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Investigations on the specificity of thiophilic interaction for monoclonal antibodies of different subclasses

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Abstract

A comparative study was carried out to investigate the influence of different mouse antibody subclasses on the chromatographic behaviour on thiophilic supports. Cell-free supernatants from different mouse–mouse hybridoma cultures in a standard medium were purified on thiophilic agarose and Fractogel EMD TA. The adsorption capacities and purification factors were monitored under optimised adsorption conditions. The different isotypes did not differ significantly regarding capacity of the thiophilic matrix, but the purity of the eluted antibody fractions was significantly lower for the IgG_{2a} subclass compared to all other murine antibodies. A significant copurification of proteins from cell culture supernatant with antibodies of the IgG_{2a} subclass indicated a restriction in the universal nature of thiophilic interaction.

Keywords: Thiophilic interaction; Adsorbents; Monoclonal antibodies; Antibodies

1. Introduction

Thiophilic adsorption has been introduced as a new chromatographic method by Porath [1] et al. in 1985. The thiophilic ligand was found to have an intriguing specificity for immunoglobulins when used in the chromatographic separation of serum [2,3], ascites fluid, cell culture supernatant [4], human colostrum [5], and bovine whey [6]. The simple synthesis, chemical stability, and high specificity of the ligand made it an interesting alternative to protein affinity ligands such as protein A or G. Although the early work concentrated on the purification of antibodies, thiophilic behavior was described for other proteins as well [7,8]. From these data no common structural element of 'thiophilic' proteins could be detected, so the mechanism of thiophilic interaction remained unclear. A mechanistic proposal, still valid today, was made by Hutchens and Porath [8]. They suggested a combined electron donating and accepting action of the ligand to be the key of thiophilic interaction and proposed that the water-structuring salt, which is necessary to promote binding, offers the correct micro environment by accumulation of proteins at the adsorbent surface in a salting-out action. The original ligand contained a sulphone-group as electron accepting species and a hetero-atom with a lone electron pair (S>N>>O) as electron donor. Novel structures retain the thioether component and replace the sulphonic group by heterocyclic compounds, thus increasing the simplicity of the ligand as well as the capacity of the respective adsorbents at very low concentrations of water-structuring salts [9,10].

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The adsorption of immunoglobulins to thiophilic matrices has been described as a universal method for the purification of these biomolecules. A discrimination of certain subclasses regarding the capacity of the sorbents has not been reported. The specificity of the purification, however, has been found to depend on the animal species [4] used as immunoglobulin source. Bridonneau and Lederer [11] described a fractionated binding of human immunoglobulin subclasses depending on the concentration of ammonium sulphate added to the serum samples under investigation. A preferential adsorption of human IgG_2 and a partial discrimination of human IgG_3 on a thiophilic agarose was shown. The observed differences in the strength of the interaction were small and a preparative separation of IgG subclasses using differential adsorption on thiophilic matrices was not possible. These are indications that the immunoglobulin – thiophilic ligand interaction is not as universal as anticipated previously. Initial investigations with thiophilic membranes in our laboratories had shown significant differences in the purification of mouse IgG_1 and mouse IgG_{2a} monoclonal antibodies, both proteins being adsorbed at similar capacities ($\approx$17 $\mu g/cm^2$ of membrane $\approx$0.85 mg/ml of sorbent) but IgG_1 being purified to a larger extent (5-fold) compared to IgG_{2a} (2.5-fold) [12]. In a study on the application of a thiophilic tentacle-type adsorbent we also found the purification of mouse IgG_{2a} from cell culture supernatant to be less efficient than with all other mouse IgG subclasses [13]. Again there was no pattern in the differences between the subclasses regarding the capacity of the adsorbent, but the IgG_{2a} antibodies were only partially purified compared to mouse IgG_1 or IgG_3, where large purification factors were found.

These observations prompted us to perform a closer investigation on the influence of IgG subclass on the purification of mouse monoclonal antibodies by thiophilic adsorption. The goal of these experiments was to extend the already existing data on thiophilic adsorption by a systematic approach trying to identify possible restrictions on the universal applicability of this purification method. The discrimination of a certain subclass or a possible deterioration of the resolving power of thiophilic adsorption together with structural details of the discriminated proteins might give additional information to reveal the mechanism of thiophilic interaction, which up to now is still unknown.

