Affinity Separation and Characterization of IgG Subfragments by Fast Protein Liquid Chromatography with HiTrap_r Protein A Column

HiTrap_r Protein A Kolon İçeren Hızlı Protein Sıvı Kromatografisi ile IgG Altbirimlerinin Afinite Ayrılması ve Karakterizasyonu

Research Article / Araştırma Makalesi

Gizem Ertürk[1], Nilay Bereli[2], Lokman Uzun[2], M. Aşkın Tümer[1]*
[1]Hacettepe University, Department of Biology, Beytepe, Ankara, Turkey
[2]Hacettepe University, Department of Chemistry, Beytepe, Ankara, Turkey

ABSTRACT

It has been previously reported that the antibody molecules can be cleaved into fragments by the proteolytic enzymes and these fragments provide information to determine an antibody's structure and function. Antibody fragments offer several advantages over intact antibodies for use in experimental applications and immunochemical techniques. These fragments have smaller sizes and higher chemical and physical resistances. In this study, papain was used for the digestion of human IgG and the resulting fragments were separated by Fast Protein Liquid Chromatography (FPLC). For this purpose, GE Healthcare HiTrap_r Protein A FF column was used, the resolution (R_s) and theoretical plate numbers (N) of the column were calculated. The unbound and bound fragments were collected by the Frac 920 fraction unit of the FPLC system and the collected fragments were analysed by enzyme-linked immunosorbent assay (ELISA) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Key Words
Antibody fragmentation, F_{ab} fragment, F_c fragment, FPLC, Protein A column.

ÖZET

Antibadi moleküllerinin proteolitik enzimlerle alt birimlerine parçalanabildiği bilinmektedir. Bu alt birimler, antibadilerin yapısı ve işlevlerinin belirlenmesi çalışmalarında önemli veriler sunmaktadır. Deneysel uygulamalarda ve immünokimyasal teknikler için antibadi alt birimleri, parçalanmamış bütün antibadi moleküllerine göre önemli avantajlar sunmaktadır. Alt birimlerin boyutları daha küçüktür, kimyasal ve fiziksel dirençleri daha yüksektir. Sunulan çalışmada, insan immunoglobulin G parçalanması için papain kullanılmıştır. Elde edilen alt birimler, hızlı protein sıvı kromatografisi (FPLC) sistemi ile ayrılmıştır. Bu amaç için GE Healthcare HiTrap_r Protein A FF kolon kullanılmış, ayırıcılık (R_s) ve teorik plaka sayısı (N) hesaplanmıştır. Kolona tutunan ve tutunmayan alt birimler, FPLC sisteminin Frac 920 fraksiyon toplama birimi ile toplanmış ve enzim-bağlı immünosorbent kit (ELISA) ve sodyum dodesil sülfat-poliakrilamid jel elektroforez (SDS-PAGE) yöntemleri ile karakterize edilmiştir.

Anahtar Kelimeler
Antibadi parçalanması, F_{ab} fragmanı, F_c fragmanı, FPLC, Protein A kolon.

Article History: Received October 15, 2010; Revised February 15, 2011; Accepted February 19, 2011; Avaliable Online: April 5, 2011.

Correspondence to: M. Aşkın Tümer, Hacettepe University, Department of Biology, Beytepe, Ankara, Turkey
Tel: +90312 297 8082 Fax: +90312 299 2028 E-Mail: tumer@hacettepe.edu.tr

INTRODUCTION

Antibodies are complex biomolecules that show specific binding capabilities for specific antigens [1]. Antibodies have a growing role in biomedical research and development. Antibody based in vivo diagnostics and therapeutics are gaining wider approval from regulatory agencies around the world [2]. Antibodies have been used for a long time for in vivo passive immunization for bacterial and viral infections or contamination with toxins. Owing to the high specificity of antibodies and the advanced techniques for the production of specific antibodies, treatment strategies based on targeting to malignant cells are developing. Furthermore antibodies are important tools for the purification of their specific antigens for pharmaceutical applications and for research purposes [3]. The specific interactions between antibody and antigen allow the functional manipulation of target antigens. The most common applications of antibodies are in immunosensors [4], immunoassays [5-7] and immunoaffinity separations [8-10]. Common point of all of these techniques is the binding of an analyte to an antibody. An antigen specific antibody fits its unique antigen in a highly specific manner. This unique property of antibodies is the key to their usefulness in all applications where only the specific antigen fits into the antibody binding site [1].

