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Quantitative investigation of the affinity properties of different recombinant forms of protein G by means of high-performance monolithic chromatography

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Abstract

The recombinantly produced different forms of protein G, namely monofunctional immunoglobulin G (IgG) binding, monofunctional serum albumin (SA) binding and bifunctional IgG/SA binding proteins G, are compared with respect to their specific affinities to blood IgG and SA. The affinity mode of the recently developed high-performance monolithic disk chromatography has been used for fast quantitative investigations. Using single affinity disks as well as two discs stacked into one separation unit, one order of magnitude in adsorption capacities for IgG and SA were found both for monofunctional and bifunctional protein G forms used as specific affinity ligands. However, despite the adsorption difference observed, the measured dissociation constants of the affinity complexes seemed to be very close. The analytical procedure developed can be realized within a couple of minutes. Up-scaling of the developed technology was carried out using another type of monolithic materials, i.e. CIM® affinity tubes.

Keywords: Monolith columns; Monolithic discs; Affinity sorbents; Stationary phases, LC; Protein G; Proteins; Immunoglobulins; Albumin

1. Introduction

Many streptococcal strains are known to bind such major blood plasma proteins as immunoglobulin G (IgG) [1–3] and serum albumin (SA) [4–6] by their surface receptors. It has been also established that protein G isolated from group C and G Streptococci cell walls had functionally separated binding regions for each of the plasma proteins. Moreover, the structure of the IgG binding domains seems to be different to the SA binding domains [7–12]. Thus, it has been shown that the SA-binding domain is located in N-terminal A and B regions, while the IgG binding is connected with its C-terminal C region [13]. The C-terminal region of albumin molecule is responsible for the binding to protein G and IgG binds to the receptor via its Fc fragments by a non-immune mechanism.

At present, protein G is widely used in different application areas, mostly as a highly specific affinity ligand to isolate IgG from crude blood media [14–17]. For these purposes, to prepare complementary to immunoglobulins product, the albumin binding domains are usually cut at the recombination step in order to keep only the IgG specificity [18,19]. Thus, there is a large number of publications dealing with the study and applications of the IgG-binding protein G, but practically no data on the SA-binding protein

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G (or its bifunctional form). However, it would be quite interesting, first to evaluate and, second to compare the quantitative characteristics of the interaction of protein G with its natural counterparts, especially taking into account that both of them can be used in modern diagnostics [20].

A significant practical interest exists in developing selective, sensitive, quantitative and, finally, fast methods to study affinity protein–protein interactions as well as isolating pharmaceutical proteins from complex media. The recently introduced high-performance monolith chromatography (HPMC) [21–26] offers possibilities for protein separations based on different types of interactions with adsorptive surface of stationary phases. The principle suggested is realized not by the use of conventional beads but monolithic macroporous layers shaped as flat disks (high-performance monolithic disk chromatography, HPMDC) or tubes [27]. HPMC combines a high capacity and selectivity with low backpressure and, as a consequence, high speed and a short time of the process. Moreover, the affinity mode of HPMC provides an excellent opportunity to study the properties of in vitro constructed biological pairs. Such investigations provide more information about biological events taking place in vivo as well as developing optimized affinity separation media for production of valuable biological substances.

In the present paper the quantitative characteristics of the interaction between three genetically designed forms of protein G (bifunctional protein G binding both to IgG and SA and genetically separated fragments of protein G binding to IgG and SA) has been evaluated with use of HPM(D)C. Additionally, the present report describes the unique possibility of simultaneous isolation of IgG and SA from human blood plasma and rabbit sera by affinity HPMDC. The attempt to scale up the developed method using several stacked disks or a monolithic tube will be also discussed.

2. Materials and methods

2.1. Chemicals

Double distilled water and analytical grade chemicals purchased from Serva (Heidelberg, Germany) or Sigma (St. Louis, MO, USA) were used to prepare buffers and eluents. γ-Globulins fraction (human, from Cohn fraction II, III) and human serum albumin (SA) were from Sigma. Three recombinant forms of protein G—IgG-binding protein G (M_r 38 000), SA-binding protein G (27 000) and IgG/SA-binding protein G (65 000)—used as specific affinity ligands, were cloned and purified at the Institute of Experimental Medicine RAMS (IEM RAMS, St. Petersburg, Russia).

