ELSEVIER

Journal of Chromatography A, 798 (1998) 65–72

JOURNAL OF CHROMATOGRAPHY A

Fast isolation of protein receptors from streptococci G by means of macroporous affinity discs

Cornelia Kasper[a], Ljudmila Meringova[b], Ruth Freitag[c], Tatiana Tennikova[d,*]

[a]*Institute of Technical Chemistry, University of Hannover, Hannover, Germany*
[b]*Institute of Experimental Medicine, Russian Academy of Medical Sciences, St. Petersburg, Russia*
[c]*Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland*
[d]*Institute of Macromolecular Compounds, Russian Academy of Sciences, St. Petersburg, Russia*

Abstract

A fast affinity method for the semi-preparative isolation of recombinant Protein G from *E. coli* cell lysate is proposed. Rigid, macroporous affinity discs based on a glycidyl methacrylate–co-ethylene dimethacrylate polymer were used as chromatographic supports. The specific ligands (here human immunoglobulin G, hIgG) were immobilized by the one-step reaction between native epoxy groups of the polymer surface and ε-amino groups of the IgG molecules. No intermediate spacer was necessary to reach full biological activity of the ligand. The globular affinity ligands are located directly on the pore wall surface and are thereby freely accessible to target molecules (here Protein G) migrating with the mobile phase through the pores. It is shown that the conditions chosen for the hIgG immobilization do not involve an active site of the protein and thus do not bias the formation of the affinity complex. Chromatographically determined constants of dissociation of hIgG–Protein G affinity complexes confirm the high selectivity of this separation method. Two different aspects of the affinity separation are discussed, which differ mostly in terms of scale. In disc chromatography, high volumetric flow velocities are possible because of the small backpressure. Since in addition the mass transfer is more efficient, it becomes possible to achieve very short analysis times. The discs proposed can be used in a single-step enrichment of Protein G from lysates of non-pathogenic *E. coli*. Gel electrophoresis data are used to demonstrate the high degree of purity achieved for the final product.

Keywords: Affinity discs; Immobilized immunoglobulins; Proteins; Protein G; Immunoglobulins

1. Introduction

The ability of the pathogenic streptococci for the interaction with human and animal plasma and serum proteins is very well known [1,2]. Currently, the possible interaction between the various immunoglobulin A and G (IgG, IgA) subtypes with certain *Streptococcus* C and G surface receptors, e.g. Protein G, has gained a prominent place in affinity chromatography. The immunoglobulins (Ig) interact with the surface receptor of the microorganism by means of their fc-fragment. Ig-Fc type receptors exposed by streptococci strain have recently attracted increasing scientific and practical attention, due to the necessity to isolate these immunoglobulins from their production environment.

The efficient isolation of proteins from complex sources such as cell culture supernatants remains a

*Corresponding author.

0021-9673/98/$19.00
PII S0021-9673(97)00981-3

challenge. The recently developed high-performance membrane chromatography (HPMC) is promising in this regard, because of its high capacity and selectivity, combined with low back pressure and short operation times. This method uses the disc configuration in the macroporous stationary phase design and thereby combines the advantages of HPLC and membrane separations [3–10]. Due to the inherent speed of the isolation it facilitates the recovery of a biologically active product, since the exposure to putative denaturing influences such as solvents, temperature, and contact time is reduced.

In conventional affinity chromatography, columns packed with swelling or rigid porous particles are used as stationary phase [11,12]. Such columns usually are severely mass transfer limited, since the molecules reach the adsorptive surface only by diffusion. For affinity interactions involving macromolecules, such as proteins, the time needed for the surface reaction may well be significantly lower than that necessary for the diffusion of the target molecules to an adsorption site at the core of a porous bead [13]. The maximum throughput is also limited by the backpressure of the column.

Membranes are employed as filters to separate biomolecules whose size differs by roughly an order of magnitude or more. Membrane filtration is widely used in bioprocessing recovery. Chemical modification of filtration membranes to adsorb biomolecules in the filtrate originally employed biospecific affinity ligands [14–21].