Proteolytic digestion provides important information to determine an antibody molecule's structure and function. Antibody molecules can be cleaved into fragments and each of these fragments have a distinct activity. A number of enzymes like papain, pepsin, ficin, bromelain and elastase can be used for this purpose, papain being the most frequently used [11]. Because of their smaller size as functional components of the whole molecule, antibody fragments offer several advantages over intact antibodies for use in certain immunochemical techniques and experimental applications. While F_{ab} fragments have the advantage of being the smallest antigen binding fragments, $F_{(ab)_2}$' fragments derive special utility from having no F_c domain and somewhat smaller size than whole IgG [12-15]. For this purpose, researchers have attempted to use smaller, more stable counterparts of antibodies and have also been searching for alternative ways to obtain antibody-like receptors [16]. Thus, bioengineered antibody fragments that are smaller than antibodies and have the ability to bind antigen bivalently have been promoted by the researchers [17].

In this study, F_{ab} fragments were obtained by the enzymatic hydrolysis of IgG molecule. For this purpose, papain was used and digestion process was performed at 37°C for 24 h. IgG fragments were separated by Fast Protein Liquid Chromatography (FPLC). GE Healthcare rProtein A column was used for separation, the resolution (R_s) and theoretical plate numbers (N) of the column were calculated. F_{ab} and F_c fragments were collected by the Frac 920 fraction unit of the FPLC system and in the last part, ELISA and SDS-PAGE analysis were performed.

MATERIALS AND METHODS

Materials

Human immunoglobulin G (IgG), papain, ethylene-diamine tetraacetic acid (EDTA), iodoacetamide were ob-tained from Sigma Chemical Co. (St. Louis, USA). All other chemicals were of reagent grade and purchased from Merck A.G. (Darmstadt, Germany). All water used in the experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed-bed system.

Preparation of F_{ab} fragments

Papain digestion of IgG

Digestion of IgG to produce F_{ab} fragments was performed in 20 mM sodium phosphate buffer (pH: 7.0) containing 20 mM EDTA. The digestion was initiated by the addition of papain to the digestion solution containing 0.5 mg/mL IgG at an enzyme to antibody ratio of 1:20. The resulting solution was incubated at 37°C for 24 h. Reaction was stopped by adding 30 mM fresh iodoacetamide to the digestion solution.

Analysis of digestion products by fast protein liquid chromatography (FPLC)

The digestion mixtures were analyzed by Fast Protein Liquid Chromatography (FPLC). FPLC

separation was performed using an AKTA-FPLC (Amersham Bioscience, Uppsala, Sweden) system equipped with a UV detection system. The system includes M-925 mixer, P-920 pump, UPC-900 monitor, INV-907 injection valve and Frac920 fraction collector. Separation was achieved on a 2.5 cm x 0.7 cm Hi_Trap_r Protein A_FF (GE, Healthcare) column. FPLC mobile phases A and B were prepared with 20 mM sodium phosphate buffer (pH: 7.0) and 0.1 M glycine-HCl buffer (pH: 3.0) respectively. The chromatographic separation was performed at 1 mL/min flow rate. After a 10 min starting period with 100% loading buffer A, a linear gradient started from 0% B to 100% B in 3 min, continued with 4 min 100% eluent B and finished last 6 min 100% buffer A. 2 mL of protein mixture was applied to the column and absorbance was monitored at 280 nm. The separation was performed at 27°C. KBr was used as the void marker. Capacity factor (k′) and separation factor (α) were calculated as $k' = (t_R - t_o)/t_o$, $\alpha = k_2'/k_1'$, where t_R is the retention time of the protein and t_o is the retention time of the void marker (KBr), k_2' is the capacity factor for F_c fragment and k_1' is the capacity factor for F_{ab} fragment. The resolution (R_s) and theoretical plate numbers (N) were calculated using the following equations:

$$N = 5.54 (t_R / w_{0.5})^2 \quad (1)$$

$$R_s = 2 (t_{R,2} - t_{R,1}) / (w_2 + w_1) \quad (2)$$

where $w_{0.5}$ is the peak height at the corresponding peak height fraction, $t_{R,1}$ and $t_{R,2}$ are the retention times of two adjacent peaks, w_1 and w_2 are the widths of the two adjacent peaks at the baseline.