Human blood plasma was donated by Clinical Microbiology Laboratory of Army Medical College, St. Petersburg, Russia. Rabbit sera was prepared at the Department of Immunology of IEM RAMS.

2.2. Instruments

Affinity HPMC was carried out using a chromatographic system consisting of a peristaltic pump (2115 Multiperpex pump, LKB, Bromma, Sweden), a UV detector (2138 Uvicord S, LKB) and a recorder (2210 Recorder, LKB).

The concentration of proteins was determined using a UV–Vis spectrophotometer SF 26 (Lomo, St. Petersburg, Russia).

The purity of the affinity isolated proteins was verified by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) carried out with use of Mini Protean II System (Bio-Rad, Hercules, CA, USA) with silver staining detection.

Enzyme-linked immunosorbent assay (ELISA) was realized in polystyrene 96-well microplate format (Medpolymer, St. Petersburg, Russia). The optical absorbency of enzyme reaction products was detected using a multiscan spectrophotometer (Titertek Multiscan® MCC instrument, Helsinki, Finland).

2.3. Stationary phases

CIM® disk monolithic columns (length 3 mm × diameter 12 mm, bed volume 0.34 ml) and a CIM® tube monolithic column (length 45 mm × 15 mm O.D. × 1.1 mm I.D., bed volume 8 ml) with the original epoxy functional groups belonging to one of methacrylate co-monomers were provided by BIA Separations (Ljubljana, Slovenia). To install the disks into a chromatographic line, a specially de-

signed cartridge also constructed by BIA Separations was used.

2.4. Methods

2.4.1. Ligand immobilisation

In all cases, the published recently [26] reproducible covalent immobilization procedure was used. The CIM® disk washed consecutively by ethanol, ethanol–water (1:1) and water was immersed into 0.1 *M* sodium carbonate buffer (pH 9.3) for 2 h and then transferred into 1 ml of a 5.0 mg/ml of the affinity ligand (protein) solution in the same buffer. The binding reaction was allowed to proceed over 16 h at 34 °C without any stirring. Then the disk was washed with the initial carbonate buffer, pH 9.3, to remove the excess of unreacted ligand from the sorbent porous volume. The carbonate buffer was then replaced by the mobile phase used in affinity HPMC, i.e. phosphate-buffered saline (PBS, 10 m*M* phosphate buffer containing 150 m*M* of sodium chloride, pH 7.0).

The CIM® tube was equilibrated with 0.1 *M* sodium carbonate buffer (pH 9.3) and washed in the same manner as the disks. After that 5 ml of the same buffer containing 5.0 mg/ml of IgG-binding protein G was pumped through the support to fill its inner space. The input and output of tube were closed with caps and the immobilization was carried out for 20 h at 34 °C. After the reaction took place, the tube was washed—pumping through 5–10 volumes (40–80 ml) of PBS.

The amount of ligand coupled to the support was determined by monitoring the decrease in absorbency at 280 nm of protein concentration before and after immobilization with the account of protein content in washing buffer volume.

The affinity sorbents were stored in the PBS solution containing 0.02% sodium azide at 4 °C.

2.4.2. Affinity chromatography

HPM(D)C affinity separations were carried out using both the zonal and frontal analysis approaches. Thus, the apparent capacity of the affinity media investigated was established by a zonal adsorption–desorption run. In this case, 1 ml of standard solution of pure IgG or SA with a concentration of 1.5 mg/ml was loaded on IgG- and SA-binding protein G disks, respectively. As to the IgG/SA-binding protein G disk, its apparent capacity was evaluated with respect to both proteins in the same manner. Uncoupled specific ligand was washed out consecutively by PBS and, to eliminate any non-specific adsorption of protein of interst, 2 *M* NaCl. The desorption was carried out by elution with 0.01 *M* HCl (pH 2.0).

The affinity characteristics of the prepared protein G–CIM® disks, such as theoretical affinity adsorption capacity (q_{max}) and dynamic dissociation constants of affinity complex (K_{diss}), were evaluated on the basis of mathematical treatment of experimental adsorption isotherms resulting from the frontal analysis procedure [28,29]. For this purpose, model solutions of commercial IgG or SA with concentrations ranging from 0.1 to 1.4 mg/ml were passed through the disk. Unbound protein was removed with PBS and, additionally, the disks was washed with 2 *M* NaCl. Affinity bound IgG was eluted with strong acid (pH 2.0). To maintain the activity of the protein isolated, the pH of its solution obtained after desorption step was immediately adjusted to 7.5 with 1 *M* NaOH.

