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Bioaffinity sorbent based on immobilized protein A *Staphylococcus aureus*: development and application

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Aim. *The obtaining of bioaffinity sorbent based on the immobilized protein A of S. aureus (SPA) using two cellulose-binding domains (CBD), and its application for purification of antibodies.* ***Methods***. *The DNA sequences encoding SPA and two CBD were genetically fused, expressed in the high-productive Escherichia coli system and the protein SPA-CBD_2 was obtained in a soluble form. The SPA-CBD_2 fusion protein was affinity immobilized on the microcrystalline cellulose.* ***Results***. *Capacity of bioaffinity sorbent (1 mg SPA-CBD_2/1 ml CC31-cellulose), dynamic capacity (3 mg mouse IgG/1 ml bioaffinity sorbent), efficiency and stability during prolonged storage were determined. The bioffinity sorbent was used for purification of antibodies. The purity of antibodies in eluted fractions was more than 95 %. The purified antibodies detected target antigens with a high sensitivity.* ***Conclusions***. *The designed bioaffinity sorbent provides obtaining pure poly- and monoclonal antibodies in functionally active form and can be use- ful for the fractionation of mouse immunoglobulin G.*

Keywords: antibodies, protein A, cellulose-binding domain, protein immobilization, affinity chromatography.

Introduction. At present antibodies are widely used in biomedical practice and in fundamental researches. The combinations of specific methods are usually used to obtain considerable amounts of antibodies with high purity degree; in particular, this is precipitation with ethanol and different kinds of chromatography: ion-exchange, hydrophobic and metal ion affinity chromatography [1]. However, these methods are not specific, moreover, the application of some of them results in partial loss of the functional activity of antibodies. A highly efficient method of obtaining antibodies is affinity chromatography with the application of sorbents on the basis of immobilized immuno-globulin-binding proteins, in particular recombinant protein A of *Staphylococcus aureus* (SPA). This method is widely used for obtaining fractions of purified antibodies out of cultural and ascitic fluid, blood sera as well as for immunoadsorption of antibodies and circulating immune complexes from blood plasma [2–4].

The specificity of SPA composition, ensuring specific binding of different species of animals and humans to IgG, is the presence of five domains (E, D, A, B, C), each of them being capable of specific interaction with Fc-fragments of antibodies [5]. One SPA molecule is capable of binding at least two IgG molecules. In spite of the application of alternative low molecular synthetic ligands with some selectivity to IgG for the purification of antibodies, the degree of purification with these ligands is considerably lower

compared to SPA. None of known low molecular ligands ensures such universal features during the purification of antibodies as SPA [6].

The advantages of SPA as a bioligand in the biotechnology are conformational resistance to the impact of physical and chemical factors and proteases, preservation of functional features in a wide pH spectrum (2.0–11.0), a possibility of renaturation after the treatment with denaturating solutions of urine (urea) or guanidine hydrochloride, and the absence of cysteine residues in its composition which facilitates the isolation and purification of SPA [7]. Besides, high affinity to Fc-fragments of antibodies and stability compared to other immunoglobulin-binding proteins, used in biotechnology (Streptococcal protein G and protein L from *Peptostreptococcus magnus*), allow positioning SPA as an efficient ligand for the purification of antibodies in laboratory conditions and in industrial scale [3, 8].

Different strategies of immobilization of protein ligands, SPA in particular, are used while creating affinity sorbents on chromatographic matrices. A classic method is indirect immobilization of chemically activated matrices (activation with bromine cyan, carbonyl diimidazol, N-hydroxysuccinimide). However, the application of these matrices often leads to a non-specific immobilization of a ligand and partial loss of its functional activity [9]. Besides, the demand for sorbents of similar kind is restricted by their high cost.

An alternative of immobilization of chemically activated matrices is genetic engineering introduction of the cellulose binding domain (CBD) from the cellulolytic complex of *Clostridium thermocellum* as a protein-partner into the composition of SPA sequence which is capable of specific interaction with carbohydrate structure of cellulose. It ensures targeted immobilization of protein on the matrix and exposure of active binding centers in the position, optimal for the interaction with immunoglobulins [10, 11]. This allows avoiding difficulties, arising during the application of chemically activated matrices.

