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Novel heterocyclic ligands for the thiophilic purification of antibodies

Alexander Schwarz[a,*], Fortune Kohen[b], Meir Wilchek[a]

[a]*Department of Membrane Research and Biophysics, Weizmann Institute of Science, 76100 Rehovot, Israel*
[b]*Department of Hormone Research, Weizmann Institute of Science, 76100 Rehovot, Israel*

Abstract

Novel thiophilic ligands based on mercaptoheterocycles were synthesized for use in one-step purification of antibodies. In order to better characterize these new structures, affinity constants were measured, as well as the influence of pH and salt on adsorption and elution. The ligand concentration was optimized for efficient and fast adsorption and elution of antibodies from ascites and serum. The purification of antibodies from cell culture supernatant proved difficult due the indicator phenol red of the growth media.

1. Introduction

Interest in monoclonal and polyclonal antibodies has increased as more applications in biotechnology, such as immunoaffinity chromatography, immunodiagnostics, drug targeting, biosensors etc., are found for these versatile molecules. For all these purposes homogeneous antibody preparations are needed. Conventional purification methods using e.g. protein A or protein G are expensive to operate due the cost of the media. Non-specific methods like hydroxyapatite or ion-exchange chromatography possess low capacity or yield antibodies contaminated with albumin and transferrin.

Thiophilic adsorption chromatography (TAC), a chromatographic method introduced by Porath et al. [1] in 1985, shows selective binding of immunoglobulins in the presence of structure-forming salts. Two structurally different sulfur-containing ligands were found to promote this type of adsorption. One basic structure derives from divinyl sulfone-activated agarose coupled with either β-mercaptoethanol [1–4] or with 2-hydroxypyridine [5]. The other structure is based on epoxy-activated agarose to which 2-mercaptopyridine is coupled [6]. Ligands, which are based on the divinyl sulfone activation, can be varied to a large extent, provided they possess some hydrophilicity and are good electron donors [1,5]. The groups attached to the divinyl sulfone structure which show the highest specificity seem to be sulfur-containing ligands [1].

We investigated the 2-mercaptopyridine ligand for the HPLC purification of antibodies and found the results unsatisfactory. We therefore examined two new mercaptoheterocycles with more than one heteroatom in the ring structure,

* Corresponding author. Present address: BioSepra, 140 Locke Drive, Marlborough, MA 01752, USA.

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as they provide a higher hydrophilicity and a higher electron density.

2. Experimental

Mercaptopyridine, mercaptopyrimidine, mercaptothiazoline, triethylamine, 3-glycidyoxypropyltrimethoxysilane and thorine were purchased from Aldrich (Milwaukee, WI, USA). Human IgM and IgA were purchased from Sigma (St. Louis, MO, USA). Polyclonal rabbit immunoglobulins against mouse immunoglobulins were purchased from Dako A/S (Glostrup, Denmark). LiChrosorb Si 1000 was a gift of Dr. K. Ernst-Cabrera (E. Merck, Darmstadt, Germany). Crosslinked agarose was purchased from Innovata AB (Sollentuna, Sweden). All other chemicals employed were of the highest grade commercially available.

The cell line secreting anti-estradiol (anti E_2) antibodies (clone 15) originated from a fusion of myeloma cells P3/NSI/1-Ag (NSI) with the spleen cells of a female C57B1/6 mouse immunized with estradiol-6-carboxymethyl bovine serum albumin [7,8]. Clone 15 was propagated in vivo as ascites in the peritoneum of (C57B1/6 × Balb/c)F_1 male mice. The titer of specific antibodies in the ascites was $>1{:}200\,000$. Clone 15 possessed a high affinity for estradiol ($K_a = 10^{10}$ l/mol) and the heavy-chain class was IgG_{2b}.

Human serum was from a local blood bank (Sheba Hospital, Tel Aviv, Israel). Tissue cell culture supernatant containing anti-CD-4 antibody was kindly provided by Allon Canaan (Department of Membrane Research and Biophysics, Weizmann Institute).

