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Preparation of highly purified human IgG, IgM, and IgA for immunization and immunoanalysis

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Summary. The authors have elaborated several protocols for isolation and purification of human IgG, IgA, and IgM using affinity chromatography and gel chromatography approaches. To control the process of globulin isolation and to evaluate globulin purity degree, the immunodiffusion reaction according to Ouchterlony and the SDS-PAGE were carried out. The immunoglobulin preparations obtained may be successfully used for animal immunization as well as for different immunoenzyme method modifications.

Key words: human IgG, IgM, and IgA, immunoglobulin isolation and purification, affinity chromatography, gel chromatography, immunodiffusion, electrophoresis.

Solving many immunological problems we need highly purified immunoglobulin preparations. First of all, IgG and their Fc-fragments, IgM, IgA, IgE, IgD, immunoglobulin λ- and κ-chains are widely used as immunogens. On the other hand, these biomolecules are necessary to obtain immunosorbent for the binding of cross-reactive antibodies; they are also needed for the affinity chromatography purification of anti-immunoglobulin antibodies from polyclonal sera. Purified immunoglobulin preparations serve often as standard ones; they are also necessary for evaluation of specific immune sera. The immunoglobulin purification is the first step for the obtaining of standard antibodies and conjugates including them. The use of highly purified antibodies leads to decreased background values in immunoenzyme analysis [1—4].

Numerous approaches of molecular immunology and biochemistry are well known for immunoglobulin isolation and purification. They all are based on the information about physico-chemical and biological properties of molecules belonging to this group (see Table 1). For isolation and separation of these molecules, their molecular masses, isoelectric points, solubility under different conditions as well as their affinity to some substances (bacterial A- and G-proteins) must be taken into consideration [4—6].

Gel chromatography, affinity chromatography and ion-exchange chromatography, dialysis, precipitation by salts and organic solvents are based on different properties of immunoglobulin molecules. The most widely used protocols described [2, 4, 6, 7] combine several approaches. However, a lot of them are not clear and lack adequate assays to control the immunoglobulin purity.

Human IgG isolation and purification are based on affinity chromatography with A- and G-protein columns; they are well described in the literature, their results are reliable, and we suppose these approaches need no further

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Table 1

Main properties of human immunoglobulins [1—4, 7]

Properties and peculiarities	Immunoglobulin isotype									
	IgG1	***IgG2***	***IgG3***	***IgG4***	***IgM***	***IgA1***	***IgA2***	***sIgA***	***IgD***	***IgE***
Heavy chain	γ_1	γ_2	γ_3	γ_4	μ	α_1	α_2	α_1/α_2	δ	ε
Light chain	κ, λ	κ, λ	κ, λ	κ, λ	κ, λ	κ, λ	κ, λ	κ, λ	κ, λ	κ, λ
Other chains	–	–	–	–	J-chain	J-chain, S-component			–	–
Quantity of main four-chained units	1	1	1	1	5	1, 2, 3	1, 2, 3	2	1	1
Sedimentation coefficient	7S	7S	7S	7S	19S	7S, 9S, 14S	7S, 9S, 14S	11S	7S	8S
Molecular mass, kDa	146	146	170	146	970	160	160	385	184	188
Average concentration in sera, mg/ml	9,0	3,0	1,0	0,5	1,5	3,0	0,5	0,05	0,03	0,00005
Half-life period, days	21	20	7	21	10	6	6	6	3	2
Portion of total serum immunoglobulins, %	50	17	5	3	10	16	2	Trace quantity	<1	Trace quantity
Intravascular pool, %	45	45	45	45	80	42	42	Trace quantity	75	50
Carbohydrate contents, %	2–3	2–3	2–3	2–3	12	7–11	7–11	7–11	9–14	12
Extinction coefficient, E_{280} (1 % 1 cm)	13,6	13,6	13,6	13,6	11,8	13,2	13,2	12,6	17,0	15,3
Complement activetion by alternative mechanism	–	–	–	+	–	+	+	–	–	+
A-protein binding	+++	+++	–	+++	–	–	–	–	–	–
G-protein binding	+++	+++	+++	+++	–	–	–	–	–	–
L-protein binding	+++	+++	+++	+++	–	–	–	–	–	–

improvement. At the same time, there are many multi-step approaches concerning the isolation and purification of human IgM and IgG; such approaches do not always lead to reproducible results and lead often to significant immunoglobulin loss. They also lack appropriate control methods evaluating the purification process and final products. That is why we tried to improve and optimize such multi-step schemes of the immunoglobulin purification, our results being described below.