Affinity HPMC using the specially designed discs with a macroporous structure identical to effective particle sorbents is likely to overcome many critical disadvantages. Most importantly, the better mass transfer mechanism (convection rather than diffusion) allows to consider only the biospecific reaction as time limiting.

In this paper we describe the use of affinity glycidyl methacrylate–co-ethylene dimethacrylate (GMA–EDMA) discs bearing human IgG ligands as affinity mediators for the recovery of IgG-binding fragment Protein G variants of human strain G148 produced by recombinant, non-pathogenic *E. coli* [22]. The isolated receptors are intended for application in practical immunology, immunochemistry and medical diagnostics.

2. Experimental

2.1. Materials

Monomers were from Sigma–Aldrich (Deisenhofen, Germany). The commercially produced GMA–EDMA discs of 10×3 mm size (CIM discs) were donated by BIA (Ljubljana, Slovenia).

Proteins and fine chemicals were from Sigma, bulk chemicals for buffer and eluent preparation were from Fluka. The human immunoglobulin G (hIgG, reagent grade) and model recombinant Protein G from *Streptococcus* sp. expressed in *E. coli* with molecular mass of 22 000 used during the optimization experiments were from Sigma (St. Louis, MO, USA).

The Institute of Experimental Medicine of the Russian Academy of Medical Sciences (IEM RAMS, St. Petersburg, Russia) kindly donated lysates of recombinant Protein G from *E. coli* and pure Protein G standard. The molecular mass of this Protein G variant was 38 000.

2.2. Instruments

2.2.1. Chromatographic system

The chromatographic system consisted of two piston pumps (Irica Σ 871, ERC, Alteglofsheim, Germany), a UV detector (Spectroflow 757, Kratos Analytical, Ramsey, NJ, USA), a mixing chamber (ERC) and a six-port valve (Valco, Houston, TX, USA). The data were processed with a Chromatography Station for Windows software program (Data Apex, Prague, Czech Republic).

For chromatographic use, the discs were installed in cartridges specifically designed for HPMC (Dr. Ing. Herbert Knauer, Berlin, Germany and BIA).

The semi-preparative affinity chromatography was carried out using a peristaltic pump, a UV–Vis detector and a plotter (all from Pharmacia Bromma, Uppsala, Sweden).

2.3. Gel electrophoresis

The Protein G purity was verified by gel electrophoresis. A Phast System (Pharmacia Biotech, Uppsala, Sweden) with silver staining detection was

used. This procedure guarantees a minimum sensitivity of 0.3–0.5 ng of protein per band. A 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel was used.

2.3.1. Quantitative protein determination

The modified Lowry protein assay [23] (Pierce, Rockford, IL, USA) was carried out according to the advisable procedure with UV–VIS colored protein registration using a spectrophotometer Unicon 922 (Kontron Instruments, Milan, Italy).

2.4. Methods

2.4.1. Synthesis of the semi-preparative GMA–EDMA discs

The macroporous polymer monoliths were prepared by free radical polymerization of a mixture of glycidyl methacrylate, ethylene dimethacrylate, 2,2′-azobis-isobutyronitrile as an initiator and porogenic solvents (cyclohexanol and dodecanol) in barrels of polypropylene syringes as published elsewhere [24,25]. The monolith was cut into 2-mm thick discs using a lathe. The discs were washed with methanol, methanol–water (50:50) and water.

2.4.2. IgG immobilization procedure

After washing with high-purity water the wet discs were immersed into a 20 m*M* sodium carbonate buffer, pH 9.3, for 1–2 h before the reaction. After that, the discs were transferred into a 20 m*M* sodium carbonate buffer, pH 9.3, containing an additional 7–10 mg/ml of hIgG. Of the IgG solution, 1.5 ml were needed for the small discs and 3–5 ml for the reaction in the case of the bigger ones. The reaction took place over 16 h at 30°C. Afterwards any remaining epoxy groups were blocked by exposure to 1 *M* ethanolamine at room temperature for 1 h. Following this, the discs were immersed overnight in a similar volume of phosphate-buffered saline (PBS, 10 m*M* phosphate buffer containing 150 m*M* sodium chloride pH 7.0) at 5°C. The amount of IgG bound to the discs was calculated from a quantification of the protein concentration in the reaction mixture before and after the immobilization. The modified Lowry protein assay was used for the determination of the immobilized protein amount.

