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Thiophilic adsorption revisited☆

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Abstract

Specific and efficient selection of serum immunoglobulins, but not other proteins, on T-gel remains difficult. T-gel capacity was determined for different activation conditions and serum loadings. Mass spectrometry analysis was used to identify the proteins found in the flow-through and in the eluted fractions. Alpha-2-macroglobulin and albumin were the major contaminants of the eluates. The influence of the competition between immunoglobulins and the other serum proteins on the adsorption was also studied. Using a serum depleted in immunoglobulins (flow-through of a first chromatography on T-gel), many serum proteins were retained on the T-gel, including albumin. We conclude that T-gel selectivity is less than absolute and may reflect for a large part the experimental conditions of the adsorption.

Keywords: Immunoglobulin; Mass spectrometry; Serum; Thiophilic adsorption

1. Introduction

Thiophilic adsorption chromatography (TAC) was developed by Porath et al. in the 1980s [1,2]. Since then, this mode of chromatography was mostly applied to the purification of serum immunoglobulins in mild conditions [3–5]. The chromatography support consists in spherical beads of divinylsulfone-activated agarose coupled with 2-mercaptoethanol. This chemical matrix was named thiophilic gel, or "T-gel". The structure of the T-gel used in this work can be represented as agarose- $(CH_2)_2$ $SO_2(CH_2)_2$ $S(CH_2)_2$ OH [6].

Hutchens and Porath [3] have shown during thiophilic adsorption the affinity of proteins for both functional groups present in the ligand structure, thioether sulphur and the adjacent sulphone group, in a cooperative manner. The immunoglobulins G seem to be the main proteins which interact with this ligand. The adsorption of IgG to the T-gel is dependent upon the presence and concentration of salt, mostly lyotropic salts, while the presence of high concentrations of sodium chloride does not promote the adsorption. But even if T-gel displays high levels of selectivity for immunoglobulins, some authors have described the presence of contaminants, like alpha-2-macroglobulins, in the eluted fraction [7]. To increase selectivity, it remains of interest therefore to determine more precisely whether the specificity of the TAC process could be experimentally altered. We report here our investigations to better control efficiency and selectivity of immunoglobulin isolation from patient and control sera. The goal of this work was both to improve the efficiency of the IgG purification from the different sera, and to better understand the selective affinity of thiophilic matrix and the precise nature of the contaminants of the eluates. TAC was investigated as a function of serum loading for a given T-gel capacity as well as of the degree of DVS activation. The protein content in flow-through fraction (non-adsorbed protein) and eluted fraction were analyzed. For this purpose, the proteins of these fractions were separated by SDS-PAGE and analyzed by mass spectrometry (MS). When flow-through fractions depleted in IgG were injected again on T-gel, different proteins currently excluded were purified on the support. This observation leads to that the specificity of the T-gel is quite large, and that the selectivity generally observed results of the competition of many proteins with different affinities for the support.

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2. Materials and methods

2.1. Samples

Sera obtained either during a routine blood test or from healthy volunteers were immediately frozen at −80 °C until used and were never refrozen. Each serum sample was mixed with an equal volume of PBS containing 1.5 M $(NH_4)_2SO_4$ for 1 h at 4 °C, centrifuged, and the supernatant was used for the TAC.

2.2. Preparation of the T-gel

In the preparation of the T-gel, the method previously described [1,7,8] was used with minor modifications. Briefly, 40 g of suction-dried Sepharose 4B gel was mixed with 40 mL of 0.5 M Na-carbonate buffer (pH 11) and either 4.0 mL or 8.0 mL of divinylsulfone (DVS). The reaction mixture was rotated for 1 h at 20 °C, and then washed copiously with water. To make the T-gel, 30 g of the DVS-activated gel was suspended in 30 mL water containing 10% 2-mercaptoethanol, and the pH was adjusted to 9.5 with 2 M NaOH. The mixture was rotated overnight at 10 °C, and washed successively with water and with 0.75 M $(NH_4)_2SO_4$. The resultant gel was stored in water with 0.01% NaN_3.