We have elaborated some purification schemes for human IgA, IgG, and IgM; our preparations are of high purity and may be used as immunogens and as antigens for different immunoenzyme assays.

Materials and methods. As a source of the human IgG, a preparation of normal human serum immunoglobulin («Biopharma», Ukraine) was used; its protein concentration was 100 mg/ml. Another IgG source was a blood serum containing no HBs-antigen and no antibodies against the human immunodeficiency virus and hepatitis C virus.

Electrophoresis. The process was carried out according to Laemmli [8] in a vertical chamber using 15 % polyacrylamide gel with SDS (PAGE-SDS). Our molecular weight markers were ovotransferrin (M_r 78,000), albumin (M_r 66,250), ovalbumin (M_r 42,700), carboanhydrase (M_r 30,000), myoglobin (M_r 16,900), and cytochrome C (M_r 12,300) («Sigma», USA). Following electrophoresis, the gels were stained by a Coomassie Blue R-250 solution.

Affinity chromatography. For the human IgG purification we used a G-protein-carrying affinity chromatography column («HiTrap Protein G», Pharmacia Biotech), its volume being 1 ml. A commercial IgG preparation or human serum were diluted twice using 0.02 M phosphate buffer (pH 7.0). This mixture was applied on the column and eluted by 0.1 M glycine-HCl buffer (pH 2.7), the elution rate being 1 ml/min; fractions were collected the non-bound to the column and neutralized them. In such a way, we removed from the mixture all IgG molecules (this procedure was repeated several times). The peaks eluted were combined

and precipitated by ammonium sulfate, the precipitate was dissolved in the minimal volume of deionized water [9].

To separate the IgG subclasses, we used an affine A-protein-carrying column «HiTrap Protein A» (1 ml, Pharmacia Biotech). The preparation was applied on the column in the 0.1 M Na-phosphate buffer (pH 7.5), the elution rate was 1 ml/min. We collected a non-bound fraction and then eluted bound immunoglobulins using a step-wise pH gradient. The pH gradient was made using two buffers — 0.1 M Na-phosphate (pH 7.5) and 0.1 M citrate buffer (pH 3.0).

Gel filtration. This chromatographic approach was chosen to change the buffer for the IgG storage and as a purification method. The buffer change was carried out on a Sephadex G-25 column (1.5x20 cm, Pharmacia Biotech). The elution rate was 2 ml/min. The protein peak was registered by a spectrophotometer (at $\lambda = 280$ nm), the buffer eluted was detected by a conductometric method.

The final immunoglobulin purification was realized on a Sephacryl S-300 column (2.5x100 cm, «Pharmacia Biotech») washed previously by the 0.05 M phosphate buffer (pH 7.2) containing 0.14 M NaCl. The elution rate was 2 ml/min, each fraction volume being 4 ml. Peak fractions were combined, the protein concentration was determined at $\lambda = 280$ nm [2, 9].

Preparation of the serum euglobulin fraction. The serum was dialyzed at 4 °C against 100 volumes of the phosphate buffer (0.002 M, pH 6.0); the buffer was changed twice. The precipitate was pelleted by centrifugation (4.000 rpm, 30 min) and washed twice with the same buffer. The supernatant was then used to obtain the serum euglobulin fraction, the pellet was dissolved in the 0.02 M phosphate buffer containing 0.15 M NaCl (pH 7.2—7.4). Insoluble particles were removed by centrifugation (4.000 rpm). The obtained euglobulin fraction was used for the IgM isolation [4, 10].

