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Journal of Chromatography A, 852 (1999) 129–140

JOURNAL OF CHROMATOGRAPHY A

Quantitative fast fractionation of a pool of polyclonal antibodies by immunoaffinity membrane chromatography

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Abstract

A new affinity method for the direct quantitative analysis of monospecific anti-peptide immunoglobulins (antibodies) and, simultaneously, their semi-preparative isolation from blood serum of the immunized animals has been developed. Immunoaffinity discs based on macroporous poly(glycidyl methacrylate–*co*-ethylene dimethacrylate) were used as the supporting stationary phase. The specifically prepared synthetic peptides with biological activity imitating that of the immunoglobulin binding sites of various proteins were used as the selective ligands instead of native proteins. These ligands were immobilized by a single-step reaction that involves epoxy groups located on the pore surface of the porous polymer disc with amine groups of the peptide molecules. A spacer between biospecific ligands and the linking site was not required to achieve good separation. These novel immunosorbents characterized by large binding capacity are well suited for high throughput screening. Dissociation constants of the peptide–antibody complexes calculated from the experimental adsorption isotherms confirm the excellent selectivity of the proposed separation method. The discs were used in a single step enrichment of antibodies both from precipitated blood fraction and crude blood serum of immunized animals. The quantitative data of the immunoaffinity disc chromatography were compared to those obtained by an enzyme-linked immunosorbent assay. Gel electrophoresis was also used to demonstrate the high degree of purity of the final product. In contrast to typical techniques that involve proteins, this immunoaffinity approach allows for the first time direct determination of concentration of specific antibodies using the immunosorbent prepared from the short peptide molecules immobilized on the internal surface of reactive porous polymer discs.

Keywords: Membrane chromatography; Immunoaffinity chromatography; Antibodies

1. Introduction

It is known that the biological interactions which take place in vivo are based on the formation of specific complexes of complementary molecules, most often proteins. Textbook examples of these complexes are the pairs; enzyme–substrate, enzyme–inhibitor, antigen–antibody, hormone–receptor, etc. Similar pairs that involve a synthetic molecule are being used successfully in both analysis and downstream processing leading to isolation of highly purified products [1]. The antigen–antibody complex is one of the most often used biocomplementary pairs in different biotechnological processes [2,3]. Similarly, a wide range of diagnostic procedures are based on these high specific interactions. The very popular separation technique immunoaffinity chromatography (IAC) finds also its origin in biocomplementary interactions. [4,5]. IAC is widely used not only in the production of valuable biological

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00672-X

substances such as monoclonal antibodies, but also seems to be quite useful in repeated routine analysis to determine the accumulation of antibodies in stressed human or animal organisms (immunomonitoring). Therefore, development of fast, simple and reliable methods of immunodiagnostics of different diseases is a serious task [4,6,7].

The fractionation of polyclonal antibodies expressed in vivo is currently also important in relation to the development of both practical and theoretical immunology. For example, the striking similarity between unique specificity of antibodies (Abs) and enzymes has been noticed recently [7,8]. The extensive immunological investigations lead to better understanding of the mechanism of specific binding between Ab and its complementary antigen (Ag). In addition, these studies also revealed genetic reasons for the large variety of the antigen binding section in immunoglobulins [2,9–11]. It was demonstrated experimentally that the typical (in vivo) immune response can be accompanied by formation of so-called catalytic antibodies [12,13]. Besides their typical participation in binding and elimination of Ag, such antibodies (abzymes) acquire catalytic activity similar to that of enzymes. These catalytic antibodies appear as a result of involvement of biological mechanisms within an immune response that concerns a wide array of antibody forming cells. Under these conditions, some immunoglobulins represent an 'image' of the original Ab and, in addition, they also mimic some of the biological functions of a specific antibody [14–17]. For example, the catalysis of transformations of biological molecules, which are carried out by antibodies in vivo, is a widespread immunological phenomenon [18].

The recent data show that abzymes may catalyze different types of biochemical reactions such as proteolysis [19–26], hydrolysis of nucleic acids [19,20], and polysaccharides [27–32]. These antibodies are often expressed by the living body suffering from autoimmune pathologies. Significant correlation between the abzyme activity of the whole pool of polyclonal antibodies or its fractions isolated using affinity techniques and the occurrence of systemic lupus erythematosus, rheumatoid arthritis, bronchial asthma, pancreatitis and other diseases has been monitored [33]. This is quite promising since the determination of the catalytic activity of antibodies isolated by fractionation from the polyclonal pool can be used in the future for effective diagnostic of specific diseases.

High-performance membrane chromatography (HPMC) is a very useful separation method that allows to carry out the separation of complex biological mixtures in fast elution mode. The basic characteristic of this method is the use of thin monolithic macroporous discs ('membranes') made of a modified glycidyl methacrylate–ethylene dimethacrylate copolymer instead of the convenient packed column [34–42]. The reactive polymer support with optimized pore size [43] and carefully balanced hydrophobic–hydrophilic matrix allows simple attachment of ligands that include biological molecules and their fragments onto the surface of pores within the monolith and the preparation of efficient media for the affinity separations [44] and solid-phase enzyme reactors [45,46].

A ligand, such as a protein immobilized on the surface of an affinity sorbent, is highly specific to its counterpart that can be present in the mobile phase. However, these ligands are often expensive and mostly labile. Therefore, substitution of the natural affinant with its more stable fragments would be better suitable. Indeed, fragments with binding clusters obtained from antibodies cloned and expressed in *E. coli* have been used, for example, as a ligand for the purification of lysozyme [47]. This enzyme has also been purified using an immobilized synthetic peptide imitating the antigen–antibody binding site [48]. Obviously, synthetic peptides, which mimic antigen determinants of immunizing protein antigens or antigens generally, are currently successfully used as highly specific affinity ligands [49–51]. It has to be emphasized that small peptides are weak immunogens and, as a rule, artificial conjugates including the peptide of interest and a macromolecular counterpart are used to produce specific antibodies. These counterparts could be proteins [52] as well as other natural and synthetic polymers [50]. Obviously, the organism 'excited' in such a way produces a wide spectrum of antibodies including anti-immunoglobulins against all fragments of the conjugate. However, the quantitative determination or preparative isolation of specific anti-peptide Ig that is a tiny part of a larger pool of polyclonal antibodies is a challenging practical problem that requires fast and reliable affinity approaches.