2.1. Synthesis of thiophilic structures

Silica

LiChrosorb Si 1000 (20 g) was washed with 0.001 *M* HCl and 0.1 *M* H_3PO_4 and then washed extensively with distilled water. The silica was dried at 180°C under oil pump vacuum overnight. The dried silica was suspended in 150 ml of anhydrous toluene and 1 ml of triethylamine and 7.5 ml of 3-glycidyloxypropyltrimethoxysilane was added and the slurry was stirred under reflux for 18 h. The slurry was filtered through a sintered glass filter and washed with toluene, acetone and double-distilled water.

The activated silica (20 g) was resuspended in 80 ml of 0.2 *M* carbonate buffer (pH 8.5) and 800 mg of the respective mercaptoheterocycle was added under nitrogen and stirred for 1 h at room temperature. The slurry was filtered through a sintered glass filter and washed extensively with methanol and with 0.1 *M* carbonate buffer (pH 8.5).

The ligand density was determined from sulfur analysis. The sulfur content was found to be 16.5 μmol/g for the mercaptopyridine, 12.5 μmol/g for the mercaptopyrimidine and 9 μmol/g for the mercaptothiazoline. Higher ligand densities were obtained by increasing the amount of mercaptoheterocycle used in the reaction.

Agarose

Suction-dried Novarose 1000/40 (40 g) was suspended in 20 ml of 4 *M* NaOH and 75 mg $NaBH_4$ were added. To this suspension was added 10 ml of epichlorhydrin and the slurry was stirred for 19 h. The slurry was filtered through a sintered glass filter and washed until neutrality with double-distilled water.

The activated agarose was resuspended in 25 ml of 50% methanol and 200 mg of the respective mercaptoheterocycle was added under nitrogen. The slurry was stirred for 8 h, filtered through a sintered glass filter and washed extensively with a 50% methanol solution and with water.

The ligand concentration was obtained from sulfur analysis. The sulfur content was found to be 17 μmol per ml of gel for the mercaptopyridine, 18 μmol per ml of gel for the mercaptopyrimidine and 14 μmol per ml of gel for the mercaptothiazoline ligand.

2.2. Analytical procedures

Sulfur analysis

Sulfur analysis was performed according to Schöninger [9]. Briefly, the modified silica or modified agarose was lyophilized and a weighted

amount of silica or agarose was enclosed in ashfree paper and the paper enclosed in a platinum mesh. A 5-ml volume of water and 200 μl of H_2O_2 were added to an Erlenmeyer flask and flushed with oxygen. The sample was burned in pure oxygen and shaken vigorously. After 1 h the Erlenmeyer flask was opened and 20 ml of 2-propanol and 200 μl of the indicator (0.04% thorine in water) were added. The titration was performed with 0.02 *M* $Ba(ClO_4)_2$ in 80% 2-propanol until the color change from orange to red occurred.

Protein determination and gel electrophoresis

The concentration of protein in column effluents was determined according to the method published by Bradford [10]. The purity of the proteins separated by HPLC was analyzed by SDS-PAGE as described by Laemmli [11] using a 10% gel. Samples were applied under reducing conditions and the proteins were visualized by staining with Coomassie brilliant blue.

Activity

The antibody activity of the clone E_2-15 was determined by time-resolved immunoassay as described earlier [7].

Affinity constants

Rabbit anti-mouse IgG was radiolabelled with ^{125}I using the chloramine-T method [12] and the radiolabelled IgG was dialyzed against the adsorption buffer (350 m*M* Na_2SO_4, 10 m*M* Hepes, pH 7.2). The resulting IgG concentration was 0.8 mg/ml. Increasing amounts of radiolabelled antibodies were incubated with the thiophilic silica. A standard experiment was performed using 0.5 mg silica and 0.5 ml adsorption solution using increasing amounts of the radiolabelled IgG solution. The mixture was incubated for 2 h, centrifuged and washed three times with adsorption buffer. The supernatant and the washing solutions were combined. The combined solutions and the remaining silica were counted for radioactivity in a gamma counter. The data were evaluated according to the method of Scatchard [13].