2.4.3. Affinity chromatography

The affinity chromatography was performed as a continuous sorption–desorption act using a stepwise gradient. Unless indicated otherwise, a PBS buffer, pH 7.0, was used for the adsorption step. Desorption was achieved in 0.01 *M* HCl, pH 2.0. No special washing procedure after protein adsorption was used. The samples of model proteins were dissolved in the sorbing buffer A (PBS) at a concentration of 1 mg/ml. The cell lysate with 20 mg/ml crude protein concentration was diluted 1:10 or 1:5 with PBS before chromatographic experiments.

The values of the effective dissociation constants (K_{dis}) of immobilized IgG–Protein G pair were measured using affinity chromatography data analysis [26]. Here, frontal analysis was carried out. Protein G concentrations of 0.17 and 0.34 mg/ml were used for the saturation step. The protein loaded was desorbed with 0.01 *M* HCl (pH 2.0). The flow-rate at both steps was 1 ml/min.

3. Results and discussion

3.1. Macroporous structure of affinity discs

The idea of using ‘membrane’, ‘filter’, or ‘disc’ configuration in a chromatographic stationary phase has been already discussed for several years. The most detailed review was presented by Roper and Lightfoot [27]. The advantages are generally assumed to be low back pressure and superior mass transfer, which concomitantly enable the use of unusually high mobile phase flow-rates compared to conventional biopolymer chromatography. As we mentioned above, the best results have been obtained with stationary phases that resemble conventional filter membranes in all aspects save for the activation of the surface with interactive groups. However, such filters, while useful, are not optimized for biopolymer chromatography. In this type of separation, issues like surface area (capacity), average pore sizes and pore size distribution are equally important. We have, for some time now, produced macroporous GMA–EDMA discs with a specially designed porous structure (pore size and pore size distribution), which are constructed to perform well under the high flow-

rate/high throughput conditions required for fast biopolymer chromatography [3–5].

The disc, or rather rod, is created by radical co-polymerization of suitable monomers (GMA–EDMA) in a tube. During the polymerization, small nodules (<1 μm) are formed, which are essentially non-porous due to the high extent of cross-linking. The non-porous nodules then aggregate into a continuous network with interconnecting channels between them, permitting high hydrodynamic flows (Fig. 1). Thus, this technique allows for an excellent combination of resolution, speed, and capacity.

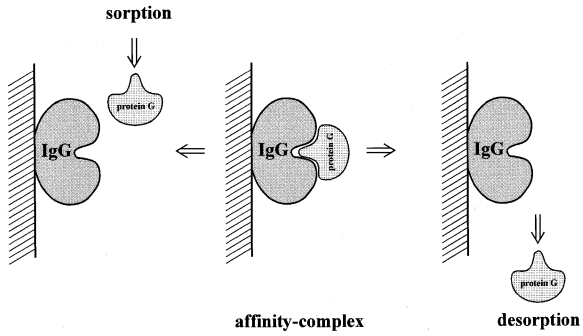

Fig. 2. Sorption–desorption act (scheme).

3.2. Optimization of affinity ligand (hIgG) binding

The most important advantage of GMA–EDMA polymer is the native epoxy groups of one of the co-monomers (GMA). Depending on the pH, these groups react with any amino group residue of the affinity ligand. No spacers are necessary in our experience, since the porous configuration of the chromatographic support allow good access to the affinity ligands even in the absence of spacers (Fig. 2). The amount of epoxy groups on the disc surface is determined by the polymerization feed, i.e. the percentage of glycidyl methacrylate and the degree of their substitution depends on the morphology of polymer.