We chose five monoclonal antibodies from different subclasses produced by mouse–mouse hybridoma cells (IgG_1, IgG_{2a}, IgG_{2b} and IgG_3) for our experiments. Supernatants of batch cultures of the different hybridoma cells were applied to a commercial thiophilic agarose gel under overload conditions and the capacity and purification for the respective subclasses were compared. Previously the conditions for the adsorption of the different monoclonals were determined with regard to optimum ammonium sulphate concentration and pH as well as protein challenge necessary for antibody breakthrough. The data obtained were compared to an extended study performed on a thiophilic tentacle-type adsorbent.

2. Experimental

Thiophilic agarose (AFFI-T) was purchased from KemEnTec (Kopenhagen, Denmark). Thiophilic Fractogel, ammonium sulphate, Tris, sodium acetate, and sodium lauroyl sarcosinate were supplied by Merck (Darmstadt, Germany). HEPES and Coomassie brilliant blue R 250 were purchased from Fluka (Neu-Ulm, Germany), anti-mouse IgG, anti-mouse IgG conjugated with alkaline phosphatase, and *p*-nitrophenyl phosphate were from Sigma (Deisenhofen, Germany).

Hybridoma culture fluid was kindly provided by the cell culture group at the Institute of Biotechnology (Forschungszentrum Jülich, Germany). The mouse–mouse hybridoma cell lines producing the antibodies of different IgG subclasses were cultivated batchwise under standardised conditions. The culture medium contained 1% fetal bovine serum and 100 mg/l bovine serum albumin (BSA).

IgG_{2a} antibody, with an unknown antigen was produced by clone VX12 from Verax (Lebanon, NH, USA) and IgG_{2a} antibody against human epidermal growth factor receptor was produced by Clone 425 from Merck. IgG_1, also with an unknown antigen, was produced by clone AX23 provided by Abion (Jülich, Germany). Hybridoma cell lines TIB174 producing Anti-rat-IgG2b-Fc IgG_{2b} as well as CL 189 producing anti-influenza virus IgG_3 were pur-

chased from the American Tissue Type Collection (ATCC).

2.1. Sample treatment

The cell culture fluid was filtered (0.45 μm) to remove cells, cell debris, and particles from the medium, then the ammonium sulphate concentration was carefully adjusted with solid salt and the pH was titrated to desired values with 25% HCl. Afterwards the medium was filtered again to remove precipitated protein (0.20 μm).

2.2. Chromatographic experiments

In order to generate comparable results the capacities of the thiophilic matrices were determined in fixed-bed mode. A 2-ml volume of moist thiophilic agarose was employed in a small column (CC10, Pharmacia, Freiburg, Germany). Sample and buffers were applied with a flow-rate of 1.0 ml/min (linear velocity: 76.4 $cm \cdot h^{-1}$). In order to describe the adsorption behaviour, the retained antibody mass during sample application (apparent adsorption) was compared to the eluted antibody mass in the desorption step (eluted antibody) after washing the overloaded gel with adsorption buffer (wash fraction). For a better comparison of the experiments the gel challenge was taken as a measure of the amount of antibody applied. It is defined as the mass of antibody applied per millilitre of adsorbent in a frontal mode. The adsorbent was regenerated with 0.1 *M* NaOH in 20% (v/v) isopropanol and reequilibrated with ten column volumes of starting buffer.

2.3. Analytical methods

Antibody concentrations were determined on a ProAnaMabs analytical HPLC column (Biolytica, Lund, Sweden) connected to a UV monitor. Calibration was carried out with a sample of pure antibody of known concentration. The results were cross-checked by anti-isotypic sandwich-ELISA tests as described elsewhere [14]. Protein concentration was measured according to Bradford [15]. The antibody purity of a sample was expressed as the ratio of antibody concentration C_{MAb} to the total protein concentration C_P. The purification factor P was calculated as the ratio of the antibody purity after the chromatographic purification steps to the initial antibody purity according to Eq. (1).

$$P = \frac{\left[\frac{C_{Mab}}{C_P}\right]_{after}}{\left[\frac{C_{Mab}}{C_P}\right]_{initial}} \quad (1)$$

Dynamic viscosity was determined with a rotational viscosimeter (Haake, Karlsruhe, Germany). The density of the cell culture fluids was measured with an electronic densitometer DMA 35 A (Paar K.G., Graz, Austria).