In the case of IgG-binding protein G–CIM® tube, 1 ml of 8 mg/ml IgG was applied to the column. Further washing and desorption steps were accomplished according to the procedure described above.

The samples of human plasma and rabbit sera were previously centrifuged and loaded on the disks and the tube without any dilution.

The protein concentration in the eluates obtained after desorption was determined by the Lowry test [30].

2.4.3. Gel electrophoresis

The IgG purity was verified by a standard SDS–PAGE procedure widely used for protein identification [29].

2.4.4. ELISA test

Wells of polystyrene microtitre plates were covered with the target protein (IgG or SA) at a concentration of 1 μg/100 μl in 0.1 *M* carbonate buffer, pH 9.6, and the plates were incubated overnight at 4 °C. Then, after removal of the non-adsorbed protein, the wells were washed three times with PBS (pH 7.2) containing 0.05% Tween 20

(PBS–T). Aliquots (100 μl) of the solution of bifunctional protein G labeled with horseradish peroxidase at an initial dilution of 1:100 were added to each well. The reactants were incubated for 1 h at 37 °C and the unbound protein G was removed by washing the wells with PBS–T. A 100-μl volume of enzyme substrate (0.5 mg/ml of *o*-phenylendiamine in 0.1 *M* sodium citrate–phosphate buffer, pH 5.0, containing 0.03% of H_2O_2) was added. The mixture was kept in darkness for 30 min, then the reaction was stopped by addition of 100 μl of 2 *M* H_2SO_4. The optical absorbency of the product was measured at 492 nm.

3. Results and discussion

3.1. Use of affinity HPMDC as a tool for investigation of functional properties of recombinant proteins G

As the recently developed HPMDC method proved to be very efficient for fast affinity protein isolations based on strong interactions between natural biological substances [28,29], the method was suggested to investigate quantitatively the interactions between three forms of genetically designed protein G, i.e. monofunctional IgG-binding protein G, monofunctional SA-binding protein G and bifunctional IgG/SA-binding protein G, with such two major blood plasma proteins as immunoglobulin G and human serum albumin.

3.1.1. Immobilization of receptorial recombinant proteins G on CIM® disks

Monolithic CIM® disks from different commercial batches were used to prepare affinity Protein G supports. All three forms of recombinant product were bound directly to the CIM® support by means of the reaction between the epoxy groups of polymeric material and protein amino groups (more probably at chosen immobilization conditions, ε-amino groups of lysine residues). The amount of ligands coupled to the disks seemed to be similar and ranged from 0.8 to 0.9 mg per disk (Table 1). The results obtained clearly show the high reproducibility of the immobilization procedure used in our experiments. Thus, the commercial sorbent demonstrated excellent batch-to-batch reproducibility of epoxy groups content as well as the porous channel-like morphology.

The described procedure was used to compare our data with those reported before. No special optimization of the method used was carried out in this paper.

3.1.2. Comparison of affinity parameters of investigated pairs

The behavior of immobilized bifunctional protein G with respect to IgG and SA binding has been quantitatively investigated using solutions of standard plasma proteins. Experimental data obtained have demonstrated that average disk's capacity to IgG binding was equal to 1.3 mg. This result was obtained for the IgG from a γ-globulin fraction which was chosen in order to make the model experiments maximum close to those carried out with crude plasma. In contrast, the apparent capacity of SA binding was established as 0.13 mg, e.g. about one order of magnitude lower (Table 2).

Since there are no reported data quantitatively comparing the interaction of the receptor discussed with IgG and SA, an ELISA test was additionally performed. For this purpose, IgG and SA at the same concentration of 1 μg/100 μl were applied onto the plate wells and the bifunctional protein G labeled

Table 1
Quantitative data on the immobilization of the proteins G on CIM disks

Recombinant protein G	Molecular mass	Amount of immobilized ligand	
		(mg/disk)	(μmol/disk)
IgG-binding protein G	38 000	0.86	0.022
SA-binding protein G	27 000	0.78	0.028
IgG/SA-binding protein G	65 000	0.93	0.014

Determinations were carried out in triplicate; RSD, 5–10%.