We now wish to report a rapid, highly selective

and reliable method for affinity isolation of monospecific antibodies from polyclonal mixtures retrieved from blood serum of immunized animals. This method is based on HPMC and utilizes synthetic ligands as biospecific ligands for the preparation of immunoaffinity sorbent discs.

2. Experimental

2.1. Materials and instruments

Synthetic peptides (i) pentadecapeptide-part of the C-chain of proinsulin, (ii) P1-type hexadecapeptide of the outside loop of the protein ATPase of Menkes' protein and (iii) nonapeptide hormone bradykinin were synthesized in solid-phase on benzhydrylamino polystyrene support using *tert*.-butoxycarbonyl (Boc)/benzyl (Bzl) strategy and purified by RP-HPLC. A chromatographic system consisting of two high pressure piston pumps, a UV spectrophotometric detector and plotter (Waters, USA) and a semipreparative column Nucleosil C_{18} (bead size, 20 μm) was used. The separations were carried out using a linear gradient from 100% 0.1% trifluoroacetic acid (TFA) in water (buffer A) to 50% (v/v) acetonitrile in buffer A 50% (buffer B). The gradient time was 30 min at flow-rate of 3 ml/min.

Mixtures of polyclonal antibodies to the synthetic ligands were produced by immunization of rabbits both by pentadeca- and hexadecapeptides with hemocyanine, or by the conjugates of bradykinin with bovine serum albumin (BSA) and 'star-like' polymeric conjugates including BSA, grafted on the protein short polymeric chains with end-bound bradykinin [53].

The macroporous glycidyl methacrylate (GMA)–ethylene dimethacrylate (EDMA) monoliths were synthesized by free-radical polymerization described elsewhere [34] and cut to discs 25 mm in diameter and 2 mm thick that have a volume of about 1 ml. The porous polymer had a mean pore size of 800 nm, a pore volume of 0.6 ml/ml according to intrusion mercury porosimetry (Micromeritics, USA) and a specific area of 15 m^2/g as determined by dynamic nitrogen adsorption/desorption (BET) (Micromeritics). The macroporous discs were fixed in a cartridge specifically designed recently by Saulentechnik Knauer (Berlin, Germany).

Buffers used in immunoaffinity chromatography were prepared by dissolving salts of analytical grade purity in double distilled water additionally purified by filtration through a microfilter Milex (Millipore, USA) with 0.2 μm pore size. All chromatographic experiments were carried out at room temperature.

Immunoaffinity membrane chromatography was carried out using a LKB (Bromma, Sweden) system consisting of a peristaltic pump (Microperpex 2132), UV detector (Uvicord S 2138) and plotter (1-Channel Recorder 2210).

The concentration of peptides were determined from the optical absorbancy of their solutions at the maximum wavelength in the UV spectrum (spectrophotometer SF-26, LOMO, St. Petersburg, Russia). Amino acid analysis was carried out using an automatic amino acid analyzer (T339M, Mikrotechna, Prague, Czech Republic). The concentration of proteins in eluates was calculated using the extinction coefficient of protein solution at 280 nm and was also determined by Lowry's method [54].

The titers of antibodies were established using an enzyme-linked immunosorbent assay (ELISA) [51] with immobilized antigen complex in the 96-well microplate format (Biohit, Finland). The optical absorption of solutions in the wells was determined at 492 nm using an immunoenzyme colorimetric analyzer KAI-TS-01 (Scientific Research Center of Biological Instrumentation, Moscow, Russia). The conjugates of peroxidase and Protein A were kind gifts of Pasteur's Scientific Research Institute of Epidemiology and Microbiology (St. Petersburg, Russia). BSA and highly purified casein were purchased from Sigma–Aldrich (Germany).

Gel electrophoretic characterizations of initial mixtures and isolated fragments were carried out using a Flat Bed Apparatus FBE-3000 (Pharmacia, Uppsala, Sweden). Gels were prepared using the set of chemicals purchased from Reanal (Hungary). Sodium dodecyl sulfate (Sigma–Adrich) and Coomassie Blue R-250 (Merck, Germany) were also used in this method.

2.2. Methods

2.2.1. Immobilization of synthetic ligands on macroporous polymeric disc

Porous polymer discs were washed with methanol, methanol–water (50:50, v/v) and water. Washed

discs were immersed into 0.1 mol/l sodium borate buffer (pH 10.0) for 24 h. The discs were then transferred into 4 ml of a 4–5 mg/ml peptide solution in the same buffer. The binding reaction was allowed to proceed at room temperature for 24–48 h without any stirring. The residual epoxide groups of the polymeric carrier were quenched with 1 mol/l 2-aminoethanol in 0.1 mol/l sodium borate buffer (pH 10.0) for 2 h at ambient temperature. After this procedure, discs were washed consequently with 0.1 mol/l sodium borate buffer and water and immersed into buffer A.

2.2.2. Quantitative determination of covalently bound ligands

The amount of immobilized peptide was determined (i) according to its content in a solution before and after the reaction with the polymeric support (disc) using RP-HPLC (column 300×4 mm I.D.; Vydac C_{18}, 5 μm, chromatographic system Waters; eluent A: 0.1% TFA in water, eluent B: 50% acetonitrile in A, gradient elution from 100% A to 50% B in 25 min at a flow rate of 1.5 ml/min); (ii) according to absorbancy of the solution at a wave length of the maximum of the UV spectrum before and after the reaction with the disc's material; (iii) according to data of amino acid analysis of a part of the polymer after its use in immunoaffinity HPMC.