2.3. Thiophilic adsorption chromatography (TAC)

All the TACs were performed using an Akta Purifier system (GE Healthcare, Uppsala, Sweden). One or two milliliters of the thiophilic gel was packed into Tricorn columns (Tricorn 5/50 and 5/100, respectively; GE Healthcare), and equilibrated with 0.75 M $(NH_4)_2SO_4$ in PBS. 0.5–2 mL of serum supernatant was passed through the column. The absorbance of the mobile phase was monitored at 280 nm. The flow rate was set at 0.5 mL/min. The column was then washed with washing buffer for an additional 6 CV, and the flow-through of the column was recovered. The adsorbed proteins were eluted with either PBS without $(NH_4)_2SO_4$ or 20 mM K_2HPO_4/KH_2PO_4, pH 8.5. The protein concentration of the eluate was determined by measurement of the absorbance at 280 nm.

To evaluate the retention of serum proteins after depletion of this serum in immunoglobulins, a flow-through obtained as described above was used as an immunoglobulin-depleted serum fraction.

2.4. Separation of the proteins eluted during TAC

To determine the precise nature of the contaminants of the eluates, proteins were separated by SDS-PAGE. For SDS-PAGE separation, the samples were deliberately overloaded to allow the detection of low abundant contaminants. Fifty micrograms of protein fractions were loaded to 6 cm long gradient gels (9–18% acrylamide), and stained with colloidal Coomassie blue [9]. Both gels and running buffer contained 0.1% SDS. Electrophoresis (200 V constant voltage) were carried out using Laemmli SDS running buffer (0.025 M Tris–HCl, 0.192 M glycine, 0.005 M sodium thiosulfate, 0.1%, w/v SDS) [10].

2.5. Tryptic digestion and MS analysis

Individual spots were excised from the SDS gels (20–25 spots per gel). Digestion was performed using the Amersham Ettan

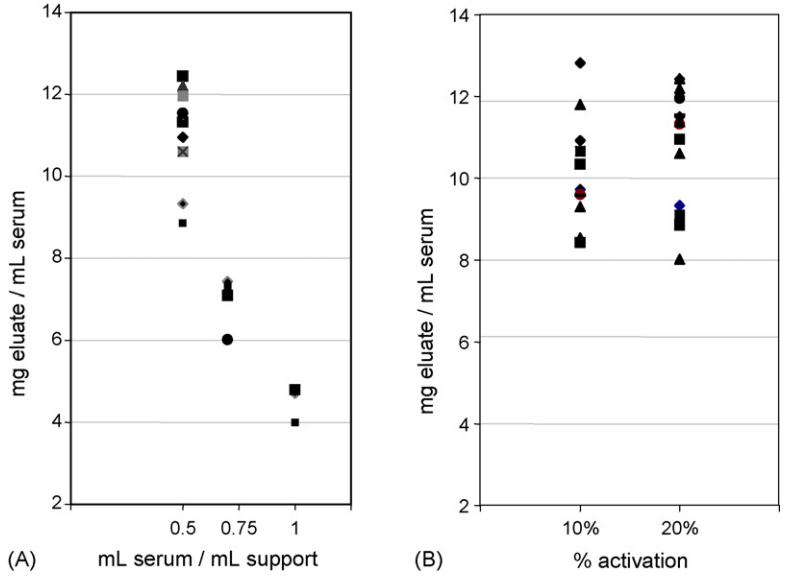

Fig. 1. Thiophilic adsorption of human immunoglobulins from human serum. General recovery as measured by spectrometry. Crude human serum was diluted with an equal volume of 1.5 M $(NH_4)_2SO_4$ and applied, at a flow rate 0.5 mL/min, to the T-gel equilibrated with 0.75 M $(NH_4)_2SO_4$ in PBS. The column was then washed with the same buffer for an additional 6 CV. Elution was performed with either PBS without $(NH_4)_2SO_4$ or 20 mM K_2HPO_4/KH_2PO_4, pH 8.5. (A) Influence of loading on binding capacity. Different amounts of serum were applied on the same T-gel column: 0.5, 0.75, or 1 mL serum per mL of support. (B) Influence of the degree of activation. Two T-gels prepared with agarose activated with either 10% or 20% DVS were compared in the conditions described above. Each point on the diagrams corresponds to an independent chromatography.