Table 2
Affinity parameters of the protein G disks

	Affinity parameters					
	IgG-binding			SA-binding		
	q_{exp} (mg/disk)	q_{max} (mg/disk)	K_{diss} (μM)	q_{exp} (mg/disk)	q_{max} (mg/disk)	K_{diss} (μM)
Disk with IgG-binding protein G	0.97	1.20	0.71			
Disk with SA-binding protein G				0.15	0.17	0.68
Disk with IgG/SA-binding protein G	1.34	1.49	0.46	0.13	0.15	0.65

K_{diss} and q_{max} values presented in the table are the average values of K_{diss} and q_{max} calculated from linearized forms (2) and (3) of the Langmuir equation (Excel). Note. Determinations were carried out in triplicate; RSD, 5–10%.

with peroxidase at the initial concentration of 1:100 was applied using twofold dilutions procedure. The comparison of the two curves presented in Fig. 1 confirms the quantitative correlation between IgG and SA binding obtained by HPMDC method.

Since the recombinant monofunctional IgG and SA binding forms of protein G are also produced at our laboratory, it was interesting to compare all the three receptorial proteins G with respect to their ability to bind two major plasma proteins discussed using the affinity HPMDC approach. From the results obtained, the immobilized monofunctional IgG-binding protein G demonstrated high specific capacity for IgG (0.9 mg of IgG) whereas the capacity of monofunctional SA-binding protein G disk was found as seven times lower (about 0.15 mg of SA adsorbed). This fact is in a good agreement with the results obtained for the bifunctional protein G used as a specific to IgG and SA affinity ligand.

The apparent dissociation constants (K_{diss}) and theoretical adsorption capacities (q_{max}) of all the affinity pairs were calculated on the basis of experimental adsorption isotherms. Fig. 2 demonstrates one typical profile. It is obvious that the present curve seems to be described by a Langmuir equation

$$q = q_{max}C/(K_{diss} + C) \quad (1)$$

where q is the amount of protein adsorbed on the affinity disk at a definite concentration C, q_{max} is the corresponding maximum adsorption capacity of the disk, C is the protein concentration, K_{diss} is the apparent dissociation constant of the affinity complex.

Eq. (1) can be rewritten in the linearized form

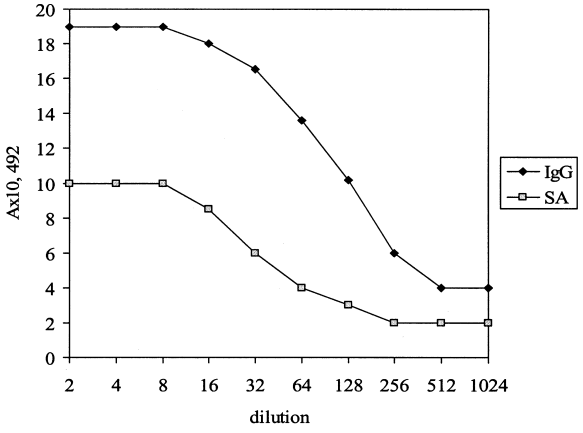

Fig. 1. ELISA test. The wells of a microplate were coated with IgG and SA at concentration of 1 μg/100 μl; ELISA was performed by using twofold dilutions of the bifunctional protein G labeled with peroxidase.

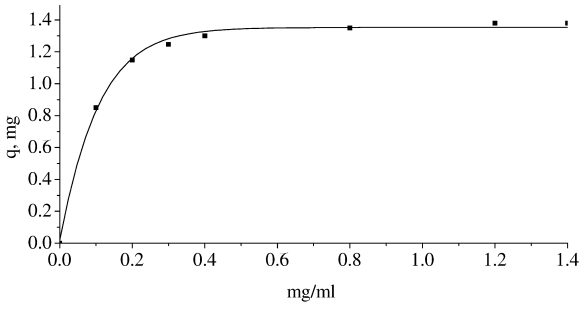

Fig. 2. Typical profile of experimental adsorption isotherm obtained by frontal analysis for IgG on the disk with immobilized bifunctional protein G. Conditions: CIM disk 12×3 mm; flow-rate, 2.5 ml/min, UV detection at 280 nm; adsorbing buffer PBS (pH 7); eluting buffer HCl (pH 2).