Preparation of the serum immunoglobulin fraction. The initial material used for the obtaining of the human immunoglobulin fraction was the IgM-free supernatant from the serum dialysis. Both IgG and IgA were precipitated from the dialyzed serum (50 ml) by the saturated ammonium sulfate solution (pH 7.0—7.2). This solution was added to the cooled serum (+4 °C) up to the sulfate final concentration 33 %, the mixture was stirred. This mixture was kept overnight at the same conditions. The precipitate was then pelleted (4.000 rpm, 30 min). The supernatant was removed, and the pellet was dissolved in deionized water (10 ml). The pelleting was then repeated, and the final pellet was dissolved in the minimal water volume to carry out the further immunoglobulin purification [4, 10].

Immunodiffusion according to the Ouchterlony protocol. The immunodiffusion was performed in the 1.25 % agar gel prepared in the borate buffer (pH 8.6) [11]. Monospecific antisera against human immunoglobulins of different classes (IgG, IgM, and IgA) used in this work were prepared by the Bacterial Preparations Division of the Ilya Mechnikov Central Research Institute of Vaccines and Sera (Russian Federation). Immune sera were poured into central wells, and serially diluted antigens were put into peripheral ones. The amido black solution was used for the gel fixation and staining [12]. The gels were washed by acetic acid solution (2 %).

Results and discussion. We isolated highly purified human IgM, IgA, and IgG preparations and obtained also individual human IgG subclasses (IgG1, IgG2, IgG3, and IgG4).

Preparation of the human IgG. A highly purified IgG fraction was obtained from a commercial preparation using affinity chromatography on a G-protein column. Carrying out our experiments, we tried to use the column capacity as fully as possible. In each experiment we applied to the column excess IgG preparation and collected a fraction non-bound to the column and a bound one; the latter fraction was eluted by the glycine buffer. Purification cycles led to the decrease of the peak height of the eluted fraction; it is indicative of the complete removal of IgG from the non-bound fraction. The chromatograms obtained following the first purification cycle and after the last one are presented in the Figures 1a and 1b, respectively. The first peak in the Figure 1b is due to the presence of both non-bound IgG and contaminating substances in the commercial human immunoglobulin sample, these substances were unable to bind with the G-protein. And the first peak in

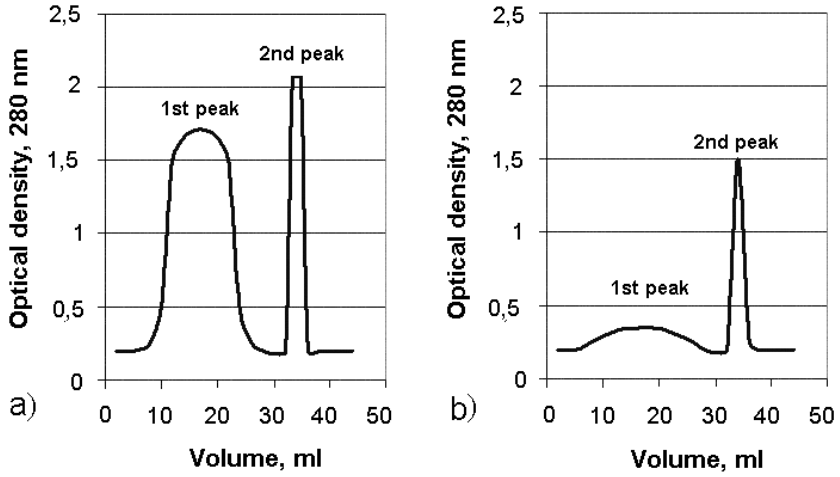

Fig. 1. Isolation of human IgG by affinity chromatography on a G-protein column (1a — first purification cycle, 1b — last one): 1st peak — fraction non-bound to the column; 2nd peak — G-protein-bound IgG eluted from the column.

the Figure 1b is only due to the presence of contaminating substances.