For hIgG, a 20 m*M* sodium carbonate buffer, pH 9.3, was the optimum immobilization buffer, while the reaction could be carried out at 30°C. This means that in the case of GMA–EDMA discs the immobilizing procedure of the biological ligand is a one-step reaction using very gentle 'biocompatible' conditions. Fig. 3a demonstrates that the reaction should be allowed to proceed for a minimum of 16 h, to ensure that the maximum amount is bound. For the same reason, the IgG concentration in the binding buffer should be at least 7 mg/ml (Fig. 3b). Under these conditions, the maximum ligand concentration (up to 15 mg/ml affinity support) is achieved and, as will be shown later, without any loss of ability for Protein G binding.

3.3. Affinity chromatography of recombinant Protein G

3.3.1. Effect of flow-rate on Protein G retention

The commercially available CIM discs (10×3 mm) were used in the investigation of the influence of flow-rate on the Protein G adsorption step as a first step in the development of a fast affinity disc chromatography procedure. It was found that a

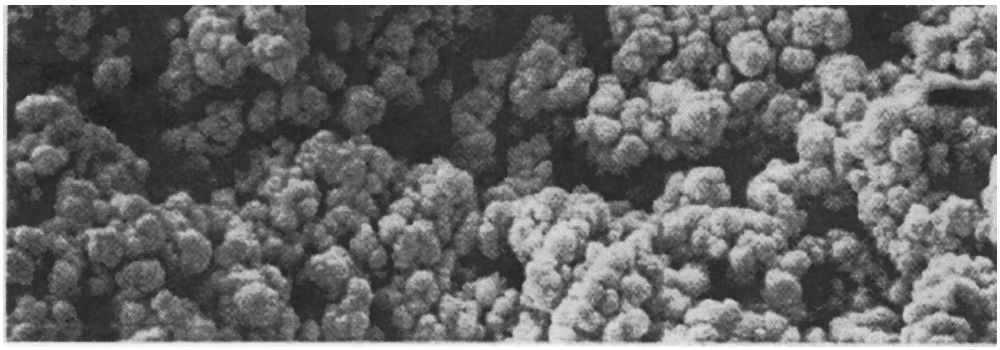

Fig. 1. SEM picture of GMA–EDMA polymer macroporous structure.

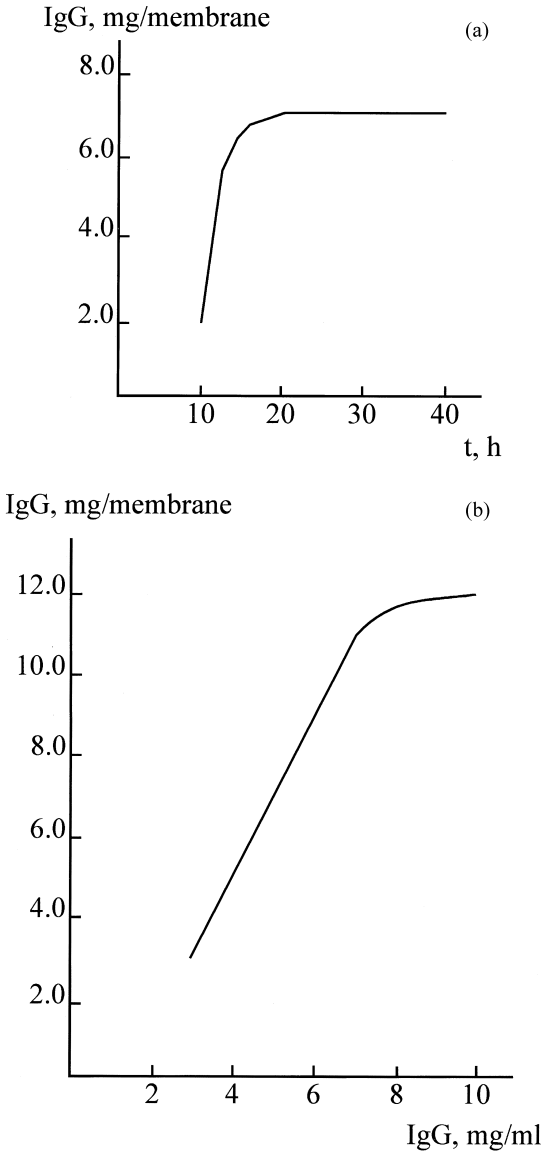

Fig. 3. (a) Dependence of the amount of hIgG bound to the GMA–EDMA disc (25×2 mm) on the reaction time. Conditions: sodium carbonate buffer, pH 9.3; 30 ml of 5 mg/ml Protein G; temperature 30°C. (b) Influence of IgG concentration on the hIgG amount immobilized. Conditions: dimensions of disc, buffer and temperature were the same as in (a); the time of reaction was 16 h.

