temperature of -20^{0}C, and then subjected to spontaneous thawing through a paper filter at room temperature. A transparent protein solution,

called a water-soluble fraction (WF), was obtained after filtration. The protein content in obtained filtrates, depending on the initial dilution of the yolk mass, are shown in Table 1.

Table 1. *Effect of the dilution multiplicity on the protein content in the filtrates*

№	Dilution multiplicity	Protein content in the filtrates, g/l
1	6	7.2
2	8	6.1

Based on the small difference between the obtained concentrations in both dilutions, it was not possible to determine the unambiguous dilution multiplicity of the yolk suspension. Therefore, further fractionation of the proteins contained in the WF was carried out by means of specific precipitation with sodium chloride salts added to filtrate solutions in concentrations equal to 5 and 10 mass %. The freshly prepared fractions were kept for a day at 4℃ to induce complexation processes. Filtration through a folded filter was chosen as the primary purification of the target product against ballast proteins and dissolved salts. The protein content of the obtained filtrates was determined using the biuret method. The experimental results are shown in Table 2.

Table 2. *Effect of the degree of dilution of the yolk mass and the content of sodium chloride on the protein yield*

№	Dilution multiplicity	Content of NaCl, mass %	Protein content , g/l
1	6	5	8.1
		10	9.7
2	8	5	10.3
		10	10.1

Based on the results obtained, it can be concluded that the highest concentration of protein is observed in the filtrate at a dilution ratio of 8 and at a concentration of sodium chloride of 5 mass %.

After that the high-molecular fraction containing immunoglobulin Y was washed from residual salts and ballast proteins using ultrafiltration (UF) on the UAM – 100 membrane. The calculated values of the protein content in solutions after UF and the integral selectivity for protein are presented in Table 3. Consequently, the best dilution of the yolk mass at which the highest value of the index φ is observed, corresponds to 6, and the concentration of the added reagent (NaCl) is 10 mass %.

Table 3. *Effect of dilution multiplicity of the yolk mass and the concentration of sodium chloride on the efficiency of ultrafiltration*

№	Dilution multiplicity	Concentration of NaCl, mass %	Protein content after cocentration, g/l	Integral selectivity φ, %
1	6	5	3.58	56
		10	3.26	66
2	8	5	4.49	56
		10	3.82	62

The dilution multiplicity equal to 8 was unsuccessful due to the presence of a strong opalescent effect in the water-soluble fraction, which indicates an incompleted separation of protein and lipid fractions.

According to the literature data, purified yolk antibodies (IgY) are able to maintain their activity for 6 months at room temperature. In addition, affine-purified and biotinylated IgY retains high activity after five years of storage at 4°C [10].

In order to confirm the efficiency of the method for isolating IgY developed at the previous stage of the study, it was advisable to evaluate the fractional composition of the obtained fraction using polyacrylamide gel electrophoresis in denaturing conditions.

During the analysis, the bands corresponding to the molecular weights of the H - and L-chains of IgY were identified. The electrophoresis data is shown on Figure 2.

Electrophoresis data show that the two bands correspond to the molecular weights of the light (28 kDa) and heavy (63 kDa) chains of IgY, which coincides with the literature information [11].

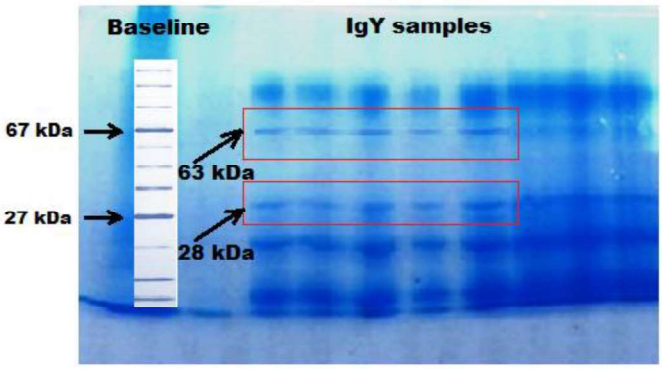

Fig. 2. *Electrophoresis data of IgY*

Since the gel contains several lower bands corresponding to impurity proteins with molecular weights of about 15 kDa, as well as fractions of proteins with molecular weights in the range of 1-100 kDa, it was concluded that the obtained immunoglobulin fraction is not sufficiently pure.