The IgG peaks eluted from the column were combinet and the IgG was precipitated by the sulfate ammonium (50 % of saturation). The precipitate was then pelleted (4.000 rpm, 30 min). The supernatant was discarded, and the pellet was dissolved in the minimal deionized water volume and filtrated (the pore size was 0.02 μm). The IgG preparation was then transferred by the gel filtration into the 0.02 M phosphate buffer containing 0.15 M NaCl (pH 7.2—7.4) (Fig. 2). The protein concentration varied from 2 up to 4 mg/ml.

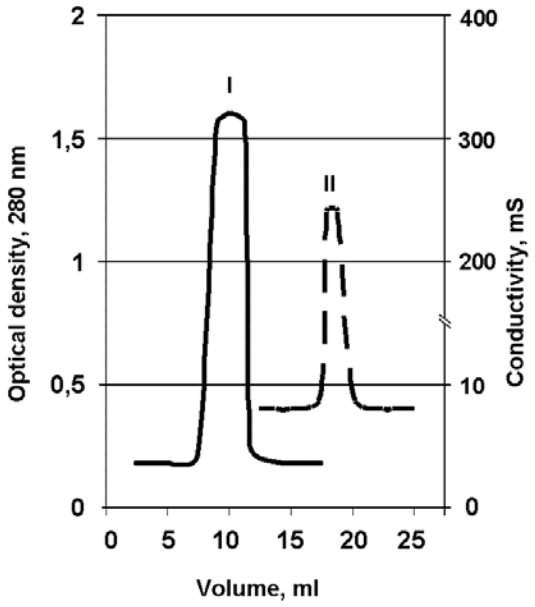

Fig. 2. Buffer change for a preparation of the human IgG by gel-filtration on a Sephadex G-25 column: I — human IgG elution from the column in the phosphate buffer; II — ammonium sulfate elution.

Separation of human IgG subclasses. The most convenient sources for the isolation of human IgG subclasses are sera of patients with various myelomas. However, such sera are not easy available. First of all, patients with the hyperglobulinemia are rare. Besides, in order to use such sera it is necessary to determine the isotypes of myeloma proteins. Such approach is impossible without reagents allowing to determine the human immunoglobulin subclasses. It is also known that the most hyperglobulinemia cases are due to monoclonal IgM, IgG-producing myelomas being especially rare.

Taking into account the literature data [1—4, 7, 10] we carried out experiments on isolation of human IgG subclasses from normal sera. Our approach is based on different biological properties of these subclasses, namely on different affinity of these isotypes to A- and G-proteins. All human IgG isotypes are known to be highly affine to the G-protein [6], so the use of the affine column «HiTrap Protein G» permits to isolate all four human serum IgG subclasses. Because of the extremely low IgG3 affinity to the A-protein, it is possible to separate it from other three IgG isotypes. The separation of IgG1, IgG2, and IgG4 is based on their different affinity to the A-protein. It is known that the

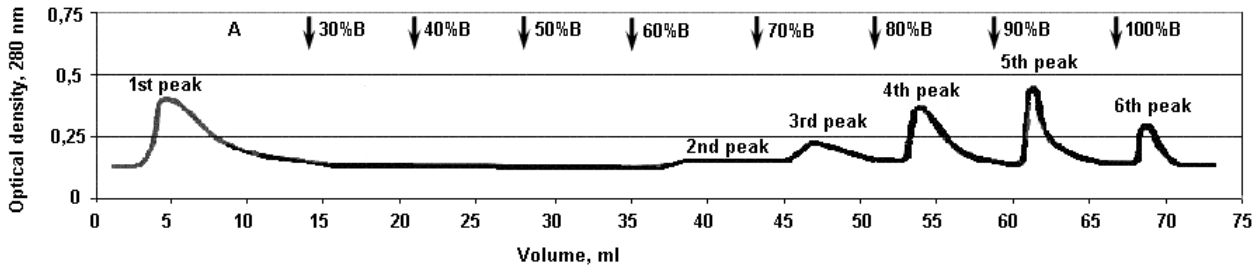

Fig. 3. Separation of different human IgG subclasses using A-protein affinity chromatography (stepwise elution): A — phosphate buffer; B — citrate buffer.