The unbound and bound fragments were collected by the Frac 920 fraction unit of the FPLC system and the solvent part of the collected fragments were removed by the freeze dryer.

SDS-PAGE analysis

For the determination of molecular weights and purities of unbound and bound fractions, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Each sample was loaded at a volume of 5 μL and then separated on a 10% SDS-PAGE at a constant current of 23 mA for 6 h and then stained with Coomasie Blue.

ELISA analysis

Intact IgG and unbound fraction of papain-digested IgG were analysed by an enzyme-linked immunosorbent assay method (ELISA). Human anti-IgG (Sigma, I-9384) diluted 1/1000 in 50 mM $NaHCO_3$, pH 9.6, was adsorbed to PVC microtitre plates at 4°C for 12 h. The plates were washed with PBS containing 0.05% Tween 20 (washing buffer) and blocked with PBS containing 0.05% Tween 20, 1.5% BSA, and 0.1% sodium azide (blocking buffer). Samples (2.5 mL, neutralized with 0.5 mL of 1.0 M trisodium citrate) or controls containing known amounts of IgG were added and incubated at 37°C for 1 h. Bound IgG was detected with the anti IgG labeled with biotin followed by peroxidase-conjugated streptavidin and o-phenylenediamine. The absorbance was measured at 492 nm.

RESULTS AND DISCUSSION

FPLC studies

Protein A is a bacterial antibody binding protein and interacts mainly with the F_c part of IgG [18]. A 24 h period of papain digestion of IgG resulted in F_{ab} and two major F_c fragments. Protein A bound to F_c fragments of papain-digested IgG and was totally non-reactive with the F_{ab} regions.

For the calibration of the FPLC system, different concentrations of IgG solutions were loaded to the column. Figure 1 shows the chromatogram of standart IgG solutions having concentrations in range of 0.05 mg/mL and 2.0 mg/mL (flow rate: 1 mL/min, T: 27°C). The 24 h papain digestion of IgG results in two major fragments which are F_{ab} and F_c fragments. Since Protein A specifically interacts with the F_c region of IgG molecule, the first peak in the unbound region in Figure 2 represents the F_{ab} fragment of digested IgG molecules.

As shown in Figure 2, a successful separation was observed and F_{ab} and F_c fragments were separately collected. The retention times of the fragments were achieved at 3.06 and 11.64 min, respectively. The calculated chromatographic parameters such as t_R, N, k′, α, R_s values are summarized and given in Table 1. R_s value was