$y = ax + b$: either

$$C/q = c/q_{max} + K_{diss}/q_{max} \quad (2)$$

or

$$1/q = K_{diss}/q_{max}C + 1/q_{max} \quad (3)$$

The determination of the coefficients a and b using the method of the least squares allows the values of K_{diss} and q_{max} to be calculated. The values of affinity parameters are summarized in Table 2. As shown, according to the values of K_{diss} obtained, the interactions of SA and IgG with all the immobilized forms of protein G can be characterized as strong affinity ones and these results are in good agreement with previous data [31,32].

To conclude this paragraph, it is necessary to underline the thermodynamic similarity of the pairs examined and the very significant difference in IgG and SA amounts bound to all forms of protein G. In this regard, it seems to be quite important that last results obtained by HPMDC were confirmed by the independent ELISA method. Taking into account that in all cases the immobilization procedure was carried out under identical conditions, the influence of the matrix on the affinity interaction of the pairs discussed should be considered the same. Thus, the observed difference in binding capacity of IgG and SA sites can be explained as a specific structural feature of the binding domains.

3.2. Fast isolation of immunoglobulin G and serum albumin from mammalian blood media by affinity HPMC of different scale

3.2.1. Direct IgG and SA production using protein G disks

One of the main goals of our investigation was to develop a new, efficient and easily operated method for the production of pure IgG and SA on the basis of a modern chromatographic approach. That is why the analytical procedures created and studied for the isolation of IgG and SA directly from blood components seemed to be very important.

The special construction of the cartridge used for installation of CIM® disks allows to use several disks of different functionalities stacked into continuous monolithic unit and, thus, to carry out a simultaneous fractionation of target molecules from crude biological fluids [33,34]. In our experiments, such a multifunctional fractionation approach was applied to recover IgG and SA from mammalian plasma. The procedure has been developed with use of a simple scheme: (1) IgG-binding protein G disk and SA-binding protein G disk were installed consecutively into the same cartridge; (2) serum was passed through the stacked disks (Fig. 3a); (3) the SA-binding disk was removed from the housing and the desorption of IgG from single IgG-binding disk was accomplished (Fig. 3b); finally (4), the SA-binding protein G disk was reinstalled into chromatographic line, and the affinity bound SA was eluted (Fig. 3c). In this way, the whole processing operation required about 15 min at 2.5 ml/min flow-rate.

The most significant observation was that we found the same dynamic capacities for the IgG and the SA isolated from human plasma or rabbit sera as those found for IgG and SA used in model experi-

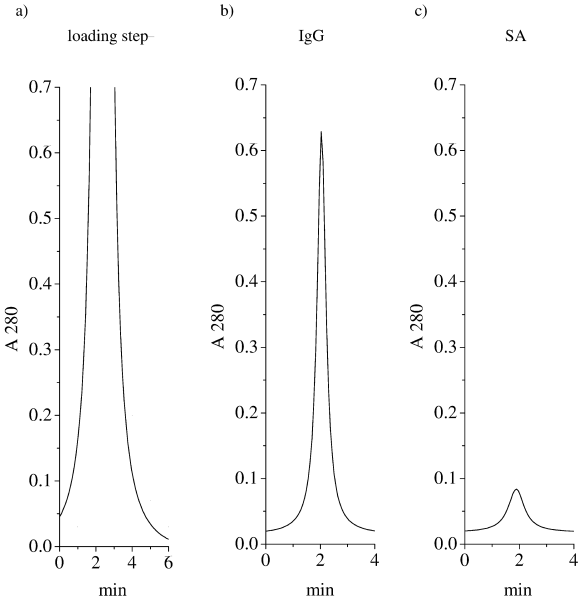

Fig. 3. Affinity fractionation of human sera on IgG-binding protein G disk and SA-binding protein G disk simultaneously installed into chromatographic line. Conditions: CIM disks 12×3 mm; flow-rate 2.5 ml/min, UV detection at 280 nm. Nondiluted human serum was passed through the two specific disks (a), loading volume 0.2 ml, unbound component were eluted with PBS; then IgG (b) and HSA (c) were consecutively desorbed from the appropriate disks with 0.01 *M* HCl.

ments. Thus, roughly 1 mg of immunoglobulin and 0.13 mg of serum albumin were isolated from blood media within a quarter of an hour.