Digester (GE Healthcare) [11]. Protein identification was carried out by peptide mass fingerprinting using a Biflex IV (Bruker Daltonique, Wissembourg, France) [12,13]. A sandwich spotting method was used [11] with α-cyano-4-hydroxy-cinnamic acid (HCCA) as matrix. For protein identification, using the Mascot Daemon software package (www.matrixscience.com), the following parameters were adopted: complete carbamidomethylation of cysteines and partial oxidation of methionines, one possible missed cleavage, databases: SwissProt/UniProt Knowledgebase and NCBInr (NCBI database was used for the identification of immunoglobulin chains).

3. Results and discussion

Two aspects can be considered for improving and characterizing the efficiency of IgG purification by TAC. First, the chromatography conditions leading to an optimal yield of purification; and second, the specificity of TAC for IgG and the characterization of the co-purified contaminants.

3.1. Chromatography parameters

To precise the influence of the serum/support ratio on protein retention during TAC, different serum/support ratio were tested for two conditions of DVS activation (10% and 20%, v/w, respectively). Independently of the percentage of activation, a ratio of 0.5 (mL serum/mL support) gave an optimal retention (11.00 ± 1.24 mg of eluate for 1 mL of serum, mean ± standard deviation) corresponding to the theoretical IgG concentration in the serum. This retention decreased for higher ratio: 6.86 ± 0.61 mg and 4.61 ± 0.41 mg, for a ratio of 0.7 and 1, respectively (Fig. 1A). Individual variations in recovery were observed that may reflect individual variations between the immunoglobulin concentrations of the different serum samples, and to a certain extent experimental variations. However, these variations remained limited compared to the differences resulting from the modification of the serum/support ratio. No significant differences were found between the supports prepared with 10% or 20% DVS (Fig. 1B).

3.2. Identification of the serum proteins purified during TAC

The efficacy of T-gel to bind immunoglobulins was generally estimated by methods that does not provide high resolution, such as cellulose acetate electrophoresis [14], SDS-PAGE [15–18] or native PAGE [2,14], and fused rocket immunoelectrophoresis [7]. These methods provided a semi-quantitative estimation of the level of immunoglobulins in the eluates, but did not allow investigating the contaminants present in these eluates. Moreover, as previously noted, the extensive molecular heterogeneity of immunoglobulins (as a result of natural variations in both amino acid sequence and carbohydrate composition) makes the evaluation of purity difficult, by definition [15]. To identify the proteins retained or not during TAC, SDS-PAGEs were deliberately overloaded (5 folds in comparison with the amount of protein currently used for analysis in the same system). Following SDS-PAGE gel plugs corresponding to the two chromatography fractions were subjected to trypsinolysis, and the resulting peptide fragments subjected to analysis using MALDI-MS. The results are summarized in Fig. 2 and Table 1. As expected, characteristic patterns of adsorption were obtained, in which mostly immunoglobulins were bound (more than 90% of total protein estimated on the SDS-PAGE) and most proteins passed through. Two major contaminants were identified in the eluate: alpha-2-macroglobulin and albumin.