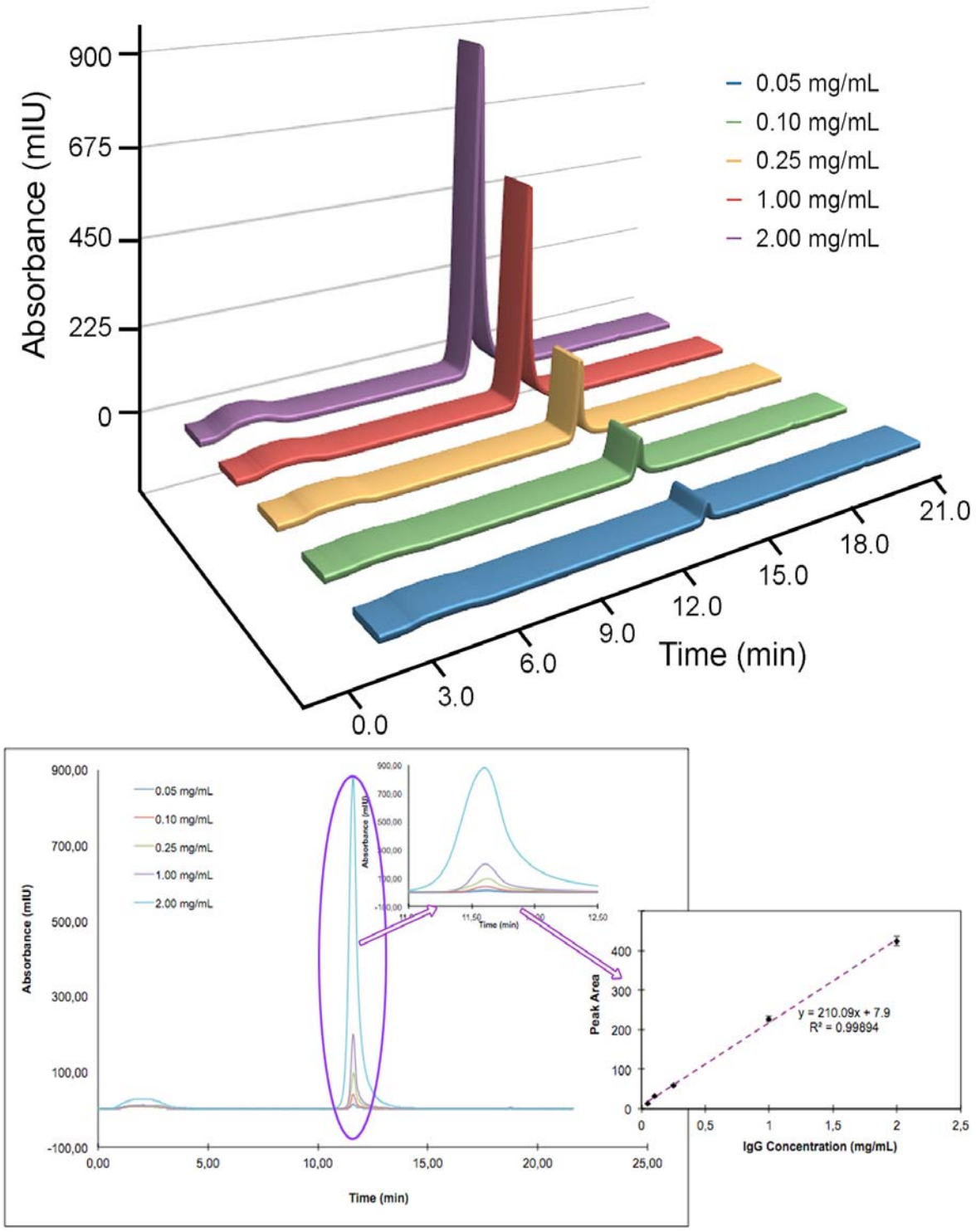

Figure 1. FPLC chromatogram of standart IgG solutions.
Flow-rate: 1.0 mL/min, T: 27°C, Detection was performed at 280 nm.

calculated as 4.70 for F_c fragment. Because the R_s value should be higher than 1.0 for a good resolution of two peaks in such a chromatography system, the results for the resolution of F_{ab} fragment/F_c fragment can be accepted as good resolution values.

SDS-PAGE analysis

The purity of unbound and bound fractions of papain digested IgG was determined by SDS-PAGE as described before. In Figure 3, Lane-1 represents the Protein A unbound fraction of papain digested

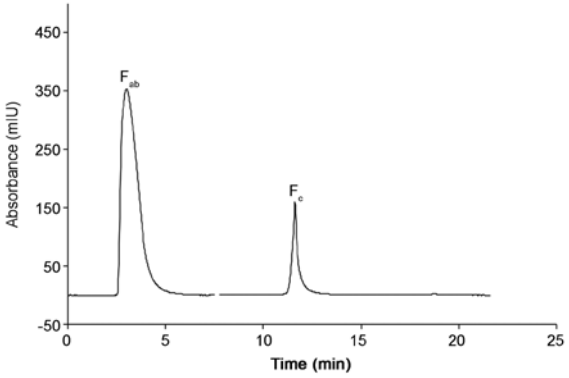

Figure 2. FPLC chromatogram of IgG after digestion with papain. IgG concentration: 0.5 mg/mL, Flow-rate: 1.0 mL/min, T: 27°C. Detection was performed at 280 nm.