The work describes the obtaining of bioaffinity sorbent on the basis of targeted immobilized SPA with the help of two cellulose-binding domains and its use for the purification of antibodies.

Materials and Methods. The strains of *Escherichia coli* XL1-blue, BL21 (DE3) and plasmid vectors *pJET*1.2 (*Fermentas*, Lithuania), *pGEM-11Zf* (*Promega*, USA), *pET-24* (*Novagen*, USA) were used in the work.

The sequences of oligonucleotides for the amplification of DNA SPA and CBD (Gene Bank Accession N X68233) were calculated using Vector NTI program (*Invitrogen*, USA) by homology with the known nucleotide sequence, presented in the database of GenBank BLAST of the National Center of Biotechnological Information (NCBI). The oligonucleotides were synthesized by Syntol company (Russian Federation).

Construction of expression vector pET-24-SPA-CBD_2. The genetically engineered tandem CBD-CBD was obtained using plasmid *pCBD*, kindly provided for our research by Professor Y. Shoham (Israel). PCR-amplification of the CBD-CBD sequence was conducted using two pairs of specific primers:

1) sn1: 5’-CATGCGGCCGCAGGCGGTGTCCG AAGGCGGTGGCAGCGAAGGTGGCGGCGCAAA TACACCGGTATCAGG-3’, asn2: 5’-GTGGGATCC GGGTTCTTTACCCCATACAAGAACAC-3’;

2)sn3:5’-CATGGATCCGGCGGTGGCTCCGAA GGCGGTGGCAGCGAAGGTGGCGGCGCAAATA CACCGGTATCAGG-3’,asn4:5’-GTGCTCGAGGG GTTCTTT ACCCCATACAAGAACAC-3’.

The first pair of primers was used to introduce the sequence of linker “-Gly3-Ser-Glu-Gly3-Ser-Glu-Gly3-” and restriction sites *BamH*I at the 5’-end and *Xho*I – at the 3’-end into the DNA composition of one CBD: PCR-product 1. The second pair was used to build the same sequence of linker as well as recognition sites for endonucleases of restriction *Not*I at the 5’-end and *BamH*I – at the 3’-end into the composition of the second CBD: PCR-product 2. PCR-products 1 and 2 (534 b.p. each) were hydrolyzed with the corresponding restrictases and cloned into plasmid vector *pGEM-11Zf* with the obtaining of plasmid *pGEM-11Zf* (CBD_2), further hydrolyzed with restrictases *Not*I and *Xho*I to obtain the fragment CBD-CBD. The latter was ligated with plasmid vector for the expression of *pET24* and construction of plasmid *pET-24-CBD_2*.

DNA-sequence of SPA was amplified from chromosome DNA of *S. aureus*.

The following pair of primers was used for PCR: Sn-SPA: 5'-ATCATATGGCGCAACACGATGAAGCTCAA C-3' and Asn-SPA: 5'-ATGCGGCCGCTTCCT CTTT TGGTGC-3', which were used to introduce restriction sites *Nde*I and *Not*I into the composition of SPA sequence.

The purified product of SPA amplification (~880 b.p.) was subcloned into the plasmid vector *pET-24-CBD$_2$*, which was used to transform *E. coli* cells, BL21 strain (DE3).

Synthesis of SPA-CBD$_2$. Recombinant SPA-CBD$_2$ was expressed in *E. coli* cells in accordance to the modified protocol of autoinduction [12]. The localization of SPA-CBD$_2$ was determined by the separation of soluble and insoluble fractions of cell proteins in 12% SDS PAGE [13].

The analysis of binding SPA-CBD$_2$ to different types of polysaccharide matrices. To bind fibrous celluloses (CFI, CFII), microcrystal granular cellulose (CC31) (*Whatman*, Great Britain) and chitin (*New England BioLabs*, Great Britain), the aliquotes of SPA-CBD$_2$ (100 µl) were incubated for 1 h with the above mentioned matrices (20 µl), precipitated by centrifugation, washed with distilled water and analyzed in 12% SDS PAGE.