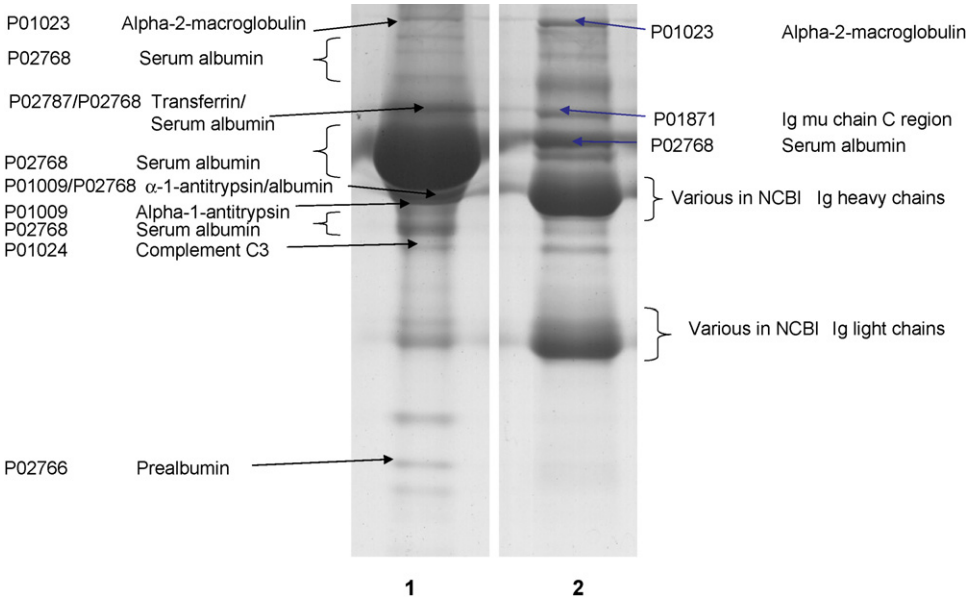

Fig. 2. SDS-PAGE (9–18%) and protein identifications on fractions of blood serum after TAC. The samples were deliberately overloaded (50 μg per lane) to detect the contaminants. Protein identification was carried out by peptide mass fingerprinting on individual spots excised from colloidal Coomassie blue-stained SDS-PAGE. Lane 1, flow-through; lane 2, eluate.

Table 1
Analysis of the T-gel fractions after separation by SDS-PAGE

Protein identified as	UniProt Acc no.	*Mr*	Molecular weight in UniProt	No of matching peptides	Score in Mascot
Flow-through					
Alpha-2-macroglobulin	P01023	170370	164600	11	76
Serum albumin	P02768	145057	71317	13	125
Serum albumin	P02768	144572	71317	9	63
Serum albumin	P02768	124753	71317	8	77
Serum albumin	P02768	99035	71317	12	123
Serum albumin	P02768	95267	71317	7	63
Transferrin/serum albumin	P02787/P02768	74889	79280/71317	12/12	160
Transferrin/serum albumin	P02787/P02768	74556	79280/71317	12/12	134
Transferrin/serum albumin	P02787/P02768	72159	79280/71317	9/13	179
Serum albumin	P02768	63507	71317	21	97
Serum albumin	P02768	63224	71317	22	150
Serum albumin	P02768	60111	71317	17	106
Serum albumin	P02768	59666	71317	19	144
Serum albumin	P02768	56141	71317	24	196
Alpha-1-antitrypsin	P01009	51584	46878	21	155
Alpha-1-antitrypsin/albumin	P01009/P02768	51431	46878/71317	11/11	164
Serum albumin	P02768	50149	71317	20	116
Serum albumin	P02768	50074	71317	20	108
Serum albumin	P02768	48056	71317	9	59
Serum albumin	P02768	44522	71317	12	70
Complement C3	P01024	41491	188585	18	74
Complement C3	P01024	30703	188585	11	58
Prealbumin	P02766	17480	15991	6	112
Prealbumin	P02766	17442	15991	5	87
Eluate					
Alpha-2-macroglobulin	P01023	166979	164600	27	201
Alpha-2-macroglobulin	P01023	166420	164600	22	145
Ig mu chain C region	P01871	73785	50210	8	82
Ig mu chain C region	P01871	73348	50210	7	57
Serum albumin	P02768	64745	71317	17	147
Serum albumin	P02768	64937	71317	20	175
Ig heavy chains	Various in NCBI	53280	∼52000*	9	ND**
Ig heavy chains	Various in NCBI	52982	∼52000*	12	ND**
Ig heavy chains	Various in NCBI	50000	∼52000*	12	ND**
Ig heavy chains	Various in NCBI	49853	∼52000*	16	ND**
Ig lambda light chains	Various in NCBI	30455	∼23000*	4	ND**
Ig kappa light chains	Various in NCBI	27969	∼28000*	10	ND**
Ig kappa light chains	Various in NCBI	27855	∼28000*	12	ND**

The left column describes the protein (protein name in Swiss-Prot/UniProt, except for Ig chains). The second column correspond to accession no. in Swiss-Prot/UniProt (except for Ig chains). In the third column, Mrs are experimentally determined on SDS-PAGE. Molecular weight with * in the fourth column are approximative due to the structural heterogeneity of Ig. For the number of matching peptides, only proteins with individual ions scores indicating identity of extensive homology ($p < 0.05$) are considered. Protein scores in Mascot are derived from ions scores as a non-probabilistic basis for ranking protein hits. ND**, the protein score was not considered for Igs, considering the high occurence of redundant sequences for these proteins.