Table 1. Chromatographic separation data.

Fragment	t_R	N	k′	α	R_s
F_{ab}	3.06	52.39	1.19	6.14	-
F_c	11.64	1089.60	7.31	-	4.70

IgG, Lane-2 is the Protein A bound fraction of papain digested IgG and Lane-3 contains proteins as markers with known molecular weights; 60 kDa bovine serum albumin, 14.7 kDa lysozyme. Molecular weight of immunoglobulin (IgG) is 150 kDa and papain digests IgG molecule from the hinge region. Therefore, polypeptide chains which have 50 kDa molecular weight were formed as the result of papain digestion in this study. In Figure 3, the bands having 50 kDa molecular weight were clearly seen. The results show that enzymatic hydrolysis and fractionation of fragments with FPLC are achieved, respectively.

ELISA results

The amount of IgG in 1:1 diluted plasma sample was found as 1280 mg/dL and the amount of IgG in the unbound fraction of papain digested IgG was found as 100 mg/dL.

CONCLUSION

Antibodies have been used for a long time as probes for research in biology and biopharmaceuticals. They are also used for immunodiagnosis and immunotherapy for several human diseases. Some understandings of the structure of antibodies are obtained on degrading them with proteolytic enzymes and examining the fragments which retain antibody activity.

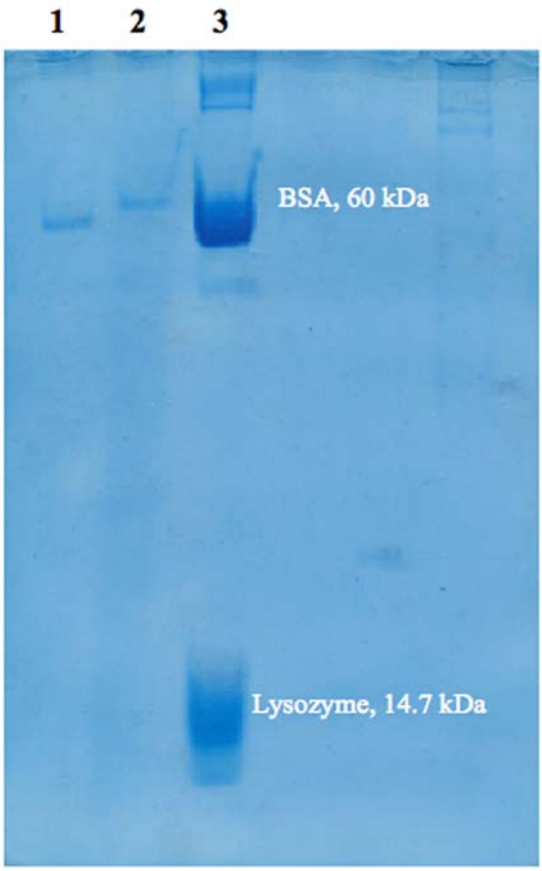

Figure 3. SDS-PAGE of IgG fragments from Protein A column. The fractions were assayed by SDS-PAGE using 10% separating gel (9 cm x 7.5 cm) was stained with 0.25% (w/v) Coomassie Brillant R 250 in acetic acid–methanol–water (1:5:5, v/v/v) and destained in ethanol–acetic acid–water (1:4:6, v/v/v). Lane 1, unbound F_{ab} fragment; Lane 2, bound F_c fragment; Lane 3, marker proteins (BSA and Lysozyme).

Fragments of antibodies are also widely used as immunochemical tools, diagnostic and therapeutic reagents because of the more rapid pharmacokinetic answer and avoided potential non-specific binding of intact antibody molecules to constant region (F_c) receptors on lymphocytes. Since antibody fragments usually retain the antigen binding activity of the original antibody, researchers have attempted to use bioengineered antibody fragments instead of intact antibody.