2.2.3. Determination of parameters of dynamic adsorption on GMA–EDMA discs

Frontal analysis was used to determine the adsorption properties of immunosorbents (immunodiscs). Solutions of antibodies with different concentrations in 0.01 mol/l sodium phosphate buffer, pH 7.0, containing 0.07 mol/l NaCl [phosphate-buffered saline (PBS buffer)=eluent A] were eluted through the disc until no further increase in the absorption density at the exit of the chromatographic cell has been monitored. The antibodies present in pores were removed by washing with the buffer and after that the strongly adsorbed part containing specific antibodies was isolated by a flow of 0.01 mol/l HCl, pH 2.0 (buffer B).

2.2.4. Immunoaffinity chromatography

Monospecific antibodies were isolated from a crude blood serum of immunized rabbits or from a serum preliminary fractionated by triple precipitation with ammonium sulfate. In the former case, 1.5 ml of serum with a typical protein concentration of 80 mg/ml were diluted with five volumes of buffer A and the adsorption at dynamic conditions was allowed to proceed (loaded sample volume 7.5 ml). After removal of ballast proteins in a washing step with 0.01 mol/l sodium phosphate buffer, pH 8.3, containing 2 mol/l NaCl, the adsorption/desorption process was repeated two more times. Adsorbed antibodies were desorbed by eluent B. The disc was washed with water, 0.05 mol/l NaOH, water again and equilibrated by the eluent A after each experiment. In the second procedure, the concentration of protein in the solution prepared from the fraction of serum precipitated by ammonium sulfate was 5 mg/ml (loaded volume 2 ml); after desorption with HCl (pH 2), the disc's were not regenerated. The flow rate at both adsorption and desorption was 2 ml/min.

2.2.5. ELISA method

Determination of titer of antibodies was carried out using a solid phase immunoenzyme analysis on 96-well microtiter plates. The diluted solutions of Ig with total protein concentration of 10 mg/ml were used as initial samples.

A 0.01 mol/l sodium phosphate buffer, pH 7.4, with 1% NaCl (PBS buffer) and 0.05% Twin-20 (PBST buffer) was used to wash the wells and dilute the Protein A–horse-radish peroxidase conjugate. PBST with 0.1 mol/l sodium phosphate was used to dilute the solutions of antibodies.

The 100 μl volume of peptide solution with a concentration of 100 μg/ml in PBS was pipetted into each well and kept for 15 min under the UV lamp. After that, 100 μ1 of 100 μg/ml BSA solution in 0.1 mol/l sodium carbonate buffer, pH 9.6, was added and the mixture incubated for 20 h at 40°C.

The wells were washed with PBS and 150 μl of 0.5% colloid solution of casein in 0.1 mol/l PBS was added immediately into each well. This mixture was incubated for 1 h at 37°C. After six-times repeated washing with PBST at 1 min intervals, solutions of antibodies (Ab) with the initial dilution of 1:20 were added into each well. The time of incubation at 37°C was 1 h. After removing the Ab solutions and washing six times with PBST, 100 μl of Protein A–peroxidase conjugate was injected into each well

and kept for 30 min at 37°C. After another washing six times with PBST, 100 μl of substrate (the 0.5 mg/ml solution of *o*-phenyldiamine in 0.1 mol/l sodium citrate–phosphate buffer, pH 5.0, containing 0.03% H_2O_2) were added. The mixture was kept for 30 min at room temperature in the dark. The reaction was terminated by addition of 100 μl of 2 mol/l H_2SO_4. Optical absorbency was determined at 492 nm and compared with the control (the wells covered by neutral casein only).

2.2.6. Gel electrophoresis

The 5 μl markers and proteins samples (concentration of 2 mg/ml) with 0.1% sodium dodecyl sulfate were applied in parallel and a plate covered with 7% polyacrylamide gel (PAAG). The fixation and staining of protein zones were carried out simultaneously after the electrophoretic run using 0.2% Coomassie Blue in methanol–acetic acid–water (30:10:60, v/v). The stained zone of Coomassie Blue dye was used as a control marker.

3. Results and discussion

3.1. Immobilization of synthetic ligands on macroporous discs

The immobilization of short peptides on the pore surface of the support has its own specifics:

(i) Small molecules of oligopeptides do not have the tertiary globular structure. Therefore, they typically need to be attached through a spacer, which removes the adsorption site from the polymer surface and allows for the optimal interactions between the ligand and the binding center of an antibody. This essentially eliminates steric effects that would otherwise be vital because of the proximity of the surface of the stationary phase.

(ii) The peptidic affinant itself has only a small volume and occupies only a small part of the space within the pore. As a result, the large molecules of antibodies (Ig) must, on average, traverse a longer distance. Since diffusional mobility of macromolecules is slower compared to small molecules, the motion of the Igs requires a longer time. This, in turn, leads to the requirement of longer residence time within the pore space of the matrix. It means that the overall flow rate of the mobile phase must be lower than it would be in the case of pairing of large molecules such as for immobilized protein and IgG [44].

(iii) Variations in the chemical structure of a peptide is largely limited by the small number of connected amino acid residues compared to a large protein. This allows essentially for only a single attachment mode common for all of the peptides: a chemical reaction of surface functionalities of the matrix with end groups of the peptide.

(iv) Although peptides may be assumed to be small protein fragments, their properties are considerably different from those of larger protein molecules. For example, peptides used for the production of antibodies are often the critical short sequences of the immunogenic part of much larger proteins. These segments may have completely different solubility than the parent polypeptide.

The solid support chemistries useful in peptide coupling include those that can react with amines, carboxyl groups, sulfhydryl groups, and phenolic hydroxyls. The choice of coupling reaction affects the orientation of the peptide at the surface and can reinforce the interactions with specific antibody molecules as a result of better orientation. Site-directed immobilization techniques are typically considered to be more suitable than methods that result in random coupling [48].