To obtain bioaffinity sorbent, SPA-CBD$_2$ was immobilized on microcrystalline cellulose CC31. Then the sorbent was washed off non-specifically bound proteins with the buffer (500 mM NaCl, 1 mM EDTA, 20 mM tris-HCl, pH 8.0) and kept at 4°C in 20% solution of ethanol.

The purification of polyclonal antibodies using the bioaffinity sorbent. Blood sera of immunized mice, diluted with the equilibration buffer, was added to the chromatographic column, containing 1 ml of bioaffinity sorbent, which was equilibrated with PBS buffer (50 mM Na_2HPO_4, 150 mM NaCl, pH 8.0). The rate of buffer flow was 0.5 ml per min. The sorbent was washed with PBS buffer, bound antibodies were eluted by 0.1 M Na-citrate buffer, pH 3.8. The purity of eluted antibodies was analyzed by electrophoresis in 12% SDS PAGE.

ELISA. The antigen rhIFN-a2b (recombinant human interferon a2b), provided by scientific and industrial complex FarmBiotek (Ukraine), was immobilized in vials of polystirol plate (10 µg/ml) (*Nunc*, Denmark). After blocking the sites of non-specific binding the vials were introduced using polyclonal antibodies to rhIFN-a2b, previously purified on bioaffinity sorbent, and incubated for 1 h at 37°C. Immune complexes were detected using secondary anti-mouse antibodies (*IMTEK*, Russian Federation), conjugated with horseradish peroxidase. TMB solution (*Sigma*, USA) was used as a chromogenic substrate. Statistical processing of results was conducted with the application of Origin 7.0 software package.

The application of bioaffinity sorbent for fractionation of mouse IgG. The chromatographic column, containing 1 ml of bioaffinity sorbent, was connected to the automated FPLC system (*Pharmacia*, USA). The column was equilibrated by the binding buffer (1.5 M glycine, 3 M NaCl, pH 8.9) and blood sera of mice, diluted with the same buffer, was applied. The rate of buffer flow was 0.5 ml per min. The sorbent was washed with the binding buffer before application. The bound antibodies were eluted using the following solutions: 0.1 M Na-phosphate, pH 6.0; 0.1 M Na-citrate, pH 4.5 and 0.1 M Na-citrate, pH 3.5. The purity and concentration of antibodies, obtained after the elution, were analyzed by electrophoresis in 12% SDS PAGE.

IsoQuick Kit for Mouse Monoclonal Isotyping (*Sigma*) was used to determine isotypes of IgG.

Results and Discussion. The criterion, determining the sorbent capacity, is the density of a ligand set on the matrix. Here the dynamic capacity of the sorbent is conditioned by the ligand orientation and availability of its active center. Therefore, the most reasonable method is a direct immobilization of the ligand, namely, the introduction of an additional thiogroup or adapter molecule into the composition of the protein ligand at the gene level [14, 15].

The adapter protein CBD, isolated from cellulolitic complex of *C. thermocellum*, providing for affinity binding of the protein on the polysaccharide matrix, was used for SPA immobilization in this work [10]. To obtain a fusion protein SPA-CBD$_2$ by genetic engineering, the interactive design was conducted in accordance to specificities of the structure of proteins-partners and the topology of their active centers [16, 17]. It was determined that the optimal design of the chimeric protein presupposes SPA at the N-end and