Alpha-2 macroglobulin was distributed between the two fractions. Due to its abundance, albumin was identified in a large portion of the SDS-PAGE of the flow-through (between 44 and 144 kDa). A focalized band of albumin was also identified in the eluate, in contradiction with previous reports indicating that it is not adsorbed to the T-gel either in the purified state of during fractionation of human serum [1,3,6]. Our results indicate that the total removal of albumin during TAC often described reflects the low sensitivity of the analytical methods currently used. However, the dramatic difference between the repartition of albumin detected by SDS-PAGE between the chromatography fractions (Fig. 3) did not allow concluding about its thiophilic affinity. In addition, Ig mu chains were also detected, reflecting to the co-purification of IgM molecules with IgG.

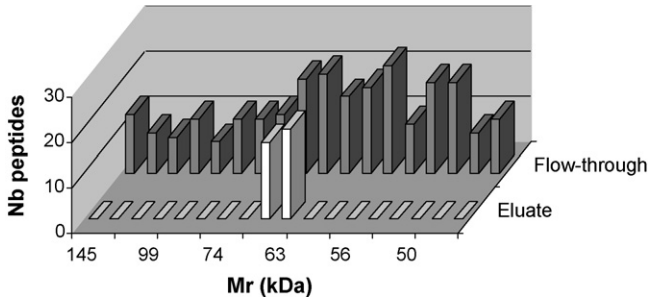

Fig. 3. Repartition of serum albumin between the flow-through and the eluate of the T-gel. On the abscissa (*x*-axis) the relative molecular mass (*Mr*) measured on the SDS-PAGE; on the *y*-axis the number of matching peptides (Nb peptides) observed during the identification by MALDI-TOF. This number of matching peptides is related to albumin abundance in the corresponding gel plug.

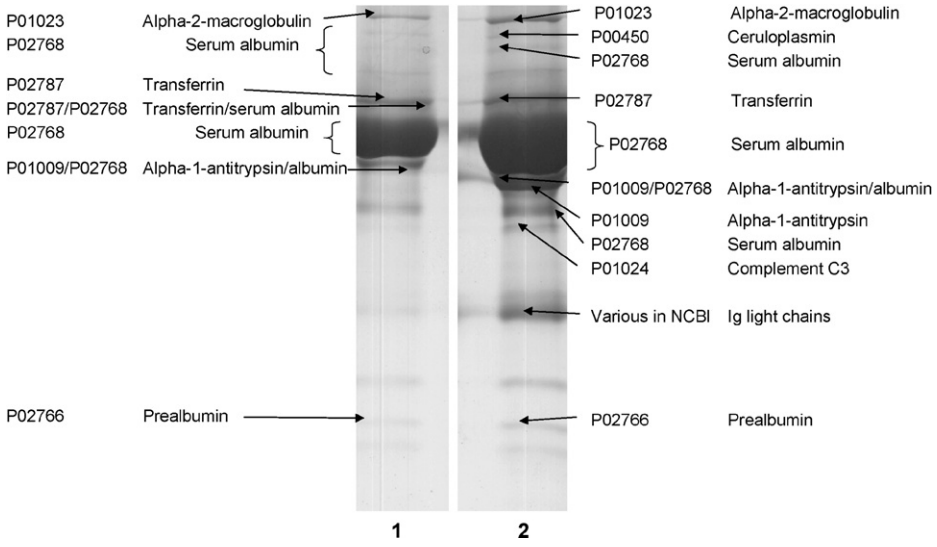

Fig. 4. SDS-PAGE (9–18%) and protein identifications on fractions of immunoglobulin-depleted serum after TAC. The samples were deliberately overloaded (50 μg per lane). Lane 1, flow-through; lane 2, eluate.