UAM-10 membrane with a lower molecular weight cut-off was tested to purify the target product from low molecular weight fragments by ultrafiltration.

After ultra-concentration, the target product was washed against mineral salts using diafiltration mode. The results of the determination of the best diafiltration multiplicity are shown in Table 4.

The experimental data show that after third diafiltration a solution with a protein content of 12.18 g/l was obtained, which indicates an increase of total protein content in 4.5 times. Based on the content of a dry substance in sample, the

resulting immunoglobulin preparation contained at least 95 mass % of the main substance.

During diafiltration the losses of the target protein is minimal, while the salt concentration in the immunoglobulin concentrate is reduced by 16 times, which allows to recommend the method of ultra-concentration and subsequent diafiltration to obtain purified immunoglobulin Y fraction.

Table 4. *Effect of the diafiltration multiplicity on the physico-chemical parameters of diafiltrates and a protein concentrate*

Diafiltration multiplicity	Residue content of NaCl in protein concentrate, g/l	Integral selectivity φ, %	Dry substance content of diafiltrate, %	Protein content in concentrate (in respect to dry substance content of diafiltrate), %
1	5.0	99.8	5.6	70.4
2	2.5	98.8	2.4	82.5
3	1.3	95.3	1.6	89.9
4	0.6	95.8	0.8	95.0

The solution obtained as a result of washing using diafiltration mode on the UAM-10 membrane with protein content of at least 98% of dry weight and with protein concentration of 16.2 g/l was tested using the gel chromatography method for the presence of low-molecular components.

Since the weight of the light chain of immunoglobulins is 26, 000 Da the G-50 sefadex was chosen as the carrier for the separation proteins and peptides, which molecular weights are in the range of 1, 500 – 30, 000 Da.

The result of the protein distribution for fractions is shown on Figure 2.

Based on collected data it follows that the light chain of immunoglobulin is present in the immunoglobulin solution as the only low-molecular compound. According to the calibration graph, its molecular weight is approximately 26 kDa, which corresponds to the literature information.

Consequently, the presence of a light chain and the absence of protein compounds with a lower molecular weight in the IgY solution was proved to be a low-molecular compound using the method of gel-chromatography, which also indicated the effectiveness of chosen purification method by diafiltration.

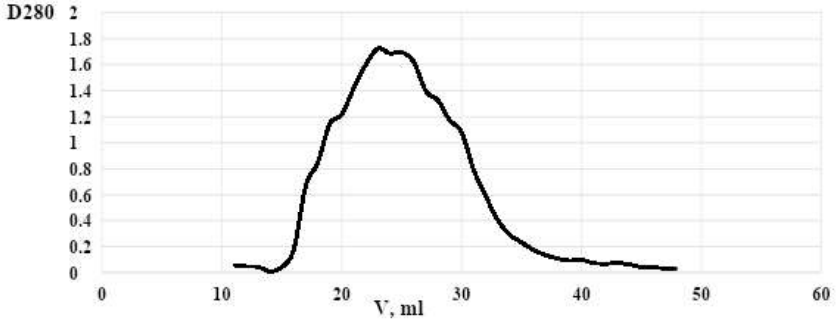

Fig. 3. *Data of gel-chromatography of light chain (L) of IgY*

IgY was proved to be immunologically active by adding bovine serum albumin (BSA) as an antigen. Results of turbidity demonstrated an intensive interaction of precipitants, which confirms the immunochemical properties of the IgY antibodies.

The final experiment was conducted to examine the process of destruction of IgY under the enzymatic conditions of the gastrointestinal tract area. Portions of enzyme preparations with adopted activity values were added into buffer solutions with IgY as previously described on Figure 1 to build the parallel profile of the gastrointestinal tract area. The collected results are shown on Figure 4.

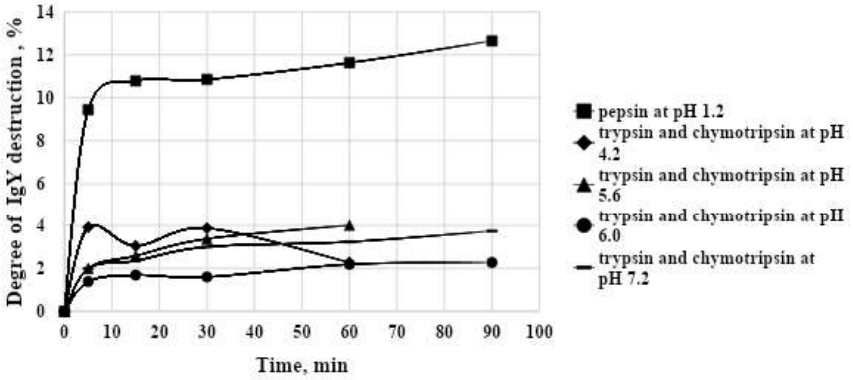

Fig. 4. *Degree of IgY destruction under the enzymatic conditions of the gastrointestinal tract area*