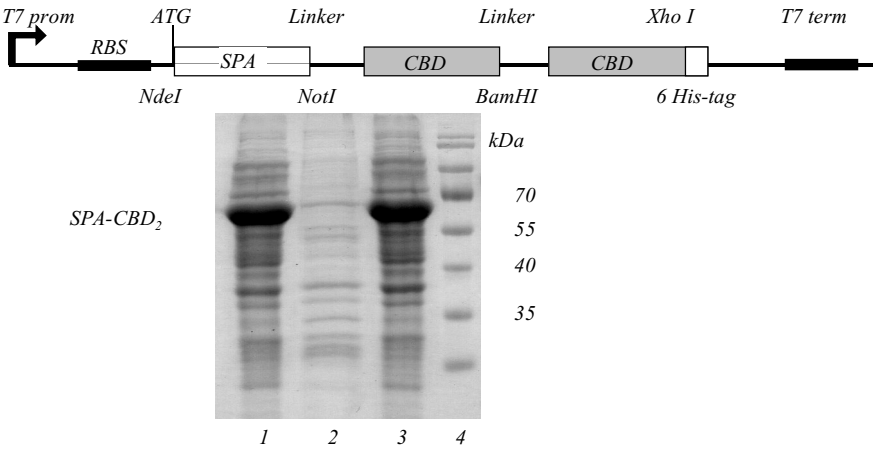

Fig.1 Schematic presentation of the cassette for the expression of recombinant fusion protein SPA-CBD_2 (*a*) and the electrophoregram of lysates of *E. coli* cells where the synthesis of SPA-CBD_2 was induced (*b*: *1* – total lysate of proteins of producent cells; *2* – fraction of insoluble proteins of the cell; *3* – fraction of soluble proteins of the cell; *4* – proteins-markers of molecular mass). 20 μl of culture were applied to each line.

two CBD with flexible linker sequences – at the C-end: "-Gly3-Ser-Glu-Gly3-Ser-Glu-Gly3-". This relationship ensures spatial availability of immunoglobulin-binding centers of SPA for the interaction with Fc-domains of antibodies. The introduction of two CBD sequences, instead of one, into the composition of recombinant protein is conditioned by the necessity of enhancing the affinity of binding the recombinant protein SPA-CBD_2 to cellulose [18]. C-terminal sequence of oligohistidine tag 6His-tag was introduced into the composition of SPA-CBD_2 to perform its chromatographic purification (Fig.1, a).

To create recombinant protein SPA-CBD_2, the DNA-sequence, encoding SPA, was amplified from chromosome DNA of *S. aureus*. The size of the amplified DNA fragment is 880 b.p. which corresponds to the aminoacid sequence of SPA five domains. The sequenation revealed its complete correspondence to SPA sequence, presented in the database of GenBank (Accession N EU695225.1) NCBI. The isolated DNA sequence, coding for SPA, was subcloned into plasmid vector *pET-24-CBD_2*, which was used to transform *E. coli*, BL21 strain (DE3). It is noteworthy that the producer of recombinant protein SPA-CBD_2 (*E. coli*, BL21 strain (DE3)), obtained by us, was sufficiently stable during long-term storage (at least one year).

Protein SPA-CBD_2 was obtained by bacterial expression according to the modified protocol of autoinduction. It was determined that SPA-CBD_2 is accumulated in the cytoplasm in soluble form (Fig. 1, b). The level of its expression is ~ 0,5 g/l (Fig.1, b) of *E. coli culture*, which is 80 times higher than in the work of Shoseyov et al. [19]. The results of CBD binding to cellulose and SPA binding to immunoglobulins demonstrated functional activity of both partner proteins SPA-CBD_2.

The next stage of our work was the selection of chromatographic matrix for the creation of bioaffinity sorbent. Since CBD has affinity to cellulose, different types of cellulose (microcrystalline and fibrous) as well as chitin were used for the analysis of binding. The binding experiments demonstrated that the capacity of SPA-CBD_2 for these sorbents fluctuates in the range from 0.4 (for CFI) to 1.2–1.5 mg/ml (for CC31 and chitin) (Fig.2). Based on these facts, for obtaining bioaffinity sorbent SPA-CBD_2 was immobilized on cellulose CC31 and chitin.

The literature data show that the affinity interaction of CBD and cellulose leads to the formation of a stable complex. It occurs due to hydrophobic interactions of several aminoacid residues of the domain with carbohydrate structure of cellulose matrix [17]. It was previously shown that one of the disadvantages of bioaffinity method of immobilizing hybrid protein on the matrix is partial dissociation of the complex in acid conditions (pH 3.0) and its enhancement at further decrease of pH to 2.0. The latter results in lower purity of the final product and narrower range of possible conditions of elution [11]. The solution of this problem was found in the introduction of genetically engineered tandem CBD-CBD into the composition of the hybrid protein SPA-CBD_2 which allowed enhancing its binding to the matrix [18].