certain portion of Protein G did not bind to the discs. This portion is constant for both samples of Protein G studied (commercially available from Sigma with M_r 22 000 and specially Protein G isolated by IEM RAMS with M_r 38 000) and in all cases was roughly equal to 10–15% of the total protein loaded. The peak shape (height, width) of this non-bound portion showed no dependency on the flow-rate (Fig. 4). This fact emphasizes the perfusion mechanism of the described affinity HPMC, where the dependence of peak broadening of non-retained marker on flow-rate is negligible. Fig. 4 also demonstrates that within the range of flow-rate studied the Protein G dynamic capacity was the same. This is different from previously reported results on membrane affinity chromatography [28]. Our results shown here argue in favor of the absence of diffusional mass transfer resistance. These data also confirm a balanced pore size dis-

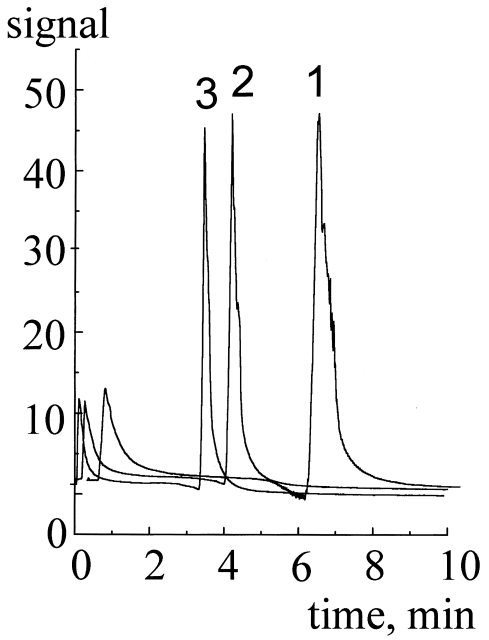

Fig. 4. Dependence of flow-rate on sorption–desorption efficiency. Conditions: flow-rate 3 (peak 3), 2 (peak 2) and 1 (peak 1) ml/min; the first three small peaks belong to the non-retained part of isolated Protein G; probe volume, 20 μl. Stepwise gradients: flow-rate, 1 ml/min: 0–4 min, 100% A; 4.0–4.5 min, 0–100% B; 4.5–8.0 min, 100% B; 8.0–8.5 min, 100–0% B; 8.5–10 min, 100% A; flow-rate: 2 ml/min, 0–3 min, 100% A; 3.0–3.5 min, 0–100% B; 3.5–7.0 min, 100% B; 7.0–7.5 min, 100–0% B; 7.5–10 min, 100% A; flow-rate 3 ml/min: 0–2 min, 100% A; 2.0–2.5 min, 0–100% B; 2.5–6.0 min, 100% B; 6.0–6.5 min, 100–0% B; 6.5–10 min, 100% A. PBS buffer, pH 7.0, was used as buffer A (adsorption step); buffer B (desorbing one) was 0.01 *M* HCl pH 2.0.