3.2.2. Scaled-up process with use of IgG-binding protein G

Since immobilized recombinant protein G demonstrated one order of magnitude bigger adsorption capacity for IgG in comparison with that for SA, this affinity support was chosen to develop a semipreparative technique for IgG recovery from blood media. CIM® technology provides different ways to resolve such a problem.

The first approach is to stack several affinity disks of the same functionality (the same affinity ligand immobilized) into the single cartridge. Thus, two IgG binding protein G disks were used as one monolithic affinity unit. In this case, the amount of IgG isolated was found to be equal to 2 mg, e.g. double value in comparison with 1 mg for single disk

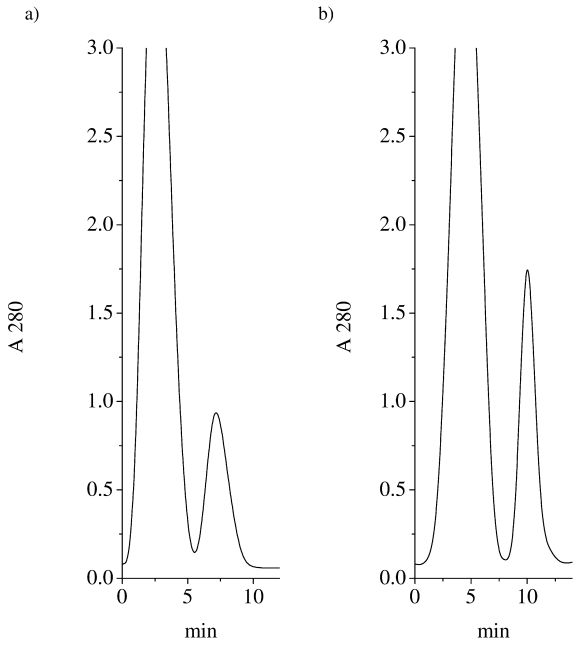

Fig. 4. Semipreparative extraction of immunoglobulin G from human sera by means of two CIM disks with immobilized protein G (a) installed into chromatographic line at the same time, and protein G CIM tube (b). Conditions: adsorption buffer PBS, pH 7.0; desorption buffer 0.01 HCl; flow-rate 2.5 ml/min for disks and 4 ml/min for tube; human serum was loaded without dilution, loaded volume 1 ml.

(Fig. 4a). The whole technological run including loading, washing and desorption steps was performed within 7 min at 2.5 ml/min flow-rate.

On other hand, an epoxy 8 ml CIM® tube was used for the preparation of IgG-binding protein G affinity column. The amount of the ligand coupled to the sorbent was 5 mg/unit. To evaluate the apparent capacity of such an affinity media, 1 ml of standard IgG solution with concentration of 8 mg/ml was loaded at 4 ml/min flow-rate and, respectively, 5 mg of IgG was measured in eluted desorption fraction.

Taking into account the results obtained, a scaled preparative process directed to extrapure IgG recovering from crude biological medium was developed. For this purpose, 1 ml of human plasma containing approximately 12 mg/ml of IgG was loaded on the column. The chromatogram presented in Fig. 4b shows the elution profile of IgG recovered from the sample. The most impressive fact was that the content of IgG directly isolated from blood plasma corresponded excellently to that found for standard immunoglobulin G (approximately 5 mg). The single technological cycle for the affinity tube is realized within 15 min at the flow-rate of 4 ml/min.

3.2.3. Quality control of protein product

The quality of immunoglobulin G (Fig. 5a) and serum albumin (Fig. 5b) affinity isolated from blood media were controlled by SDS–PAGE. For all procedures discussed above, the results clearly demonstrate very high level of purity of proteins isolated by very simple and fast one-step procedure.

3.2.4. Technological characteristics of protein G–CIM® supports

In designing a bioaffinity separation, particular attention must be paid to the productivity of the immunosorbents. Since the media used have improved mass transfer and mass transport characteristics, it was possible to process six to eight affinity-based purification cycles within 1 h using monolithic protein G–CIM® units. As a result, the productive efficiency was found to be equal to 8 mg/h for the single disk and 25 mg/h for the tube in the case of IgG extraction. It is significantly high.