The next required step was the determination of the conditions of elution, ensuring selective dissociation of antibodies and avoiding the leaking of the ligand. The

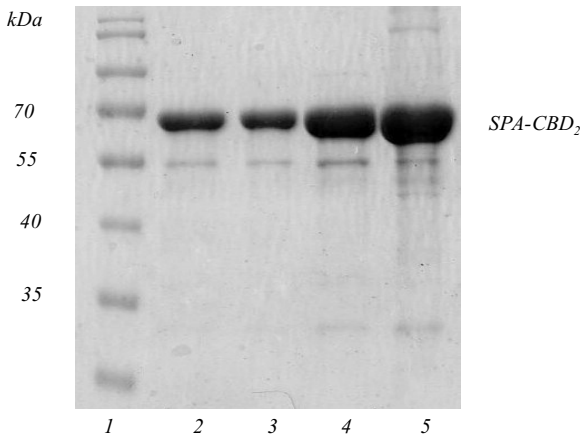

Fig.2 Electrophoregram of SPA-CBD_2 after binding to different types of polysaccharide matrices: *1* – proteins-markers of molecular mass; *2*, *3* – fibrillar cellulose CFI and CFII, respectively; *4* – microcrystalline cellulose CC31; *5* – chitin. 3 µl of sorbent were introduced to each line

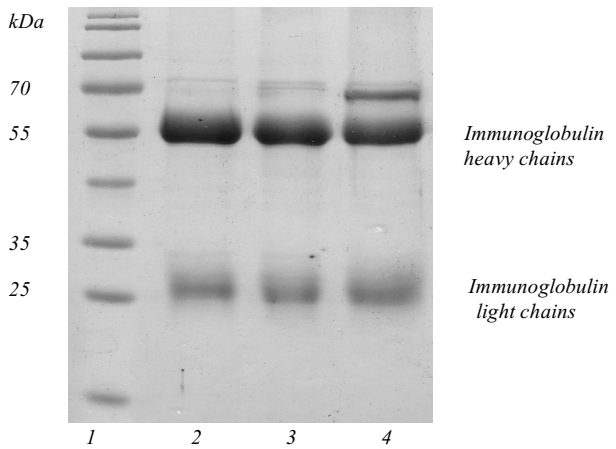

Fig.3 Electrophoregram of fractions of antibodies, purified on the bioaffinity sorbent, obtained during elution with buffer solutions with different pH values: *1* – proteins-markers of molecular mass; *2* – elution with Na-citrate buffer, pH 3.8; *3* – elution with glycine buffer, pH 3.0; *4* – elution with 2 M arginine, pH 4.3

following solutions were used for elution: 0.1 M glycine, pH 3.0; 0.1 M Na-citrate buffer, pH 3.8 and 2 M arginine, pH 4.3 [20]. After electrophoretic separation of obtained eluates it was determined that only 2 M arginine causes a considerable leaking of immobilized SPA-CBD_2 into the eluate. While using glycine and citrate buffer the end product is completely eluted with a high degree of purity. Based on this fact, further purification was conducted with the citrate buffer, pH 3.8 (Fig.3).

The obtained bioaffinity sorbent was used for isolation of antibodies from blood sera and ascite fluid of mice (the data are not presented). For this reason 3 ml of 30% suspension of CC31-SPA-CBD_2 were introduced into the 1 ml analytical column. After the equilibration of the sorbent with phosphate saline solution we applied diluted sera of mice, immunized with recombinant IFN-б2b, washed from non-specifically bound proteins, and eluted the antibodies with Na-citrate buffer, pH 3.8. As a result IgG of high purity (more than 95%) was obtained (Fig.4). The dynamic capacity of bioaffinity column for binding antibodies was determined by the concentration of isolated antibodies. It was found to be 3 mg of mouse antibodies per 1 ml of precipitated bioaffinity sorbent. Also we established the constancy of functional characteristics of the bioaffinity sorbent while storing during one year at 4°C and the possibility of its repeated application for several cycles of the isolation of antibodies without decreasing the dynamic volume.