2.3. Chromatographic procedure

The HPLC system consisted of a HPLC pump 2150, HPLC controller 2152 and a solvent delivery system 2156, a double channel recorder 2210 (Pharmacia-LKB, Uppsala, Sweden) with a variable-wavelength monitor (Knauer, Berlin, Germany). The methanolic slurry of the thiophilic silica was packed into a 50 × 4.6 mm I.D. column (Alltech, Deerfield, IL, USA) at 75 MPa.

Samples of 50–250 μl of ascites fluid, sera from various species and tissue cell culture supernatant of antibody-secreting clones were injected in a mobile phase containing 350 m*M* Na_2SO_4 and 10 m*M* Hepes (pH 7.4). The column was washed with adsorption buffer until the absorbance was < 0.01. The adsorbed protein was eluted either with 10 m*M* Hepes (pH 7.4) or 10 m*M* Tris-HCl (pH 7.4). All experiments were carried out at room temperature.

3. Results and discussion

Porath and Oscarsson [6] reported that 2-mercaptopyridine-modified agarose will efficiently purify antibodies from serum. We tried to apply this ligand for the fast one-step purification of antibodies on silica-based matrices, but found the result not satisfactory in terms of recovery of activity as shown in Table 1. We therefore synthesized two new structures and in order to quantitatively compare the newly synthesized structures, affinity constants were measured and are shown in Fig. 1.

The thiophilic structures investigated have affinity constants in the useful range of 10^{-7} *M* for antibodies. The differences in the respective affinity constants between the different structures are not significant and we found no difference in their chromatographic behavior other than the minimum amount of salt needed for adsorption of antibodies. The minimum amount of sodium sulfate required for almost complete adsorption is 350 m*M* for the mercaptopyrimidine and the mercaptothiazoline ligand, while the mercaptopyridine ligand required 450 m*M* as shown in Fig. 2. The higher amount of

Table 1
Activity of clone E_2-15 against estradiol in effluent and eluent and capacity of the columns

Mercapto-	Activity in Effluent (%) Silica	Activity in Eluent (%) Silica	Capacity	
			Silica (mg/g)	Agarose (mg/ml)
Pyridine	37.5	62.5	25.7	17.9
Pyrimidine	1.1	98.9	26.3	18.8
Thiazoline	2.1	97.9	25.4	19.2

Adsorption: 350 m*M* Na_2SO_4, 10 m*M* Hepes (pH 7.4); elution: 10 m*M* HEPES (pH 7.4).

sulfate needed for complete adsorption of antibodies on the mercaptopyridine structure was detrimental to the purity of the antibody eluted, as at this amount of salt, other proteins also adsorbed to the matrix. In order to achieve a pure antibody preparation, a lower amount of salt had to be applied, but this in turn led to a low recovery of the antibody in the eluate as shown in Table 1.

All structures investigated showed a broad pH optimum (pH 5–9) for the adsorption of antibodies. However, complete elution could only be achieved after optimization of the ligand concentration. The higher the ligand concentration, the higher the nonspecific adsorption of other proteins and the lower the recovery of proteins from the column. We also found a correlation between the elution pH and the ligand concentration as shown in Fig. 3. The higher the ligand concentration, the lower the elution pH had to be in order to achieve complete elution. It turned out, that for the most specific adsorption and the mildest elution conditions, a ligand concentration between 10 to 15 μmol/g for silica and 15 to 20 μmol per ml of gel for agarose were optimal. In view of the recovery of antibody activity, the mercaptopyridine structure was the least efficient as only 62.5% of the activity was recovered in the eluate, while the mercaptopyrimidine and mercaptothiazoline structure recovered 99% and 98% of the activity, respectively, as shown in Table 1. Although chemically quite different, the purification results of the

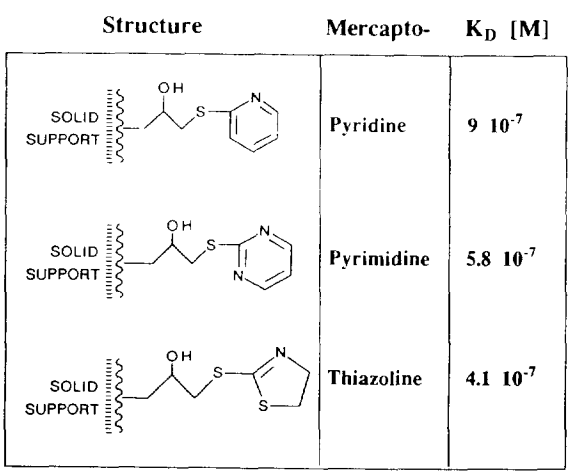

Fig. 1. Structure, name and K_d of thiophilic structures synthesized.