dissociation of the immunoglobulin-protein A complex occurs at pH 4.3—5.0 for the IgG2, 3.9—5.0 for the IgG4, and 3.99—4.6 for the IgG1 [7]. Having used a stepwise pH gradient, we separated partly these three IgG subclasses. However, their complete separation is impossible because of the overlapping of pH intervals for different IgG-protein A complexes. The stepwise IgG elution from the «HiTrap Protein A» column is shown in the Figure 3. The first peak is the IgG3 fraction which has failed to bind to the column. Other peaks contain immunoglobulins of other three subclasses. Fractions 2 and 3 contain mostly the IgG2, and fractions 4—6 — almost pure G1-subclass immunoglobulins. The subclass IgG4 was represented by admixture of fractions in peaks 3—5.

The available data published in the literature do not provide any clear protocols permitting the separation of IgG subclasses, the general separation principle is pH gradient-dependent elution [2, 4]. The approach described here for the separation of normal serum immunoglobulins allows to obtain fractions, containing mostly molecules of certain IgG isotypes. The use of both A- and G-proteins permits to isolate the pure IgG3 fraction. The preparations obtained in such a way may be taken for the determination of monoclonal antibody reactions with different human IgG subclasses.

Preparation of the human IgM. To isolate the human IgM, the euglobulin blood serum fraction was taken, containing, in addition, α_2-macroglobulin [1].

Some authors recommend to obtain the IgM molecules by the filtration of serum euglobulin through the Sephacryl S-300 gel [1, 4]; in such case the obtained IgM fraction is contaminated by the α_2-macroglobulin; it is well proved by the data of immunodiffusion by the Ouchterlony method (Fig. 4).

To separate these two serum proteins, the IgM precipitation by the polyethylene glycol (PEG 8.000) was carried out. The IgM molecules become almost completely insoluble when the PEG concentration in the solution reaches 5 %; they form precipitate pelleted later by centrifugation. At the same time, the α_2-macroglobulin remains in the supernatant, its precipitation occurring at PEG concentrations 10—12 %. The IgM pellet was dissolved in the minimal volume of the phosphate buffer, the non-dissolved particles were removed by centrifugation (4.000 rpm, 15 min); the IgM solution was then filtered, the filter pores being 0.2 μm. The last purification step was the gel-filtration on the Sephacryl S-300 column (2.5x100 cm). It is clear from the Figure 5a that the IgM molecules form the first elution peak; contaminating proteins are present in the second one. The first peak fractions were colected and concentrated (by 10 times) by the reverse dialysis against the PEG 40.000. In the preparation obtained after the first gel-filtration cycle the contaminating proteins may form aggregates with molecular

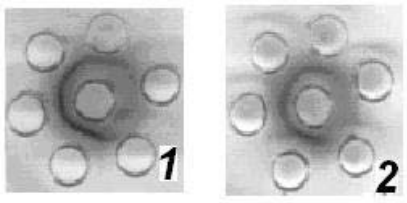

Fig. 4. Purity evaluation of a human IgM sample isolated from euglobulin fraction: lines obtained by Ouchterlony immunodiffusion method: 1 — human IgM fraction reacting with an anti-IgM serum; 2 — human IgM fraction reacting with a serum against the α_2-macroglobulin.