Table 2
Analysis of the T-gel fractions after depletion in IgG

Protein identified as	UniProt Acc no.	*Mr*	Molecular weight in UniProt	No of matching peptides	Score in Mascot
Flow-through					
Alpha-2-macroglobulin	P01023	174999	164600	14	80
Alpha-2-macroglobulin	P01023	172669	164600	18	92
Serum albumin	P02768	148003	71317	9	62
Serum albumin	P02768	125171	71317	8	55
Serum albumin	P02768	98715	71317	8	64
Serum albumin	P02768	97447	71317	8	64
Transferrin	P02787	74445	79280	14	65
Transferrin/serum albumin	P02787/P02768	74004	79280/71317	14/11	96
Serum albumin	P02768	65130	71317	19	91
Serum albumin	P02768	64745	71317	24	199
Serum albumin	P02768	62663	71317	21	125
Serum albumin	P02768	62200	71317	24	172
Serum albumin	P02768	59225	71317	24	174
Serum albumin	P02768	59137	71317	21	146
Alpha-1-antitrypsin/albumin	P01009/P02768	55561	46878/71317	8/8	130
Alpha-1-antitrypsin/albumin	P01009/P02768	55478	46878/71317	19/17	276
Prealbumin	P02766	17707	15991	4	67
Prealbumin	P02766	17669	15991	4	78
Eluate					
Alpha-2-macroglobulin	P01023	171515	164600	24	163
Ceruloplasmin	P00450	146522	122983	8	56
Serum albumin	P02768	127286	71317	8	66
Serum albumin	P02768	124753	71317	8	87
Serum albumin/complement factor B	P02768/P00751	101690	71317	19/14	176
Serum albumin	P02768	100000	71317	12	67
Transferrin	P02787	75976	79280	13	75
Transferrin	P02787	75731	79280	13	66
Serum albumin	P02768	64075	71317	19	181
Serum albumin	P02768	58006	71317	23	117
Alpha-1-antitrypsin	P01009	51814	46878	16	124
Alpha-1-antitrypsin/albumin	P01009/P02768	51661	46878/71317	16/13	194
Serum albumin	P02768	48410	71317	14	103
Serum albumin	P02768	48339	71317	13	84
Complement C3	P01024	41920	188585	14	63
Ig kappa light chains	Various in NCBI	28930	∼28000*	7	ND**
Prealbumin	P02766	17593	15991	6	106
Prealbumin	P02766	17518	15991	7	132

Same legend as in Table 1.

3.3. Retention of the proteins after depletion of the serum in immunoglobulins

Considering that mostly immunoglobulins are adsorbed to the T-gel in current conditions, a question remains open. How can be interpreted the presence of low concentrations of contaminating proteins in the eluates? Is it related to the non-specific binding of traces of abundant proteins to the affinity matrix? Or is it due to the affinity of these proteins – lower that the affinity of immunoglobulins – for the thiophilic support? If the second hypothesis is correct, an excess of "thiophilic" sites will induce a risk to promote the interaction of different proteins, other than immunoglobulins. To test this hypothesis, a flow-through of the T-gel was used as an immunoglobulin-depleted serum fraction. This fraction was chromatographied on the same T-gel and in the same conditions than previously. An eluate quantitatively comparable to that obtained during TAC was obtained. The flow-through and the eluate of this second chromatography were analyzed as described above. The results are reported in Fig. 4 and Table 2. Surprisingly, the major protein adsorbed by the T-gel from the immunoglobulin-free serum was albumin. Albumin was distributed between the two fractions, but the major part was found in the eluate. In contradiction with what may be expected, alpha-2-macroglobulin, generally considered as the main thiophilic protein after the immunoglobulins [7], was apparently distributed in the same proportions than after the first TAC. Some other proteins were also identified in the two fractions, such as transferrin, alpha-1-antitrypsin, and prealbumin. Ceruloplasmin and complement C3 were found only in the eluate. In addition, a faint band of Ig light chains suggests that traces of immunoglobulins remained in the depleted serum fraction.