The study on the formation of the protein A -immunoglobulin complex demonstrated that the main role in SPA binding to IgG is attributed to hydrophobic interactions between б-helix I of SPA and Fc-domains of IgG (over 80% of free energy of binding) while the selectivity of SPA to different subclasses of IgG is determined by electrostatic interactions between б-helix II of SPA and Fc-domains of IgG (20%) [21]. Therefore, the application of buffer solutions with high salt concentration for the binding of IgGs to the column promotes their more complete binding to SPA. It should be noted that IgG from SPA-based sorbents are eluted in milder conditions comparing with the application of other affinity sorbents (for instance, sorbents with immobilized protein G). It ensures obtaining pure fractions of immunoglobulins without significant loss of their functional activity.

Considering different affinity of SPA to specific isotypes of murine IgG, the latter were fractioned into subclasses by means of alternate diminution of pH value of the elution buffer: IgG1 was eluted at pH 6.0; IgG-2a – pH 4.5; IgG-2b – pH 3.5 (Fig.5). The presence of predominantly specific subclasses of murine IgG was confirmed using IsoQuick Kit for Mouse Monoclonal Isotyping. It is also known that human IgG1, IgG2, IgG4 interact with SPA with the affinity constant $K_A = 10^8\ M^{-1}$, while IgG3 demonstra-

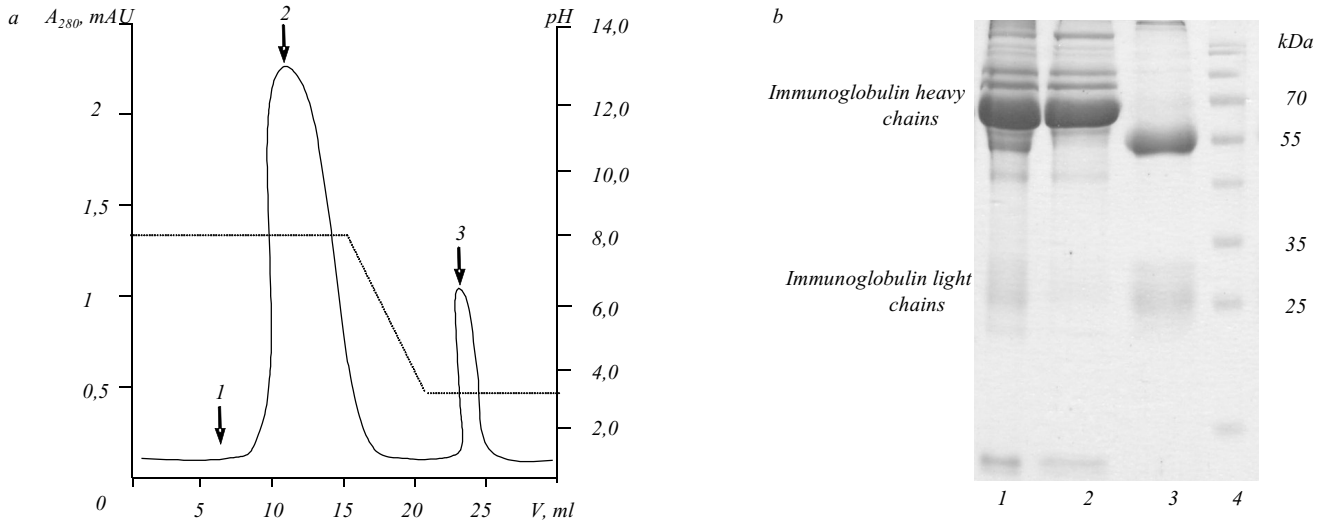

Fig.4 Isolation of polyclonal antibodies against human IFN- 2b from blood sera of immunized mice on the bioaffinity sorbent: *a* – chromatogram (*1* – application of proteins of blood sera of immunized mice; *2* – proteins, eliminated during the sorbent washing; *3* – elution of bound antibodies); *b* – electrophoregram of protein fractions (*1* – application of proteins of blood sera of immunized mice; *2* – proteins, eliminated during the sorbent washing; *3* – chromatographically purified fraction of polyclonal antibodies against human IFN- 2b; *4* – markers of molecular mass)