High reactivity of the epoxide groups of the macroporous GMA–EDMA discs together with their high content (3–5 mmol/g) and opened porous channel-like morphology [43] allows to carry out immobilization in a single step under mild conditions using amine groups of the ligand while forming strong C–N bonds [44].

Various short synthetic peptides such as the pentadeca- and hexadecapeptides corresponding to the hypothetical antibody-binding domains of the C-chain of proinsulin and of the outside membrane loop of ATPase of P-1-type (Menkes' protein that participates in copper transport in the human body), and the nonapeptide hormone bradykinin were used as highly specific affinity ligands for the isolation of antibodies throughout our research. Table 1 shows that the amount of bound ligand that has been determined by both RP-HPLC and from the difference in absorbency of the peptide solution before and

Table 1
Amounts of peptide ligands immobilized on GMA–EDMA discs

Ligand	Immobilized ligand	
	mg/ml	μmol/ml
Pentadecapeptide (M_r 1600)	2.6	1.6
Hexadecapeptide (M_r 2000)	5.9	3.0
Nonapeptide (bradykinin) (M_r 1060)	2.9	2.7

after the reaction was in the range 1.6 to 3.0 μmol/disc. The amino acid analysis of the sorbent confirms these values. The immobilization reaction was typically completed after 20 h since no increase in capacity was observed even after another 24 h of reaction. Similar binding kinetics has been recently observed for the immobilization of macromolecular ligands (IgG) on the GMA–EDMA discs [44].

The matrix surface coverage by the immobilized peptide ligands can be simply calculated using a cross-section of the peptide molecule and its molar concentration. Thus, the pentadecapeptide occupies 2 m^2, hexadecapeptide 4.8 m^2, and bradykinin 1.8 m^2. Since the specific surface area of the matrix is 10 m^2/g and its mass 0.9 g, the surface coverage data indicate that the surface of transport channels is occupied almost completely in the case of hexadecapeptide, while the other two peptides occupy only half of the surface. This difference can be explained by the different reactivity of peptides at chosen conditions. However, these differences do not affect the activity of the immunisorbents (see below). It is also necessary to note that the immobilization of peptide ligands was carried out without any use of a spacer usually recommended in conventional affinity chromatography. For example, such spacers were used recently in the flow-through rod columns [55]. It is likely that the role of a spacer is played by the fragment $-COO-CH_2CHOH-CH_2-$ which is formed after the opening of the epoxide ring of the glycidyl functionality. The length of this bridge (about 0.9 nm) seems to be sufficient to provide the optimal interaction of peptide ligand with an antibody. This confirms the optimal porous structure of the polymeric support in which the formation of the affinant–ligand complex occurs without steric limitations.

3.2. Test of non-specific interactions

Fig. 1 shows the single peak of the two-step affinity chromatography of BSA using the disc with immobilized hexadecapeptide as the affinity ligand. After the second elution step (desorption) no peak of desorbed fraction occurs in the chromatogram. This indicates the absence of specific affinity binding of the protein marker and peptide ligand. The amounts of loaded and recovered BSA are equal. This further confirms the absence of non-specific (hydrophobic or ionic) interactions between protein and polymer matrix. This is also why no other eluents except for buffer B were tested in this step.

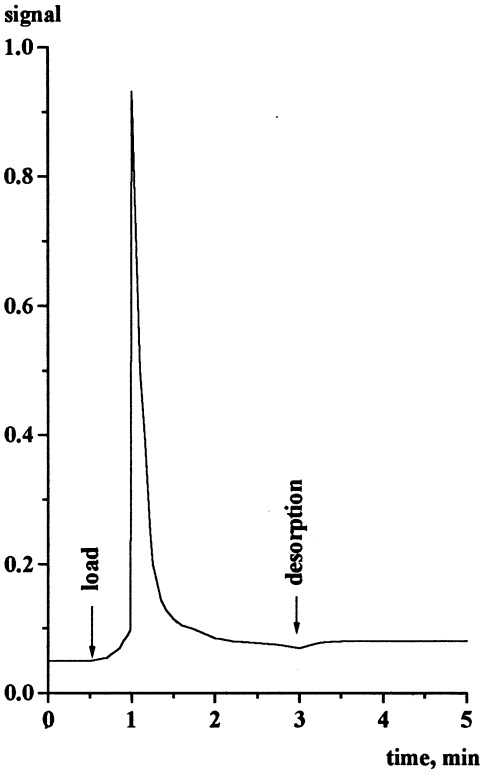

Fig. 1. Determination of non-specific adsorption of bovine serum albumin (BSA) on GMA–EDMA disc with immobilized hexadecapeptide as the anti-peptide ligand. Conditions: 25×2 mm disc, flow rate 2 ml/min; BSA concentration 3 mg/ml PBS buffer, sample volume 200 μl; mobile phase of adsorption step was PBS buffer, pH 7, desorption was carried out with 0.01 M HCl, pH 2.0 (the arrow shows the change of buffers).

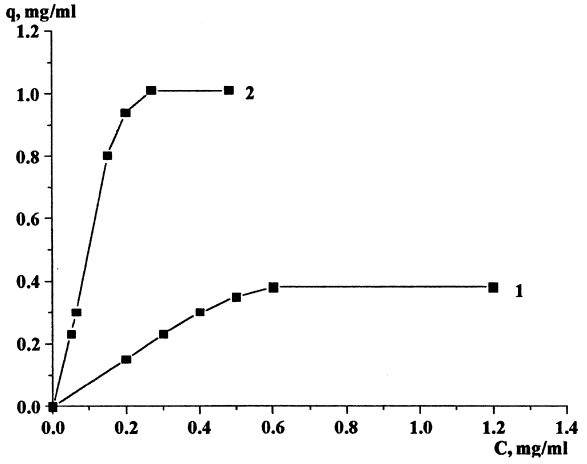

Fig. 2. Adsorption isotherms of specific antibodies on GMA–EDMA discs with the immobilized pentadecapeptide (curve 1) and hexadecapeptide (curve 2). Conditions: 25×2 mm discs, flow rate 2 ml/min; frontal analysis; concentrations of antibodies used for a saturation of immunodisc were varying from 0.3 to 1.2 mg/ml PBS buffer (pentadecapeptide immobilized, 1.6 μmol/disc) and from 0.04 to 0.46 mg/ml PBS buffer (hexadecapeptide immobilized, 3.0 μmol/disc); mobile phase of the adsorption step was PBS buffer, pH 7; desorption was carried out with 0.01 M HCl, pH 2.0.