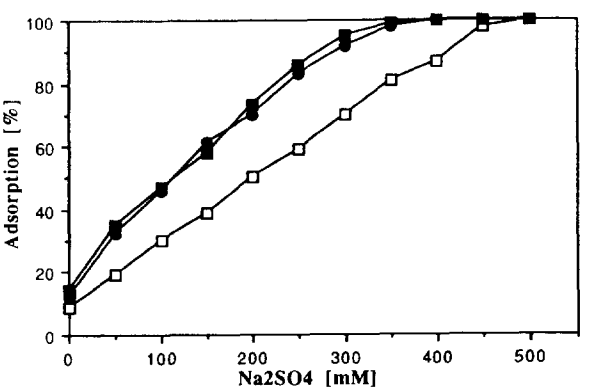

Fig. 2. Influence of the salt content on adsorption of human IgG. Experimental conditions: 1 mg human IgG in 100-μl volume containing corresponding Na_2SO_4 concentrations and 10 m*M* Hepes (pH 7.4); flow-rate 1 ml/min; (■) mercaptopyrimidine, ligand concentration 112.5 μmol/g; (●) mercaptothiazoline, ligand concentration 9 μmol/g; (□) mercaptopyridine, ligand concentration 16.5 μmol/g; every point is the average of three measurements.

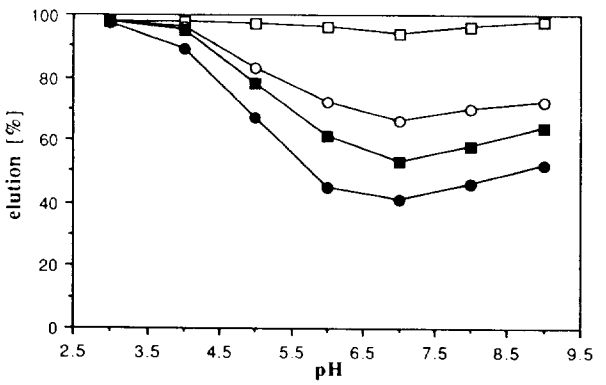

Fig. 3. Influence of the ligand concentration and pH on the elution from mercaptopyrimidine-silica. Experimental conditions: adsorption of 2.5 mg/ml human IgG in 250-μl volume containing 350 m*M* Na_2SO_4 and 10 m*M* Hepes (pH 7.4). Elution conditions: flow-rate 1 ml/min; 0.1 *M* citric acid/NaOH for pH 3 and 5; 0.1 *M* Hepes between pH 5 and 8; 0.1 *M* Tris-HCl for pH 9; (□) 17 μmol/g; (○) 25 μmol/g; (■) 39 μmol/g; (●) 62 μmol/g.

mercaptopyrimidine and the mercaptothiazoline ligand did not differ as judged from the SDS-gel in Fig. 4. The purification of ascites with respect to activity was very good and the purity was ca. 90%. The purity of the IgG fraction from human serum was judged to be about 75%. The capacity was measured by overloading the thiophilic matrices with human IgG at a flow-rate of 1 ml/min until the flowthrough had the same absorbance as the starting solution. The column was then washed with adsorption buffer until baseline values were obtained and the protein bound was then eluted with a low-salt buffer solution. The concentration of the antibody was determined in the eluted fraction. The capacity was determined to be 26 mg per g of silica and 19 mg per ml of agarose for all three ligands investigated.