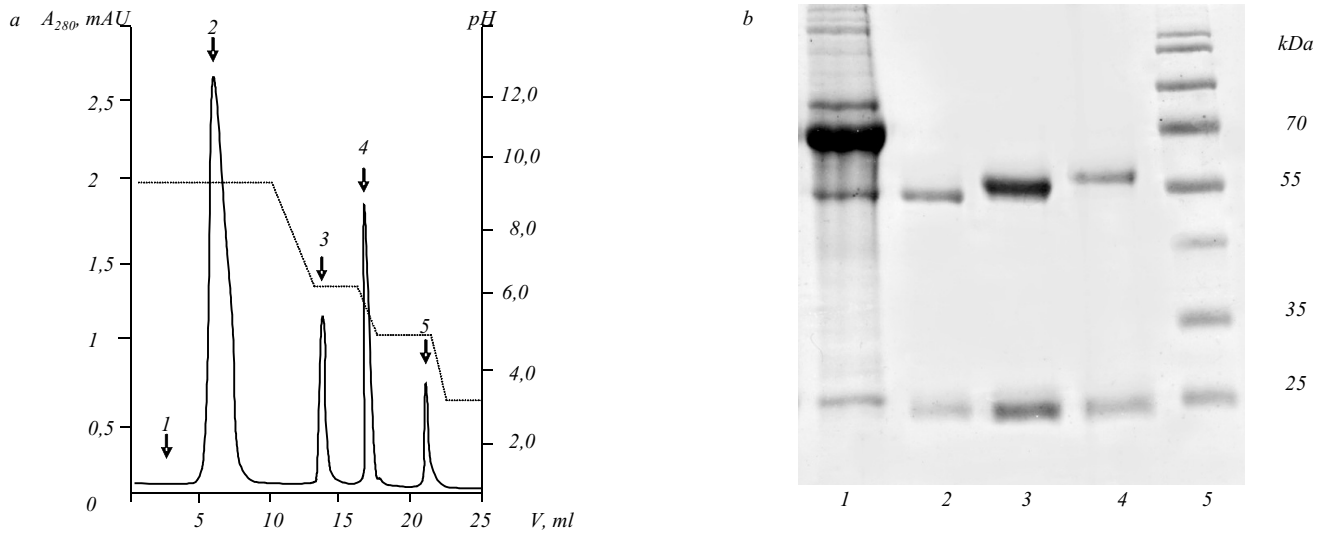

Fig.5 Fractionation of mouse IgG subclasses on the bioaffinity sorbent: *a* – chromatogram (*1* – application of proteins of blood sera; *2* – proteins, eliminated during the sorbent washing with the buffer; *3–5* – elution of bound antibodies with buffer solutions, containing 0.1 M Na-phosphate, pH 6.0 (*3*); 0.1 M Na-citrate, pH 4.5 (*4*) and 0.1 M Na-citrate, pH 3.5 (*5*)); *b* – electrophoregram of protein fractions (*1* – application of proteins of blood sera of immunized mice; *2–4* – purified fractions of antibodies, obtained after gradient elution; *5* – markers of molecular mass)

tes weak binding to SPA. It allows distinguishing IgG3 among the other subclasses of human immunoglobulins G.

Conclusions. The results obtained testify to the promising perspective of applying the bioaffinity sorbent, constructed by us, for highly efficient purification and fractionation of poly- and monoclonal antibodies from the complex biological mixtures in laboratory conditions. The antibodies, purified on the bioaffinity sorbent, demonstrated their stability during storing and sufficient sensitivity for the detection of corresponding antigens by ELISA method (Fig.6).