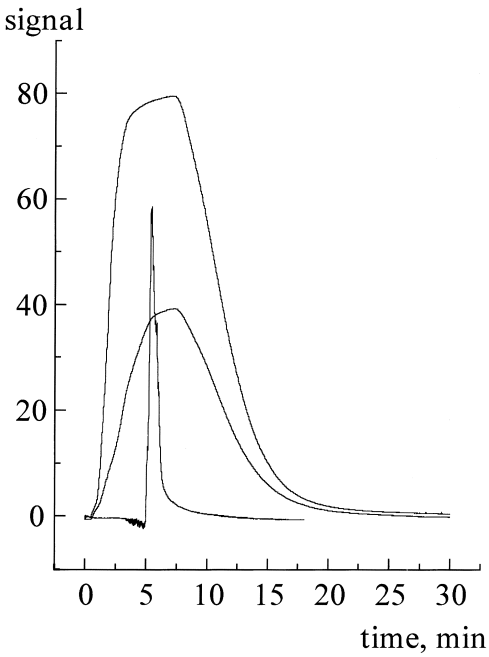

Fig. 5. Frontal analysis of Protein G capacity. Conditions: CIM disc, flow-rate, 1 ml/min; sample volume, 5 ml (loop); total amount of Protein G in sample loaded, 0.83 mg (lower curve) and 1.67 mg (upper curve); 0.18 mg were desorbed in both cases (one sharp peak which means the same quantity of desorbed Protein G). Mobile phase of adsorption step is PBS buffer, pH 7; desorption was caried out with 0.01 *M* HCl, pH 2.0.

tribution within the macroporous structure of GMA–EDMA polymer, which is characterized by a negligible part of small (less than 250 nm) pores on one hand and, on other hand, the absolute priority of very large transport channels (up to 1000 nm) [29].

3.3.2. Affinity characteristics of GMA–EDMA-IgG discs

Fig. 5 presents the data on the determination of dynamic Protein G (IEM RAMS) capacity using the analytical GMA–EDMA–IgG disc (10×3 mm). Two different concentrations of Protein G were used for the loading. It seems to be very important that, in both cases discussed, the same amount of protein was isolated after the desorption step. It is necessary to add here that no difference in chromatographic behavior of protein analyzed, available from the sources mentioned earlier, was noticed. This means that the identical molar amount was isolated in both cases.

The residual biological activity, i.e. affinity interaction of surface-bound IgG with recombinant Protein G from two different sources, was measured by affinity chromatography data analysis. The effective constants of dissociation (K_{dis}) of the affinity complexes clearly showed that these interactions should be characterized as strong affinity ones (Table 1). As shown in Table 1, the optimized method of IgG immobilization was successfully applied both to the smaller, analytical GMA–EDMA discs (10×3 mm) available from a commercial source (BIA) and the home-produced bigger (semi-preparative) ones of 25×2 mm. The affinity supports compared had very similar specific characteristics, such as capacity per unit of disc volume and order of magnitude of affinity constants.

3.3.3. Fast affinity Protein G isolation from E. coli cell lysate

Taking into account all of the results discussed above, a rapid affinity chromatographic process was developed using the GMA–EDMA macroporous discs for Protein G recovery from *E coli* cell lysate. Fig. 6 shows the results of an isolation of Protein G from cell lysate in analytical scale using the 10×3-mm disc. The amount of Protein G isolated corresponded exactly to its content in crude proteins determined recently by immunoassay.

It was possible to apply the same procedure of

Table 1
Affinity characteristics GMA–EDMA-IgG discs

	Analytical device (CIM disc)	Semi-preparative device (25×2-mm disc)
Amount IgG immobilized (mg)	2.2	10.2
Dynamic affinity capacity (mg)	0.2	0.9
Dynamic affinity capacity (mol)	5.3×10^{-9}	2.4×10^{-8}
Affinity constant, *K* (mol)	3.3×10^{-8}	1.1×10^{-7}