3. Results and discussion

3.1. Physical and chemical parameters of the cell culture supernatants

Physical and chemical parameters of the employed cell culture supernatants are listed in Table 1. The protein concentrations in the cul-

Table 1
Details of the cell culture supernatants used

Clone	IgG	Protein concentration (mg/l)	Antibody concentration (mg/l)
AX 23	IgG_1	510	26
VX 12	IgG_{2a}	1000	42
425	IgG_{2a}	940	38
TIB 174	IgG_{2b}	1000	45
CL 189	IgG_3	1000	40

ture supernatants were between 510 mg/l and 1000 mg/l, the respective antibody concentrations were between 26 mg/l and 45 mg/l. pH, density and viscosity were found to be equal for all samples (pH 7.4, density 1010 kg/m^3, viscosity 1 mPa s).

3.2. Adsorption behaviour on thiophilic matrices

3.2.1. Frontal adsorption

The antibody adsorbed during sample application (apparent capacity or retained antibody) was measured as a function of matrix challenge and was compared to the amount of antibody eluted. Conditions were chosen as advised by KemEnTec for antibody purification from cell culture media [20] (adsorption: 20 m*M* sodium acetate (pH 5.2) +1.0 *M* ammonium sulphate; elution: 20 m*M* Tris–HCl pH 8.0). The data are shown in Fig. 1 with each symbol representing a single experiment. The difference between apparently adsorbed and eluted antibody represents the amount of antibody bound by non-thiophilic interactions which is removed from the matrix during the washing step with adsorption buffer. As can be taken from Fig. 1 the amount of non-specific binding is increased with increasing antibody challenge of the adsorbent. At very high challenges the amount of antibody which can be eluted approaches the saturation or equilibrium capacity of the matrix, this value has not been reached in our experiments as indicated by the course of elutable capacity vs. antibody challenge. Total recoveries (including washed and eluted antibody) of 95% to 100% were reached.

Table 2
Recommendations for optimised thiophilic adsorption

Buffer ion	Reference	Range of pH
Sodium acetate	[20]	5.2
Sodium phosphate	[4]	8.0
HEPES	[10]	7.4
Tris–HCl	[21]	7.6

3.2.2. Systematic analysis of adsorption conditions

Because there are various equilibration methods proposed in the literature (see Table 2) they all were systematically tested for the antibody purification. For capacity determination exclusively the eluted antibody from overloaded columns was taken as a basis. In Fig. 2 IgG_{2a} binding capacities from clone 425 under various conditions are summarised. With rising ammonium sulphate concentration, capacities can be increased. With acetate buffer pH 5.2 and with HEPES between pH 7.0 and 8.0, capacities between 790 $\mu g/ml_{matrix}$ and 520 $\mu g/ml_{matrix}$

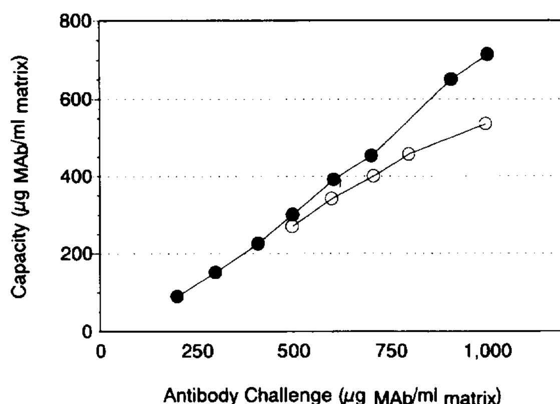

Fig. 1. Apparently adsorbed (●) and eluted antibody (○) vs. antibody challenge on thiophilic agarose (hybridoma supernatant clone 425, pH 5.2, 1 *M* ammonium sulphate).