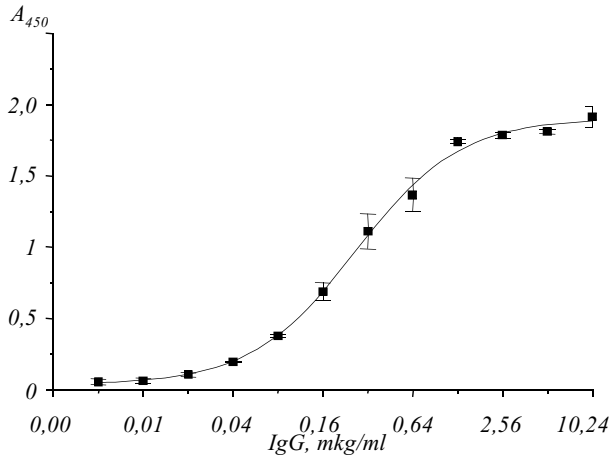

Fig.6 The curve of binding of polyclonal antibodies, purified on the bioaffinity sorbent, to rhIFN- 2b antigen at ELISA method ($M \pm m$; $n = 3$)

Besides, cellulose is a cheap and available material, which does not require additional chemical modifications, that is another evidence of appropriateness of the presented investigation.

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Биоаффинный сорбент на основе иммобилизованного белка А *Staphylococcus aureus*: создание и использование

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Sammary

Цель. *Создание биоаффинного сорбента на основе ориентированно иммобилизованного белка А Sta- phylococcus aureus (SPA) через два целлюлозосвязывающих домена (CBD) Clostridium thermocellum и его применение для очистки антител.* ***Методы***. *С использованием последовательностей ДНК, кодирующих SPA и два CBD, сконструирован ген слитного белка SPA-CBD_2 и обеспечено его получение в растворимой форме экспрессией в клетках Escherichia coli. Целевой белок иммобилизовали методом биоаффинного связывния на микрокристаллической целлюлозе.* ***Результаты***. *Установлены такие характери- стики биоаффинного сорбента, как емкость (1 мг SPA-CBD_2 /1 мл целлюлози), динамическая емкость (3 мг иммуноглобулинов мыши/1 мл сорбента) и продуктивность, а также показана его стабильность при долгосрочном хранении. С помощью биоаффинного сорбента выделены фракции иммуноглобулинов с чистотой более 95 %. Определено, что очищенные таким способом антитела с высокой чувствительностью выявляют соответствующие антигены.* ***Выводы***. *Предложенный биоаффинный сорбент позволяет получать очищенные функционально активные поли- и моноклональные антитела, а также проводить фракционирование на подклассы иммуноглобулинов G мыши.*

Ключевые слова: антитела, белок А, целлюлозосвязывающий домен, иммобилизация белка, аффинная хроматография.

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Біоаффінний сорбент на основі іммобілізованого білка А *Staphylococcus aureus*: створення і використання

Резюме

Мета. *Створення біоафінного сорбента на основі орієнтовано іммобілізованого білка А Staphylococcus aureus (SPA) через два целюлозов'язувальних домени (CBD) Clostridium thermocellum та його застосування для очищення антиті.* ***Методи***. *З використанням послідовностей ДНК, кодуючих SPA і два CBD, сконструйовано ген злитого білка SPA-CBD_2 та забезпечено його отримання в розчинній формі експресією у клітинах Escherichia coli. Цільовий білок іммобілізовано методом біоафінного зв'язування на мікрокристалічній целюлозі.* ***Результати***. *Встановлено такі характеристики біоафінного сорбента, як ємність (1 мг SPA-CBD_2/1 мл целюлози), динамічна ємність (3 мг імуноглобулінів миші/1 мл сорбента) і продуктивність, а також показано його стабільність при тривалому зберіганні. За допомогою біоафінного сорбента виділено фракції імуноглобулінів з чистотою понад 95 %. Визначено, що очищені у такий спосіб антитіла з високою чутливістю виявляють відповідні антигени.* ***Висновки***. *Запропонований біоафінний сорбент дозволяє отримувати очищені функціонально активні полі- і моноклональні анттіла, а також проводити фракціонування на підкласи імуноглобулінів G миші.*

Ключові слова: антитіла, білок А, целюлозозв'язувальний домен, іммобілізація білка, афінна хроматографія.

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