4. Concluding remarks

The use of thiophilic agarose beads as a separation medium is well established, especially for the purification of immunoglobulins or immunoglobulin fragments from many different sources [3–5,8,19,20]. Binding and elution conditions for T-gel are mild and involve the use of neutral pH buffers throughout. However, the demonstrated potential for modifications of experimental conditions to vary T-gel selectivity [6] may make the adsorption of immunoglobulins, but not other serum proteins, difficult according to the degree of purity required. Then several parameters affecting T-gel selectivity have been explored, such as starting salt concentration [6,7,21] or pH [6,15]. In this study, varying the conditions of TAC, we show that the interaction of definite proteins (typically serum immunoglobulins) to the T-gel may be considered in terms of competition with other proteins of the biological fluid. If we consider the major serum proteins, many of them have the ability to be retained by the T-gel, even in the salt conditions currently used for the selective adsorption of immunoglobulins. During TAC performed in current conditions, immunoglobulins are competing successfully with the other serum proteins, more than interacting specifically by a biospecific mechanism. On the opposite, in the absence of the immunoglobulin competitor, the "thiophilic" sites are free for the other protein competitors. This observation has at least two consequences. First, studying the specificity of TAC with pure proteins can conduct to unpleasant surprises when the same proteins are present in complex mixtures such as biological fluids. Second, we would like emphasize that T-gel selectivity appears to be less than absolute and may reflect for a large part the experimental conditions of the adsorption. Trying to maximize efficiency by increasing the volume of affinity support compromises absolute selectivity. Conversely, it is possible to improved selective adsorption by adjusting the sample/support ratio, even if there will be some decrease of capacity. Taking that into account, qualities such as low gel costs, functional integrity of the immunoglobulins isolated on T-gel, and a purity similar to that obtained when using protein A beads [14], make that TAC remains an effective procedure for the isolation and purification of immunoglobulins.

Acknowledgements

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References

[1] J. Porath, F. Maisano, M. Belew, FEBS Lett. 185 (1985) 306.
[2] J. Porath, J. Chromatogr. 376 (1986) 331.
[3] T.W. Hutchens, J. Porath, Anal. Biochem. 159 (1986) 217.
[4] M. Belew, N. Juntti, A. Larsson, J. Porath, J. Immunol. Methods 102 (1987) 173.
[5] E. Boschetti, J. Biochem. Biophys. Methods 49 (2001) 361.
[6] T.W. Hutchens, J. Porath, Biochemistry 26 (1987) 7199.
[7] A. Lihme, P.M. Heegaard, Anal. Biochem. 192 (1991) 64.
[8] R. Joubert-Caron, M. Caron, P. Bochet, A. Chadli, P. Delaporte, E. Schuller, D. Bladier, Int. J. Biochem. 26 (1994) 813.
[9] N.L. Anderson, D.C. Copple, R.A. Bendele, G.S. Probst, F.C. Richardson, Fundam. Appl. Toxicol. 18 (1992) 570.
[10] U.K. Laemmli, Nature 227 (1970) 680.
[11] L. Canelle, C. Pionneau, A. Marie, J. Bousquet, J. Bigeard, D. Lutomski, T. Kadri, M. Caron, R. Joubert-Caron, Rapid Commun. Mass. Spectrom. 18 (2004) 2785.
[12] C. Pionneau, L. Canelle, J. Bousquet, J. Hardouin, J. Bigeard, M. Caron, R. Joubert-Caron, Cancer Genom. Prot. 2 (2005) 199.
[13] L. Canelle, J. Bousquet, C. Pionneau, L. Deneux, N. Imam-Sghiouar, M. Caron, R. Joubert-Caron, J. Immunol. Methods 299 (2005) 77.
[14] C.C. Dawes, P.J. Jewess, D.A. Murray, Anal. Biochem. 338 (2005) 186.
[15] T.W. Hutchens, J.S. Magnuson, T.T. Yip, J. Immunol. Methods 128 (1990) 89.
[16] B. Nopper, F. Kohen, M. Wilchek, Anal. Biochem. 180 (1989) 66.
[17] P. Konecny, R.J. Brown, W.H. Scouten, J. Chromatogr. A 673 (1994) 45.
[18] E. Juronen, J. Parik, P. Toomik, J. Immunol. Methods 136 (1991) 103.
[19] T.W. Hutchens, J.O. Porath, Clin. Chem. 33 (1987) 1502.
[20] D. Lutomski, R. Joubert-Caron, P. Bourin, D. Bladier, M. Caron, J. Chromatogr. B Biomed. Appl. 664 (1995) 79.
[21] P. Bridonneau, F. Lederer, J. Chromatogr. 616 (1993) 197.