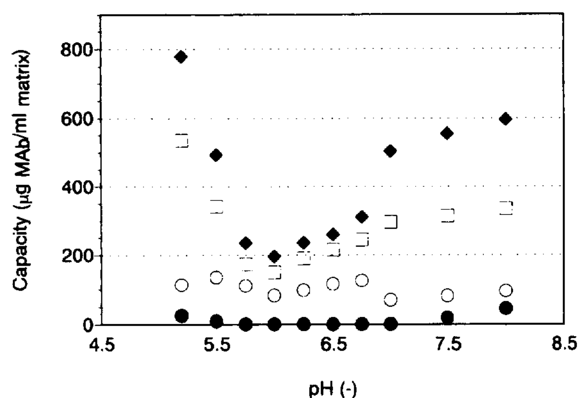

Fig. 2. Eluted capacity from thiophilic agarose for IgG_{2a} (clone 425) at different pHs and ammonium sulphate concentrations (●=0.5 *M* ammonium sulphate, ○=0.75 *M* ammonium sulphate, □ 1 *M* ammonium sulphate, ♦=1.5 *M* ammonium sulphate).

were found. An adsorption minimum can be found at pH 6.0. Corresponding results were obtained for the same antibody employing phosphate buffer for the whole pH range. Although pH-dependent capacity differences could be observed for certain ammonium sulphate concentrations, average values can be determined for the concentration and purification factors of a given antibody as shown in Table 3. Averaging over the whole range of pH values becomes possible as a higher antibody adsorption, caused by changes in pH, is always accompanied by an increased coadsorption of other proteins, mainly BSA. Thus the elution volume as well as the degree of non-specific binding of contaminating proteins is increased so that the concentration and purification factor for a given pH value is nearly constant. In conclusion, the ammonium sulphate concentration – and neither the pH nor the ions of the equilibration buffer – turn out to be the crucial parameter influencing the selectivity of a thiophilic antibody purification.

3.2.3. *Influence of the antibody idiotype on adsorption*

To test the influence of the antibody idiotype on the thiophilic adsorption, a second antibody of the subclass IgG_{2a} (clone VX12) was employed for purification experiments carried out under various equilibration conditions (Fig. 3). In contrast to the behaviour of the IgG_{2a} derived from clone 425 it is not influenced by the pH of the equilibration buffer. The maximum capacities are reached with the highest ammonium sulphate concentration and are in the same range as the maximal value for the IgG_{2a} of clone 425. Averaging the concentration and purification factors as described above shows increased values for the VX 12 antibody compared to clone 425 (see Table 4).

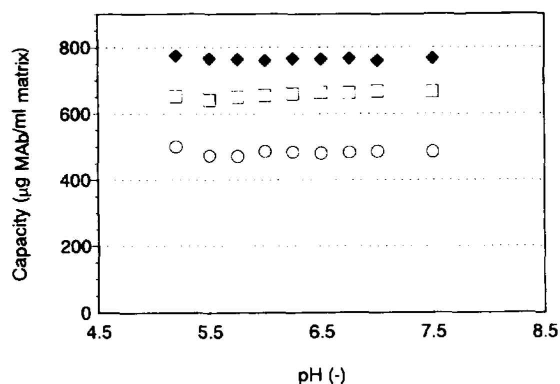

Fig. 3. Eluted capacity from thiophilic agarose for IgG_{2a} (clone VX 12) at different pHs and ammonium sulphate concentrations (○=0.75 *M* ammonium sulphate, □=1 *M* ammonium sulphate, ♦=1.5 *M* ammonium sulphate).

3.2.4. *Influence of the antibody subclass on adsorption*

As reports from literature [4,11] as well as own experiments [12] had given indications that the adsorption efficiency of antibodies from different subclasses or species differs, we decided to test thiophilic adsorption of three further antibodies (IgG_1, IgG_{2b}, IgG_3). The adsorption behaviour of the antibodies was investigated in a pH range between 5.2 to 8.0 with the buffers introduced earlier. The ammonium sulphate concentrations employed were 0.5 *M*, 0.75 *M*, 1.0 *M*, and 1.5 *M*. For the purification factors, the concentration, and the capacity average values could be obtained. The purification factors of all tested antibody subclasses are shown in Fig. 4. Each data point represents the average value of the purification factor of the indicated ammonium sulphate concentration over the whole pH range

Table 3
Average concentration and purification of IgG2a (clone 425) on thiophilic agarose