3.3. Adsorption capacity of GMA–EDMA immunodiscs

Fig. 2 shows the adsorption isotherms of antibodies on discs with immobilized pentadecapeptides and hexadecapeptides. The adsorption can be described by the Langmuire equation [56]. The number of μmols of antibody adsorbed on the disc with immobilized affinity ligand, q, at equilibrium is

$$q = \frac{q_m C}{K_d + C}$$

where K_d is the dissociation constant of the pair ligand–antibody, C is the concentration of antibodies in the solution used for the adsorption, and q_m is the maximum adsorption capacity of the immunosorbent. The linearization of this equation in the $C/q - C$ ordinate system allows calculation of K_d and q_m (Table 2). A simple calculation reveals that one molecule of Ig may cover an area that would accommodate up to 600 and 300 molecules of immobilized pentadecapeptide and hexadecapeptide, respectively. This result corresponds to the data on the surface coverage with immobilized ligands discussed above and indirectly confirms the fact that the process of immune pair formation occurs in the space of flow-through channels where the biospecific ligands are mainly located. The values of constants confirm the high affinity of ligands to the antibodies to be isolated and are comparable with those found previously with affinity disc chromatography [44].

Table 2
Immunoaffinity characteristics of GMA–EDMA discs

Ligand	K_d		q_m	
	mg/ml	mol/l×10^6	mg/ml	mol/l×10^9
Pentadecapeptide	0.31	1.9	0.42	2.6
Hexadecapeptide	0.18	1.1	1.50	9.3

3.4. Direct quantitative analysis and isolation of anti-bradykinin antibodies

The disc with the immobilized hormone bradykinin was used for direct quantitative analysis and, simultaneously, for semi-preparative isolation of anti-bradykinin antibodies from the serum of immunized rabbits. In order to increase the response for such a poor immunogen as this peptide, both succinylized BSA as a protein support with bound bradykinin and the ‘star-like’ polymeric derivatives of BSA which have the bradykinin in the polymeric part [53,57] were used as the immunogens. Fig. 3 shows examples of immunoaffinity chromatograms for the isolation of anti-bradykinin antibodies. The first chromatogram presents further chromatographic fractionation of a pool of immunoglobulins obtained by sulfate ammonium precipitation of blood serum, whereas the second chromatogram shows the affinity isolation of monospecific anti-bradykinin antibodies from a crude serum of immunized rabbits. The purity of isolated products was proved by SDS PAGE method and results are presented in Fig. 4. It should be emphasized that our method allows the separation of only the antibodies specific to bradykinin from the whole pool of polyclonal antibodies expressed in vivo. This method also makes possible to quantita-