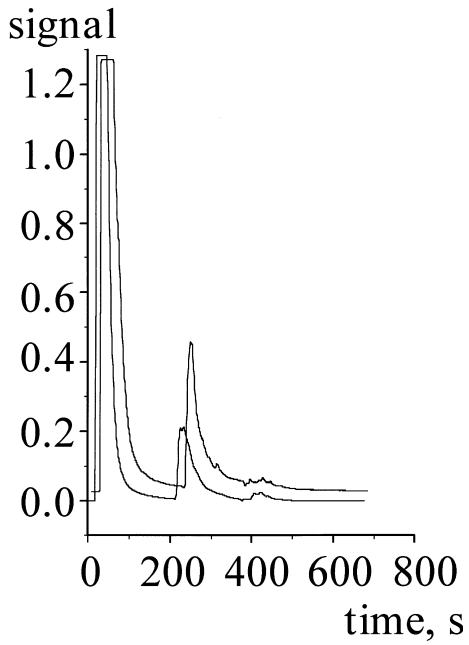

Fig. 6. Fast isolation of Protein G from 1:5 and 1:10 diluted cell lysate (solid and dotted elution curves, respectively). Conditions: buffer A (adsorption) was PBS, pH 7.0; buffer B (desorption) was 0.01 *M* HCl, pH 2.0; flow-rate, 3 ml/min; CIM disc; probe volume, 5 μl. Stepwise gradient: 0–2 min, 100% A; 2.0–2.1 min, 0–100% B; 2.1–6.0 min, 100% B; 6.0–6.1 min, 100–0% B; 6.1–10.0 min, 100% A.

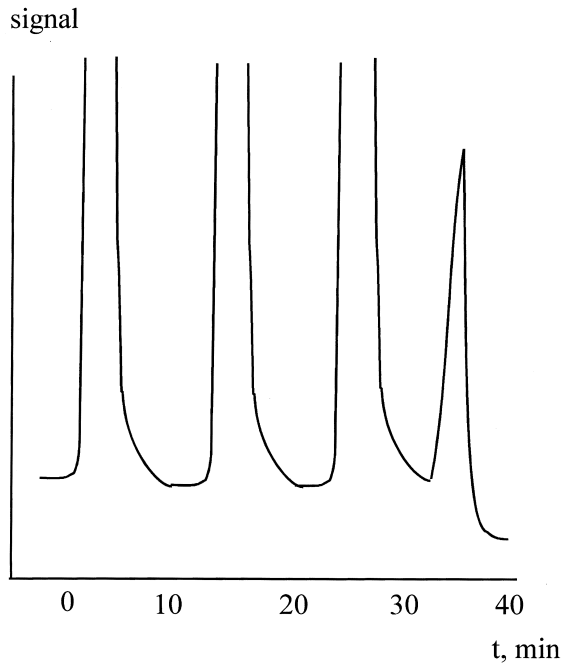

Fig. 7. Use of 'three sorption–one desorption' approach to increase the Protein G capacity. Conditions: semi-preparative GMA–EDMA disc of 25×2 mm dimensions; 1:10 diluted cell lysate; flow-rate, 2 ml/min; the volume of cell lysate loaded was 1×3 ml (three portions). Chromatographic procedure: 1st loading, 0–10 min, 100% A; 2nd loading, 10–20 min, 100% A; 3rd looding, 20–30 min, 100% A; 30.0–30.1 min, 0–100% B; 30.1–35.0 min, 100% B (desorption); 35.0–35.1 min, 100–0% B; 35.1–40 min, 100% A (equilibring). PBS buffer, pH 7.0, was used as buffer A (adsorption) and 0.01 *M* HCl pH 2.0 served as buffer B (desorption).

Protein G affinity isolation to the bigger discs. As a result, about 0.4 mg was recovered during one sorption–desorption affinity run at 2 ml/min flow-rate and 1:5 cell lysate dilution. The chromatographic system used was very simple and consisted merely of a peristaltic pump and a UV detector only. Using the 'three-adsorptions–one-desorption'-approach it was possible to isolate up to 1 mg of pure Protein G within minutes (Fig. 7).

It is necessary to add that the purity of the isolated Protein G was controlled by gel electrophoresis. It was proved that a Protein G of high purity is isolated the very simple, fast, one step procedure proposed here.

4. Conclusions

Disc affinity chromatography thus helps to minimize the total time spent on affinity isolation procedures, a feature that also holds exciting prospects for the use of affinity discs in various state-of-the-art monitoring processes [30].

Acknowledgements

This paper is the result of a collaboration between the University of Hannover, Germany, and the Russian Academy of Science, which has been made possible by DfG-grants to Prof. Ruth Freitag BIA (Ljubljana, Slovenia) kindly donated the analytical CIM discs. The authors also thank Acad. Professor A.A. Totolian and Dr. T.V. Gupalova (IEM RAMS, St. Petersburg, Russia) for kindly presenting the samples of recombinant Protein G and cell lysate.

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