Another aspect, which is very important for modern biotechnological processes, is the ability of affinity sorbents to withstand high flow-rates as well

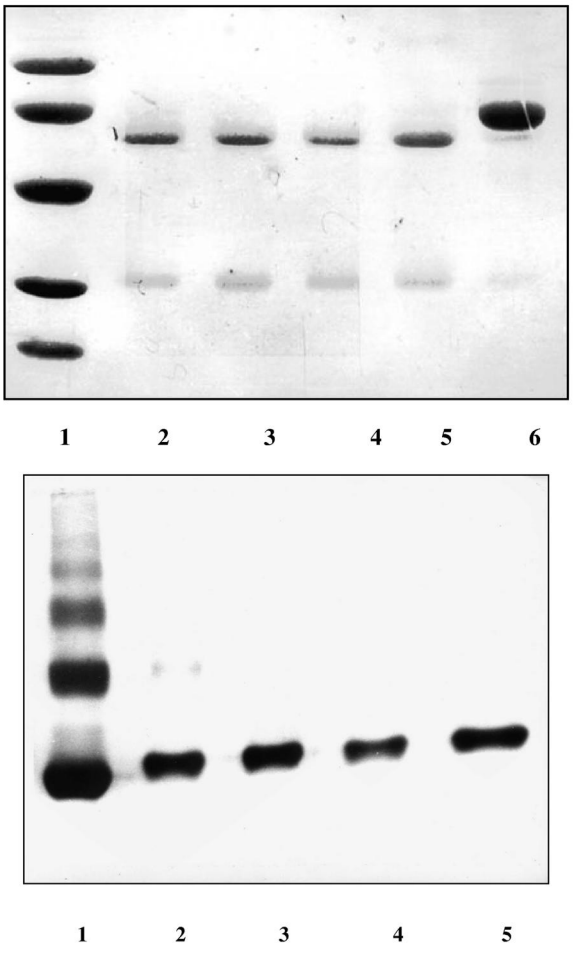

Fig. 5. SDS–PAGE quality control of IgG (a) and SA (b) isolated from human plasma. Lanes: (a) 1, molecular mass markers: 97 000, 66 000, 36 000, 21 000, 14 000; 2, commercial IgG (Sigma); 3, commercial IgG (Pharmacia, Sweden); 4, laboratory extracted IgG, using CNBr-activated agarose column with immobilized protein G; 5, laboratory extracted IgG, using protein G CIM disk; 6, crude plasma. Lanes: (b) 1, crude plasma; 2, commercial human SA (Sigma); 3, commercial human SA (Pharmacia, Sweden); 4, laboratory extracted human SA, using CNBr-activated agarose column with immobilized protein G; 5, laboratory extracted human SA, using protein G CIM disk.

as pH and temperature variations, in other words, their mechanical and chemical stability. After 2 years of operating, control experiments to determine adsorption capacity of protein G-CIM® supports were carried out. The apparent capacity found for both disks and tube was 60% of initial one. For this time period, such a stable level of affinity ligand activity can be deemed sufficient.

4. Conclusions

Three recombinant forms of protein G with different functional activities—IgG-binding, SA-binding, IgG/SA-binding—were compared with respect to their extraction capacity for immunoglobulin G and serum albumin. The affinity-high performance monolith disk chromatography, used in the present investigations, demonstrated many advantages. The main one is the possibility to carry out analytical processes at high speed and, consequently, to develop fast quantitative investigations. Thus, it was shown that bifunctional protein G as well as monofunctional ones expressed ten times greater isolation capacity for IgG then for SA. Despite the difference in the affinity adsorption, the dynamic dissociation constants of the complexes examined seemed to be of the same magnitude.

The combination of high selective and specific recombinant proteins G having unique IgG-binding properties with high-performance monolith chromatography allowed to realise a multifractionation approach for the purification of IgGs and SA from mammalian blood media.

Extremely high affinity capacity of the monofunctional IgG-binding protein G and an epoxy 8 ml CIM® tube allowed to develop a powerful semi-preparative method for the extraction of IgG from crude biological liquids. The results of SDS–PAGE demonstrated high purity level of isolated IgG. The affinity protein G–CIM® supports exhibited both high productivity and stability during 2 years of operation.

The method suggested could be considered as a successful example of the processes intended for ‘‘biotechnology-on-table’’ and this experience can be recommended as a basis for further developments of similar procedures.

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