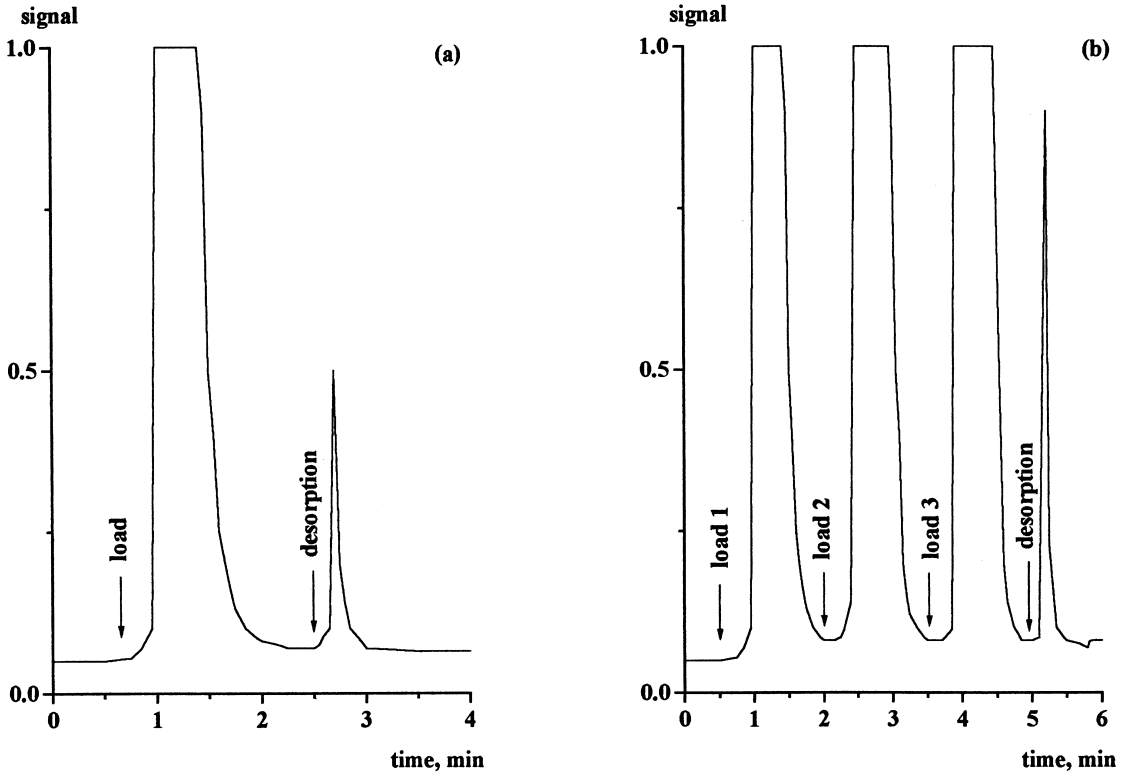

Fig. 3. Isolation of anti-bradykinin IgGs: (a) affinity fractionation of the preliminary purified pool of polyclonal antibodies (precipitated by ammonium sulphate fraction of serum); (b) isolation of anti-bradikiin antibodies from crude serum of immunized rabbit. Conditions: 25×2 mm disc with immobilized bradykinin (2.7 μmol/disc), flow rate 2 ml/min; protein concentration 0.6 mg/ml PBS buffer and sample volume loaded 2 ml (a); crude blood serum with protein concentration 80 mg/ml diluted five times by PBS buffer and sample volume loaded 7.5 ml (the loading procedure was repeated twice) (b); no washing procedure was carried out in the case (a) and the disc was washed with 2 ml of 0.01 mol/l sodium phosphate buffer, pH 8.3, with 2 mol/l sodium chloride salt in (b) experiment; mobile phase of the adsorption step was PBS buffer, pH 7; desorption was carried out with 0.01 M HCl, pH 2.0 (the arrows show the change of buffers).

tively evaluate the concentration of these antibodies in the analyzed pool (or in the crude serum).

Table 3 presents the results of immunoanalysis (ELISA method) of some examples of blood sera of immunized rabbits. These results show the quantitative contents of monospecific anti-bradykinin (anti-BK) antibodies both in the precipitated fraction of blood serum and immunoaffinity HPMC. As follows from the data presented the titres of anti-BK antibodies practically coincide in both cases. It is obvious that the main advantage of the immunoaffinity HPMC method is not only fast and efficient isolation of the antibodies of interest. The chromatographic method proposed allows to separate the anti-BK antibodies from those against BSA. The titres of antibodies against the protein part of the complex immunogen (anti-BSA Abs) are two to three orders of magnitude lower in the case of the analysis of immunoaffinity HPMC data in comparison with those obtained for the precipitated serum fraction. It seems to be possible to prepare really monospecific antibodies using immunoaffinity HPMC. This peculiarity seems to be quite useful for the modern immunomonitoring processes needed in such kinds of simple and reliable methods.

The data demonstrated show very clearly that high performance immunoaffinity chromatography using discs (‘membranes’) with immobilized synthetic peptides – mimicking antigen determinants of proteins – allows to carry out fast quantitative analysis

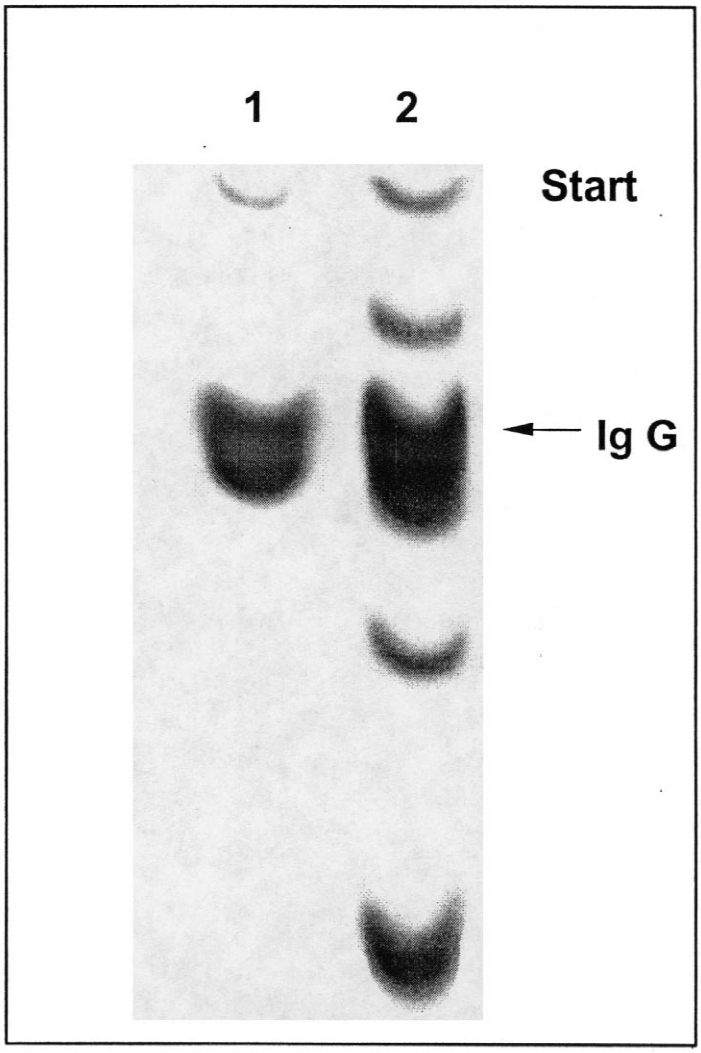

Fig. 4. Gel electrophoresis analysis of product isolated by immunoaffinity HPMC. Conditions: 5 μl of both anti-bradykinin serum (precipitated by ammonium sulfate fraction) (1) and lyophilized product of immunoaffinity HPMC (2) isolation with a concentration of 2 mg/ml with 0.1% sodium dodecyl sulfate were applied; 7% polyacrylamide gel; the fixation and staining of protein zones by 0.2% Coomassie Blue in methanol–acetic acid–water (30:10:60, v/v); the arrow shows the monospecific IgG position.

and simultaneous isolation of specific antibodies from both precipitated fraction and crude blood serum.