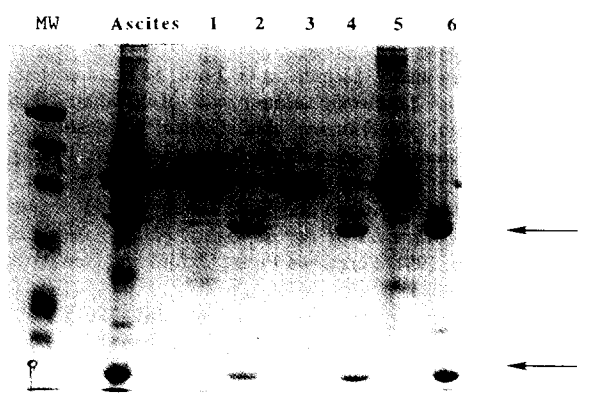

Fig. 4. SDS-PAGE analysis of effluent and eluent of ascites E_2-15 purification with the thiophilic ligands synthesized. Lane 1 and 2: flowthrough and elution from mercaptopyridine; lane 3 and 4: flowthrough and elution from mercaptopyrimidine; lane 5 and 6: flowthrough and elution from mercaptothiazoline; arrows indicate heavy and light chain, respectively.

We found no indication, that under these adsorption conditions there is any difference in the chromatographic behavior between different IgG subclasses, as even a combined pH and salt gradient eluted the antibodies in one peak. We also investigated other antibodies from different classes and species. Human IgA bound as tightly to the thiophilic columns as human IgG, while only 40% of human IgM bound under the conditions optimized for IgG. We found that IgGs from other species, such as rat, mouse, bovine and goat, also bound to the thiophilic matrices investigated. Also the antibodies from egg yolk, IgY, bound to the column, but at least one other major protein, presumably ovalbumin, copurified with the IgY, requiring an additional chromatographic step for its purification.

A third major source of monoclonal antibodies is hybridoma culture supernatant. Similarly to our investigation of divinyl sulfone based ligands [14], we again encountered serious problems due to the indicator phenol red. This indicator, which is a triphenylmethan dye, is strongly retarded on the thiophilic matrices and elutes after the flowthrough portion of the media. This effect reduces the capacity of the thiophilic matrices from above 25 mg per g of silica to approximately 2 mg per g of silica, as the indicator presumably competes with the antibodies for binding sites on the column. No problems were encountered with hybridoma culture supernatant that did not contain the indicator. The purity and the recovery of monoclonal antibodies from hybridoma culture supernatant was above 90%.

Due to the simplicity of the synthesis of these thiophilic ligands, we also investigated these ligands on agarose as the purification of larger volumes from hybridoma cell culture is usually

performed on agarose. We also wanted to investigate whether there are any differences in the chromatographic behavior of these two matrices. We found no significant differences between the behavior on silica or on agarose both with respect to the salt concentration needed for adsorption or elution and the same low dependence on the adsorption pH. One major difference was that the capacity on agarose was found to be lower than on the silica matrix, although the optimized ligand concentration was higher on the agarose matrix. With the agarose-based ligands, we performed stability tests. It was found that after 6 months at 4°C in various storage solutions, such as 0.5 *M* NaOH supplemented with 0.1% (v/v) β-mercaptoethanol, no reduction in the capacity of the column was observed as shown in Table 2.

It is interesting to note, that two chemically very different structures, one based on divinyl sulfone, the other based on mercaptoheterocycles, show the same chromatographic behavior towards proteins in general and towards antibodies in particular. Even the affinity constants for the heterocycle-based ligands investigated here and the divinyl sulfone based ligands investigated in our earlier study are of the same order of magnitude of 10^{-7} *M* for antibodies. All our present results were basically also obtained using divinyl sulfone based ligands and no chromatographic differences were found between the two structures. The strong interaction of phenol red and aromatic peptides with the thiophilic matrices might indicate that aromatic amino acids of the antibodies are partly or totally responsible for the interaction observed.

Table 2
Stability of mercaptopyrimidine-agarose in various storage solutions at 4°C for 6 months

Conditions	Capacity (mg/ml)
Start	18.4
Double distilled water	18.8
0.1 *M* NaOH	19
0.5 *M* NaOH + 20% (v/v) ethanol	18.2
0.5 *M* NaOH + 0.1% (v/v) mercaptoethanol	18.7
1 *M* NaOH + 0.1% (v/v) mercaptoethanol	12.1
10 m*M* Hepes (pH 7.4)	18.9
0.1 *M* Acetic acid	18.5

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