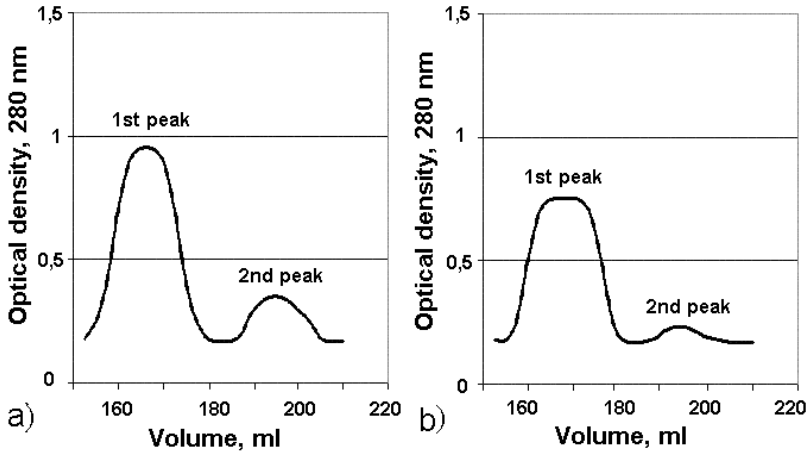

Fig. 5. Purification of the human IgM by gel-filtration on a Sephacryl S-300 column: 1st peak — human IgM; 2nd peak — ballast proteins.

masses reaching about 900 kDa. It is evident that such aggregates were eluted together with the IgM molecules; it is proved also by the immunodiffusion data (see Fig. 6). So the IgM fraction was contaminated by the human IgG. To remove the contaminating molecules, the IgM preparations were with by Triton X100 (1 %, v/v) and run once more through the Sephacryl S-100 column (Fig. 5b). The presence of the detergent prevents the molecule aggregation in the solution, so the second chromatography cycle enables to remove some contaminating proteins (see the peak 2 in the Fig. 5b). The first peak fractions were collected and used for the final evaluation of the IgM purity.

Obtaining of the human IgA. To isolate the human IgA fraction, we used the serum immunoglobulin fraction containing IgG (about 80 %), IgA (about 20 %) and negligible quantities of other proteins. Most authors recommend to carry out the separation of different immunoglobulin classes using ion-exchange and gel chromatography [1, 2, 4, 10]. According to these data, we realized our experiments to purify the IgA using the ion-exchange chromatography (DEAE-cellulose) and gel-filtration on a Sephacryl S-200 column.

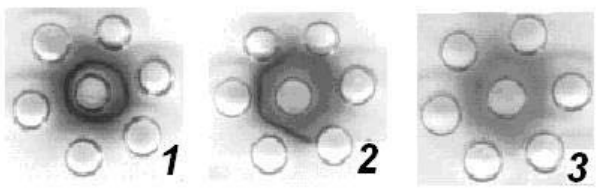

Fig. 6. Purity evaluation of a human IgM preparation following the first purification step: lines obtained by Ouchterlony immunodiffusion method: 1 — human IgM fraction reacting with an anti-IgM serum; 2 — human IgM fraction reacting with an anti-IgG serum; 3 — human IgM fraction reacting with an anti-IgA serum.

In our hands, the use of ion-exchange chromatography was found to lead to significant loss of the target protein, the IgA yield being as low as 7—8 % comparing to its total content in original preparations. So the use of such approach needs high quality original raw material. Besides, it is noteworthy that all three fractions — IgG, IgM, and IgA — possess the same range of isoelectric points [3, 13]; this fact doesn't allow to obtain highly purified IgA by ion-exchange chromatography as a single method. Additional purification steps also decrease the target product yield. Taking all these circumstances into consideration, we refused to purify our IgG preparations by this method. In addition, we carried out some experiments aiming at separation of IgG and IgA from the blood immunoglobulin fraction on a Sephacryl S-300 column (2.5x100 cm) as recommended by some authors [1, 2, 14]. Our data (see Fig. 7) demonstrate it is impossible to separate these two fractions by a single chromatography cycle because of their similar molecular masses. Three-four identical cycles are to be carried out; it complicates experiments and causes significant target product loss.