3.5. Stability of GMA–EDMA immunodiscs

While designing a test system, the attention must be paid to the chemical stability of the affinity sorbents. The discs based on the macroporous GMA–EDMA copolymer exhibit both high chemical and mechanical stability compared to commercially available rigid immunosorbents based on alkyl modified silica [58,59] and soft BrCN activated polysaccharide-based gels. The use of GMA–EDMA–peptide conjugates during 1 year and their storage for 12–24 months at 4°C was not accompanied by any significant loss of ligands and, accordingly, in a decrease in adsorptive capacity. Control experiments

Table 3
The data of ELISA method for the comparison of antibodies isolated by immunoaffinity HPMC and $(NH_4)_2SO_4$ precipitation

Serum	Prepared by immunoaffinity HPMC			Prepared by $(NH_4)_2SO_4$ precipitation	
	mg/mg serum[a]	Anti-BK Abs	Anti-BSA Abs	Anti-BK Abs	Anti-BSA Abs
1	$8.1 \cdot 10^{-2}$	2500	1000	1280	100 000
2	$3.1 \cdot 10^{-2}$	320	40	640	2560
3	$3.6 \cdot 10^{-2}$	160	1000	640	11 000

[a] Quantitative part of monospecific antibodies (Abs) in rabbit's blood serum.

with the Igs isolations from blood serum show that the amount of monospecific antibodies in the desorbed peak stays constant over the whole covered time range (Fig. 5).

4. Conclusions

Our research demonstrated that a single-step fast affinity method employing the recently developed HPMC works safely in the simultaneous quantitative analysis and micropreparative isolation of monospecific antibodies from both precipitated fraction and straight blood serum. Therefore, a quantitative affinity membrane technique can become a method of choice for the fine fractionations of pools of polyclonal antibodies from blood serum and find a broad use in medical diagnostics of various diseases for which the presence of enzymatically active antibodies (abzymes) in blood has to be simply and quickly determined. It is very important that the discs presented here are identical to the commercially available CIM products from BIA Separations d.o.o. (Ljubljana, Slovenia) which can be successfully used for such kinds of the immunomonitoring processes.

Acknowledgements

The preparation of peptides by N.N. Skvortzova and S.V. Burov (Department of Physiologically Active Polymers of the Institute of Macromolecular Compounds of the Russian Academy of Sciences, St.

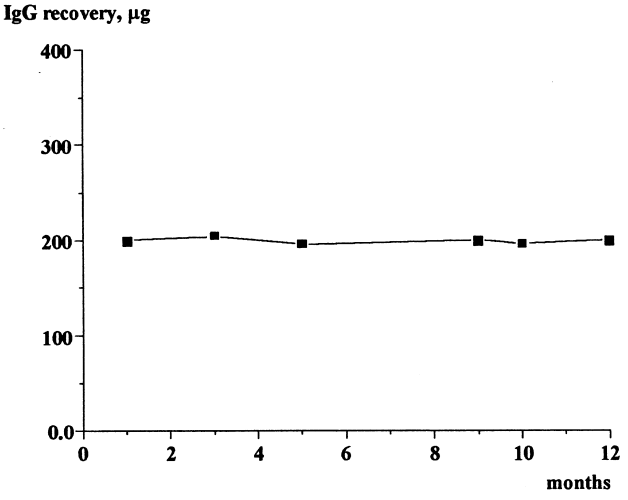

Fig. 5. Stability control of the GMA–EDMA–bradykinin immunoaffinity disc. Conditions: 25×2 mm discs with immobilized bradykinin (2.7 μmol/disc), flow rate 2 ml/min; frontal analysis; protein concentration of anti-bradykinin serum (precipitated fraction) was 0.5 mg/ml PBS buffer; mobile phase of adsorption step was PBS buffer, pH 7; desorption was carried out with 0.01 M HCl, pH 2.0.

Petersburg, Russia) and sera from immunized rabbits by L.V. Puchkova, M.M. Shavlovskii, T.D. Olejnikova and S.B. Ivanova (Department of Molecular Genetics of the Institute of Experimental Medicine of the Russian Academy of Medical Sciences, St. Petersburg, Russia) is gratefully acknowledged. This project is funded by grant 'Scientific Schools' (96-1597393) of the Russian Basic Research Foundation.