Ammonium sulphate (mol/l)	Concentration (*n*-fold)	Purification (*n*-fold)
0.5	0.3±.0	0.9±.0
0.75	1.49±0.23	1.34±0.08
1.0	4.27±0.16	2.29±0.13
1.5	4.35±0.32	2.67±0.25

Table 4
Average concentration and purification of IgG_{2a} (clone VX 12) on thiophilic agarose

Ammonium sulphate (mol/l)	Concentration (n-fold)	Purification (n-fold)
0.75	5.05±0.33	3.1±0.28
1.0	6.3±0.45	3.54±0.0.26
1.5	6.6±0.37	4.27±0.21

(n=10). For the IgG_{2a} isotypes the purification factor is improved with increasing ammonium sulphate concentration. The immunoglobulin of clone 425 could be purified with a factor of 2.7 and IgG_{2a} (clone VX12) with a factor of 4.3 from cell culture supernatant. The opposite behaviour can be observed for the adsorption of IgG_{2b} (clone TIB174) and IgG_3 (clone CL198). Their purification is reduced with increasing ammonium sulphate concentration. The maximum purification factor for IgG_{2b} (13.7) is reached with an ammonium sulphate concentration of 0.5 *M*, it drops to 3.8 for a salt concentration of 1.5 *M*. For IgG_3 an eleven-fold purification is achieved with ammonium sulphate concentrations between 0.5 *M* and 1.0 *M*. A strong decrease can be observed at 1.5 *M* salt. IgG_1 could not be bound to thiophilic agarose at the lowest ammonium sulphate concentration (0.5 *M*). A maximum purification factor (14.5) could be reached at 1.0 *M* salt, the efficiency using 0.75 *M* ammonium sulphate is slightly lower (11.5). A further decrease in purification factors was observed at 1.5 *M* ammonium sulphate. Summarising the data shown above, antibodies from the subclasses IgG_1, IgG_{2b}, and IgG_3 can be purified to a significantly higher degree from cell culture supernatants by thiophilic adsorption compared to the tested IgG_{2a} antibodies.

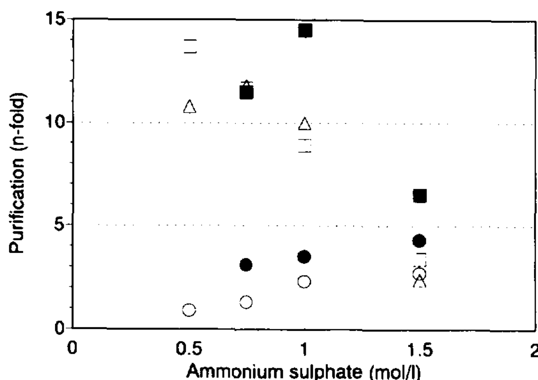

Fig. 4. Average purification factors for IgG_{2a} (VX 12, ●) IgG_{2a} (425, ○), IgG_1(AX 23, ■), IgG_{2b}(TIB 174, □) and IgG_3(CL 189, △).

These results are supported by data summarised in Table 5. Additional murine IgG subclasses were investigated for their adsorption to thiophilic Fractogel. In this study all immunoglobulins could be purified using the thiophilic adsorbent. The antibody content in the flow-through and wash fraction after frontal application of the culture supernatants varied between 0.5% and 23%. The purity of the immunoglobulins eluted under desorption conditions varied depending on the subclass. While IgG_1 and IgG_3 were purified successfully – the antibody content in the elution peak ranged from 100% to 31% – the purification of IgG_{2a} resulted in antibody contents of only 22% and 9.2%. The different purification qualities were proven by SDS gel electrophoresis.

Table 5
Purification of different antibodies on Fractogel EMD TA-650

Antibody	Purity of eluted fraction (%)
IgG_1 a	100
IgG_1 b	71
IgG1 c	67
IgG_1 d	66
IgG_1 e	31
IgG_{2a} a	9.2
IgG_{2a} b	22
IgG_3 a	86
IgG_3 b	92

3.2.5. *Influence of phenol red*

As stated in the literature [16] the indicator phenol red can cause a severe capacity decline during thiophilic adsorption of antibodies from cell culture supernatant. To investigate the influence of the indicator, clone 425 was cultivated in a medium lacking phenol red and applied to a thiophilic agarose in experiments carried out under identical conditions as described above with phenol red containing supernatant. The data showed no significant difference between the purifications. The deviations were lower than 5%. So an influence – as observed for the HPLC adsorbent Lichrospher cannot be stated for thiophilic agarose. As described by Schwarz et al. [16] the dye binds also to the agarose adsorbent but this does not influence capacity or resolution of the thiophilic adsorption process.