These data led us to the conclusion that a new experiment scheme, simple and effective, was necessary. Here we describe this scheme pro-

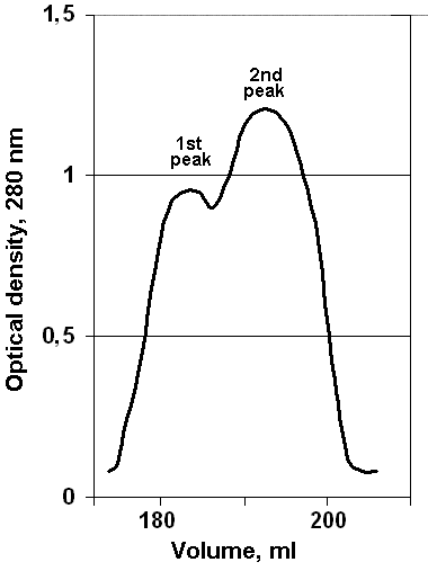

Fig. 7. Purification of the human IgA by gel-filtration on a Sephacryl S-300 column: 1st peak — human IgA; 2nd peak — human IgG.

posed in our laboratory. The serum immunoglobulin fraction obtained following ammonium sulfate precipitation was transferred into the 0.02 M phosphate buffer (pH 7.0) by gel-filtration. The IgG molecules may be removed from this fraction on a column «HiTrap Protein G». We carried out several affinity chromatography cycles, the column stopped to bind human IgG molecules. The proteins non-bound to the column were precipitated by ammonium sulfate,

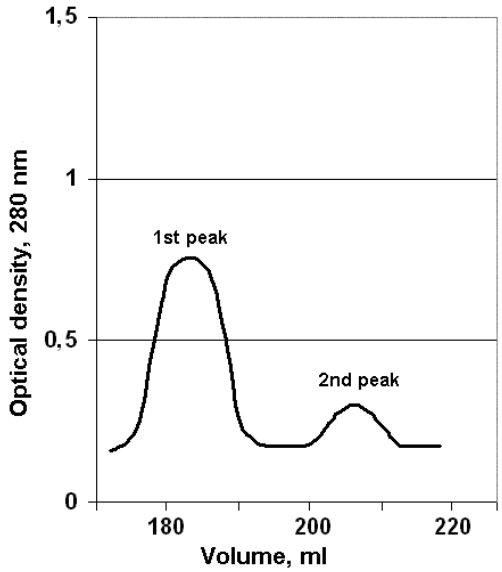

Fig. 8. Purification of the human IgA by gel-filtration on a Sephacryl S-300 column: 1st peak — human IgA; 2nd peak — ballast proteins.

dissolved in minimal water volume and filtered (the pore size was 0.2 mm). The last purification stage was the gel filtration using a Sephacryl S-300 column (2.5x100 cm) as described above for the human IgM preparation. The IgA molecules were eluted in the first peak fractions (Fig. 8). The second peak contained other serum proteins of lower molecular weights. The chromatographic profile resembles the pattern obtained for the IgM (Fig. 5); the IgA molecules being eluted a little later. The first peak fractions were collected and used for the final evaluation of the IgA preparation purity.

This scheme elaborated for the first purification step enabled the solving of our main problem — separation of IgA and IgG molecules with similar molecular weights and charge values. Such separation became possible due to G-protein affinity differences for different immunoglobulins. During the gel-filtration, some serum proteins were removed that contaminated immunoglobulin fraction obtained by ammonium sulfate precipitation.

Process evaluation on different steps of immunoglobulin isolation. To control the process of immunoglobulin purification and to evaluate the purity of the samples, we used the immunodiffusion approach and PAGE-SDS. All peaks obtained by chromatographic procedures were investigated by the immunodiffusion using monospecific anti-immunoglobulin antibodies. Such approach was necessary to determine the peak contents after the gel-filtration. It was also useful for determination of immunoglobulin cross-contamination. Our results concerning the IgA preparation purity are presented in the Figure 9. As it is seen the precipitation lines were

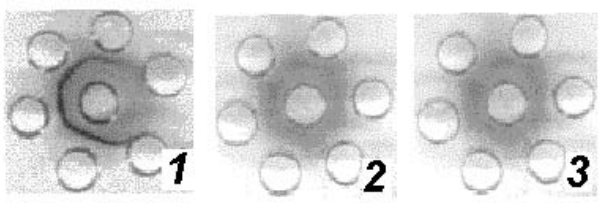

Fig. 9. Purity evaluation of a human IgA preparation: lines obtained by Ouchterlony immunodiffusion method: 1 — human IgA fraction reacting with an anti-IgA serum; 2 — human IgA fraction reacting with an anti-IgG serum; 3 — human IgA fraction reacting with an anti-IgM serum.