References

[1] N. Labrou, Y.D. Clonis, J. Biotechnol. 36 (1994) 95.
[2] P.G. Schultz, R.A. Lerner, S.J. Benkovic, Chem. Eng. News 68 (1990) 26.
[3] R.D. Davies, E.A. Padlan, S. Sheriff, Ann. Rev. Biochem. 59 (1990) 439.
[4] H. Ehle, A. Horn, Bioseparations 3 (1990) 47.
[5] D.S. Pepper, in: A. Kenney, S. Fowell (Eds.), Practical Protein Chromatography, Humana Press, Totowa, 1992, p. 135.
[6] V.I. Pokrovskii, S.V. Shabalina, Zh. Vsesojuzn. Khim. Obshch. 24 (1) (1989) 3.
[7] L.N. Padyukov, B.F. Semyonov, Zh. Vsesojuzn. Khim. Obshch. 24 (1) (1989) 11.
[8] L. Pauling, Am. Scientist 36 (1948) 51.
[9] P.G. Schultz, Science 240 (1988) 426.
[10] E.Je. Marx, in: W. Paul (Ed.), Immunologiya, Mir, Moscow, 1987, p. 255, in Russian.
[11] R.A. Lerner, S.J. Benkovic, P.G. Schultz, Science 252 (1991) 659.
[12] E.J. Marx, Science 234 (1987) 1497.
[13] M. Zouali, D.E. Hansen, Trends Biotechnol. 12 (1994) 73.
[14] A.G. Gabibov, G.V. Gololobov, O.I. Makarevich, D.V. Schourov, E.A. Chernova, R.P. Yadov, Appl. Biochem. Biotechnol. 47 (1994) 293.
[15] C.M. Fraser, J.C. Venter, Allerg. Clin. Immunol. 74 (1984) 661.
[16] A.D. Strosberg, Springer-Semin., Immunopathol. 6 (1983) 67.
[17] A. Tramontano, K.D. Janda, R.A. Lerner, Science 234 (1986) 1566.
[18] S. Paul, A. Gabibov, R. Massey, Mol. Biotechnol. 1 (1994) 109.
[19] S. Paul, S.I. Said, A.B. Thompson, D.J. Volle, D.K. Agrawal, H. Foda, S. de la Rocha, J. Neuroimmunol. 23 (1989) 133.
[20] S. Paul, D.J. Volle, C.M. Beach, D.R. Johnson, M.J. Powell, R.J. Massey, Science 244 (1989) 1158.
[21] E.G. Goetzl, K.T. Kodama, C.M. Turck, S.A. Shiogolev, S.P. Sradharan, Immunology 66 (1989) 554.
[22] M. Sun, Q.S. Gao, L. Li, S. Paul, J. Biol. Chem. 269 (1994) 734.
[23] M. Sun, Q.S. Gao, L. Li, S. Paul, J. Immunol. 153 (1994) 5121.
[24] R. Kalaga, L. Li, J.R. O'Dell, S. Paul, J. Immunol. 155 (1995) 2695.
[25] A.Y. Kulberg, Y.V. Docheva, Progr. Immunol. Stand Milan 4 (1968) 26.
[26] L. Li, S. Paul, S. Tyutyulkova, M.D. Kazatchkine, S. Kaveri, J. Immunol. 154 (1995) 3328.
[27] A.Y. Kulberg, I.M. Petyaev, Immunologiya 6 (1988) 10.
[28] A.Y. Kulberg, I.M. Petyaev, A.G. Zamotayeva, Immunologiya 3 (1988) 37.
[29] I.M. Petyaev, A.Ya. Kulberg, Immunologiya 5 (1989) 12.
[30] K.S. Azarenok, I.I. Generalov, E.A. Dotsenko, V.K. Okulich, Immunologiya 2 (1989) 15.
[31] G. Johnson, S.W. Moore, Eur. J. Immunol. 25 (1995) 25.
[32] Yu.Ya. Kit, D.V. Semyonov, G.A. Nevinskii, Molek. Biol. 29 (1995) 893.
[33] I.I. Generalov, D.K. Novikov, Usp. Sovrem. Biol. 118 (1998) 178.
[34] T.B. Tennikova, B.G. Belenkii, F. Svec, J. Liq. Chromatogr. 13 (1990) 63.
[35] T.B. Tennikova, M. Bleha, F. Svec, T.V. Almazova, B.G. Belenkii, J. Chromatogr. 555 (1991) 90.
[36] T.B. Tennikova, F. Svec, J. Chromatogr. 646 (1993) 279.
[37] H. Fbou-Rebyeh, F. Korber, K. Schubert-Rehberg, J. Reusch, Dj. Josic, J. Chromatogr. 566 (1991) 341.
[38] D. Josic, J. Reusch, K. Loster, O. Baum, W. Reutter, J. Chromatogr. 590 (1992) 59.
[39] D. Josic, Y.-P. Lim, A. Strancar, W. Reutter, J. Chromatogr. B 662 (1994) 217.
[40] J. Luksa, V. Menart, S. Molicic, B. Kus, V. Gabero-Porekar, D. Josic, J. Chromatogr. A 661 (1994) 161.
[41] A. Strancar, P. Koselj, H. Schwinn, D. Josic, Anal. Chem. 68 (1996) 3483.
[42] R. Giovannini, R. Freitag, T. Tennikova, Anal. Chem. 70 (1998) 3348.
[43] M.B. Tennikov, N.V. Gazdina, T.B. Tennikova, F. Svec, J. Chromatogr. A 798 (1998) 55.
[44] C. Kasper, L. Meringova, R. Freitag, T. Tennikova, J. Chromatogr. A 798 (1998) 65.
[45] D. Josic, H. Schwinn, A. Strancar, A. Podgornik, M. Barut, Y.-P. Lim, M. Vodopivec, J. Chromatogr. A 803 (1998) 61.
[46] G.A. Platonova, M.I. Surzhik, A.L. Timkovskii, G.P. Vlasov, T.B. Tennikova, Bioorg. Khim. 25(3) (1999) 189 (in Russian).
[47] M. Berry, J. Davis, S. Smith, I. Smith, J. Chromatogr. 587 (1991) 161.
[48] G. Weilig, J.V. Gorkum, R. Damhof, J. Wouter-Drijghout, W. Bloemhoff, S. Welling-Wester, J. Chromatogr. 512 (1990) 337.
[49] S. Kato, M. Terashima, presented at the 6th European Congress on Biotechnology, Florence, 1992, Vol. 1, p. 539.
[50] I.P. Briand, S. Muller, M.H.V. van Regenmortel, J. Immonol. Methods 78 (1) (1985) 59.
[51] T.T. Ngo, H.M. Lenhoff (Eds.), Enzyme-Mediated Immunoassay, Plenum Press, New York, 1985.
[52] H.J. Geerligs, W.J. Weijer, G.W. Welling, S. Welling-Wester, J. Immunol. Methods 124 (1989) 95.
[53] G.P. Vlasov, N.G. Illarionova, N.L. Izvarina, I.G. Denisov, Macromol. Chem. 9 (1985) 239.

[54] O.H. Lowry, N.I. Posebrough, A.L. Farr, P.I. Randall, J. Biol. Chem. 193 (1951) 265.
[55] M. Petro, F. Svec, J.M.J. Frechet, Biotechnol. Bioeng. 49 (4) (1996) 355.
[56] P. Langlotz, K.H. Kroner, J. Chromatogr. 591 (1992) 107.
[57] I.N. Nikonova, S.B. Ivanova, A.M. Kotin, in: C.H. Schneider, A.N. Eberle (eds.), Proceedings of the 22nd European Peptides' Symposium, Escom, 1993, p. 842.
[58] I. Krasilnikov, V. Borisova, J. Chromatogr. 446 (1988) 233.
[59] N. Kitagawa, J. Chromatogr. 443 (1988) 133.