The data of the graph demonstrate brightly expressed degradation of IgY under the influence of pepsin at pH 1.2 and stability of IgY molecule under the influence of trypsin and chymotrypsin, which corresponds to literature [10].

CONCLUSION

A method for selective isolation of immunoglobulin Y from egg yolk was developed, based on the technology of freezing and spontaneous thawing under conditions that do not cause strong opalescent effect. It is possible to obtain a protein fraction with a protein content at least 9 g/l by diluting the yolk suspension

solution in 6 times and adding sodium chloride as a precipitating agent in amount of 10 mass %.

IgY was purified from low-molecular impurities using diafiltration mode on the UAM-10 membrane, which ensures the protein content at least 98% of dry substance in the preparation.

The purity of the immunoglobulin fraction of the yolk was electrophoretically tested. The presence of a light chain (L) in IgY solution was proved to be a low-molecular compound using method of gel-chromatography.

The immunochemical properties were tested using precipitation with BSA as an antigen.

The construction of a parallel profile of the gastrointestinal tract area proved the destruction of IgY under the influence of pepsin at pH 1.2 and enzymatic resistance of IgY under the influence of trypsin and chymotrypsin at different pH values correspond to the acidity values of the human small intestine.

REFERENCES

[1] Kaplin V.S., Kaplina O.N. IgY technologies in medicine. Avian yolk antibodies in immunotherapy, International Reviews: Clinical Practice and Health. PP. 59-75. 2016.

[2] Kaplin V.S., Kaplina O.N. IgY technologies. Avian yolk antibodies, Biotechnology, vol.33/issue 2. PP. 29-40. 2013.

[3] Wei Nie, Chao Zhao, Xiaoxiao Guo et. al. Preparation and identification of chicken egg yolk immunoglobulins against human enterovirus 71 for diagnosis of hand-foot-and-mouth disease, Analytical Biochemistry. vol. 573. PP. 44–50. 2019.

[4] E.P.V. Pereira, M.F. van Tilburg, E.O.P.T. Florean, M.I.F. Guedes. Egg yolk antibodies (IgY) and their applications in human and veterinary health: A review. International Immunopharmacology. vol 79. PP. 293-303 2019.

[5] Warr, G.W., Magor, K.E. and Higgins, D.A. (1995). IgY: clues to the origins of modern antibodies, Immunol. Today. vol. 16, PP. 392–398. 1995.

[6] Efremova M.S. Isolation of immunoglobulin Y from blood serum of hens and egg yolk. Mat. conf. "State and prospects for the development of veterinary science in Russia" Veterinary medicine and feeding. vol. 4. PP.34-35. 2013.

[7] P. Hodek, P. Trefil, J. Simunek et. Al. Optimized Protocol of Chicken Antibody (IgY) Purification Providing Electrophoretically Homogenous Preparations, J. Electrochem. Sci. vol.8. P. 113 – 124. 2013.

[8] Yudina A.N., Krasnoshtanova A.A. Selection of the optimal scheme for the isolation of immunoglobulins from the yolk of poultry eggs. "Biochemical Physics", Moscow, PP. 247-250. 2019.

[9] Yudina A.N. A.A. Krasnoshtanova Methods for the isolation of immunoglobulins from the yolk of poultry eggs. Congress materials "Advances in chemistry and chemical technology", D. Mendeleev University of Chemical Technology of Russian. Moscow, vol. 33, PP. 49-50. 2019.

[10] E.P.V. Pereira, M.F. van Tilburg, E.O.P.T. Florean et. al. Egg yolk antibodies (IgY) and their applications in human and veterinary health: a review, International Immunopharmacology. vol. 73. PP. 293-303. 2019.

[11] Hartmann C., Wilhelmson M. The hen's egg yolk: a source of biologically active substances, World's Poultry Sci. J. vol. 57/issue 1. PP. 14–28. 2001.