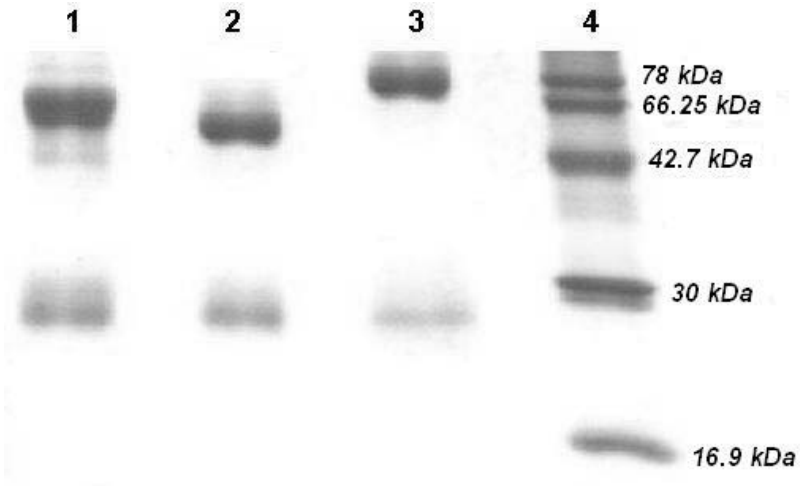

Fig. 10. Electrophoregrams of purified immunoglobulin samples: 1 — human IgA; 2 — human IgG; 3 — human IgM; 4 — molecular mass markers.

formed only with an antiserum specific for the human IgA; it is a proof of high IgA purity. Similar results were obtained with IgG and IgM preparations interacting only with their specific antisera.

The final purity evaluation of immunoglobulin samples was carried out by electrophoresis reducing conditions. For all preparations of three immunoglobulin classes only two bands were found in electrophoregrams (for heavy and light chains, respectively) (Fig. 10). These results prove the samples to be of high purity, the contents of target immunoglobulins to be about 100 %.

Conclusions. We obtained human IgG, IgA, and IgM samples of high purity reaching about 100 % according to the PAGE-SDS data. These samples may be used without reserve for animal immunization to obtain hybridomas as well as for testing of hybridoma-produced antibodies in immunoenzyme analysis. The IgG, IgM, and IgA concentrations were 2.7 mg/ml, 0.7 mg/ml, and about 2 mg/ml, respectively. A well-known scheme of the IgM isolation was modified to increase the preparation quality. A simple and effective approach is proposed for the IgA purification. The authors elaborated also a protocol of normal human serum IgG fractionation and separation of IgG subclasses. All these schemes are simple and reproduceable.

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Одержання високоочищених IgG, IgM та IgA людини, придатних для імунізації та імуноаналізу

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Резюме. Відпрацьовано схеми виділення й очистки IgG, IgA та IgM людини із використанням афінної та гельхроматографії. Для контролю процесу виділення імуноглобулінів та перевірки їх чистоти використовували імунодифузію за Оухтерлоні та електрофорез у поліакриламідному гелі (ПААГ) із додецилсульфатом натрію (ДСН). Одержані препарати імуноглобулінів цілком придатні для імунізації тварин та застосування у різноманітних варіантах імуноаналізу.

Ключові слова: IgG, IgM та IgA людини, виділення та очистка імуноглобулінів, афінна хроматографія, гельфільтрація, імунодифузія, електрофорез.

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