In this study, we digested IgG molecule with papain for gaining F_{ab} and F_c fragments. For this purpose, we utilized Fast Protein Liquid Chromatography system that has been introduced as one of the most preferred methods for purification of antibody fragments that are prepared

by enzymatic digestion of whole Immunoglobulins. For the separation of F_{ab} and F_c fragments, we used HiTrap_r Protein A column and we collected the fragments by the fraction unit of the FPLC system. In the last part, we performed the SDS-PAGE and ELISA analysis of the fragments. As a result, we can digest human IgG molecule with papain and we can separate the IgG subfragments from each other by Fast Protein Liquid Chromatography. So that, we can obtain F_{ab} and F_c fragments which have several advantages over intact IgG for use in immunochemical and therapeutic applications.

REFERENCES

1. T. Vo-Dinh, B. Cullum, Biosensors and biochips: advances in biological and medical diagnostics, Fresenius J. Anal. Chem., 366 (2000) 540.
2. L. Uzun, R. Say, A. Denizli, Porous poly(hydroxyethyl methacrylate) based monolith as a new adsorbent for affinity chromatography, React. Functl. Polym., 64 (2005) 93.
3. K. Huse, H. Bohme, G.H. Scholz, Purification of antibodies by affinity chromatography, J. Biochem. Biophys. Methods, 51 (2002) 217.
4. C.L. Morgan, D.J. Newman, C.P. Price, Immunosensors: technology and opportunities in laboratory medicine, Clin. Chem., 42 (1996) 193.
5. D.S. Hage, Immunoassays, Anal. Chem., 67 (1995) 455R.
6. R.S Yalow, The Nobel lectures in immunology. The Nobel Prize for Physiology or Medicine, 1977, awarded to Rosalyn S. Yalow Scand., J. Immunol., 35 (1992) 4.
7. T. Porstmann, S.T. Kiessig, Enzyme immunoassay techniques: an overview., J. Immunol. Methods, 150 (1992) 5.
8. M.L. Yarmush, A.M. Weiss, K.P. Antonsen, D.J. Odde, D.M. Yarmush, Immunoaffinity purification basic principles and operational considerations, Biotechnol. Adv., 10 (1992) 413.
9. S. Özkara, H. Yavuz, A. Denizli, Purification of Immunoglobulin G from human plasma by metal-chelate affinity chromatography, J. Appl. Polym. Sci., 89 (2003) 1567.
10. N. Bereli, S. Akgöl, H. Yavuz, A. Denizli, Antibody purification by Concanavalin A affinity chromatography, J. Appl. Polym. Sci., 97 (2005) 1202.
11. Q. Luo, X. Mao, L. Kong, X. Huang, H. Zou, High-performance affinity chromatography for characterization of human immunoglobulin G digestion with papain, J. Chromatogr. B, 776 (2002) 139.
12. D. Beale, Molecular fragmentations. Some applications in immunology., Dev Comp. Immunol., 11 (1987) 287.
13. S.J. Boguslawski, Improved procedure for preparation of F(ab')2 fragments of mouse IgGs by papain digestion, J. Immunol. Meth., 120 (1989) 51.
14. K.M Wilson, A new enzymatic method to obtain high-yield F(ab')2 suitable for clinical use from mouse IgG1., Mol. Immunol., 28 (1991) 69.
15. R.S. Kerbel, B.E. Elliot, Detection of Fc receptors., Method. Enzymol., 93 (1983) 113.
16. K. Haupt, K. Mosbach, Plastic antibodies: developments and applications, Tibtech November, Vol 16, 1998.
17. R. Cortese, Identification of biologically active peptides using random libraries displayed on phage, Curr. Opin. Biotechnol., 6 (1995) 73.
18. L. Uzun, D. Türkmen, V. Karakoç, H. Yavuz, A. Denizli, Performance of protein A based affinity membranes for antibody purification, J. Biomat. Sci. Polym. Ed., 2011, In Press, doi: 10.1163/092050610X538731.