4. Conclusions

The investigation presented here is aimed at the examination of the universal applicability of thiophilic adsorption for the purification of all kinds of antibodies. Therefore a broad range of monoclonal antibodies from different subclasses was tested for capacity and resolution (purification factor) on thiophilic matrices. Regarding the capacity of the matrices no tendency of favoured or discriminated subclasses could be detected. Nevertheless differences in the 'thiophilicity' of certain monoclonals became obvious when examining the purity of the fractions eluted under desorption conditions, which depended on the murine IgG subclass. Purification deteriorated if mouse IgG_{2a} was isolated compared to mouse IgG_{2b}, IgG_1 and IgG_3 although in all cases presumably the same proteins derived from the medium components and hybridoma metabolism were present in excess. The differences in the purification factors were three- to four- fold. In the literature a high specificity of thiophilic ligands for the purification of all IgG subclasses from different species is stated frequently [10,16,17]. The only antibody class reported previously to interact only weakly with thiophilic adsorbents only is human and murine IgM [4,18]. Our data support the observations reported by Bridonneau and Lederer [11] as well as the remark made by Belew et al. [4] that the thiophilic interaction is not as universal as it is commonly understood. The individual capacity of the thiophilic matrix may vary significantly because of different antibody and total protein concentrations in the applied sample. The relative efficiency, measured as the purity of the antibody eluted from the adsorbent, shows drastic differences. With all IgG_{2a} antibodies we found a significant co-adsorption of foreign proteins which was not as severe with the other subclasses. This effect may be caused by the mechanistic details of thiophilic adsorption. Although the exact mechanism of this method is still unknown, several theories have been published which will now be discussed with reference to our data.

The mechanistic theory of Hutchens and Porath [8] proposes electronic effects as the basis of thiophilic interaction. The striking coadsorption of foreign proteins during IgG_{2a} purification is difficult to explain by this electron donating and accepting mechanism. An alternative approach may be the interpretation of the thiophilic structure as a concerted interaction of hydrophilic (sulphone) and hydrophobic (thioether) domains. The interaction of antibodies with this structure might then be a mixed mode hydrophilic–hydrophobic interaction, which is promoted by the addition of the water-structuring salt similar to a salting-out action. As the coadsorption of proteins does not reduce the adsorbent capacity, it might be concluded that the binding of additional protein does not occur at the 'thiophilic' ligand but is more related to the three-dimensional structure of the antibody in terms of a protein–protein interaction. According to Burton et al. [19] secondary functions of antibodies rely on that kind of interaction, which often is due to contact with hydrophobic patches of the proteins. The coadsorption in the case of mouse IgG_{2a} which is not observed with other mouse IgG subclasses might now be attributed to potential secondary functions of this subclass which are not present in the case of the other mouse IgG subclasses. As thiophilic inter-

action is performed in the presence of water-structuring salts, which usually lead to an increased exposure of hydrophobic patches on the protein surface, the capability of mouse IgG_{2a} to interact with other proteins in a secondary function could then give rise to a coadsorption of proteins from the cell culture supernatant because of an increased amount of hydrophobic patches available for interaction. This "package" of interacting proteins would then be eluted together as an antibody fraction of decreased purity, as observed with all mouse IgG_{2a} under investigation. The main difference in thiophilic behaviour, different purification factors at comparable capacity, might then be attributed to the structural properties of a certain subclass – possibly any secondary functions, the nature of which remains to be elucidated. Further efforts are needed to reveal the exact mechanistic basis of the phenomenon observed. Nevertheless, from the data presented the versatility of thiophilic interaction for the isolation of antibodies of high purity appears somewhat restricted. In the case of murine antibodies IgG_{2a} deviate from the expected behaviour.

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