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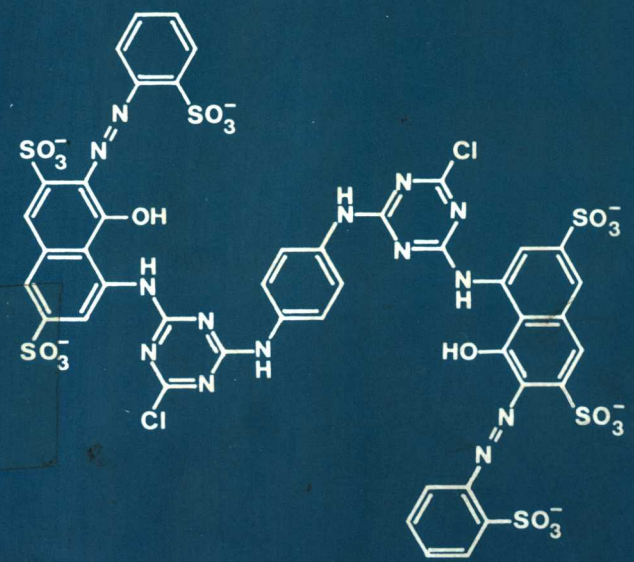
PRACTICAL PROTEIN CHROMATOGRAPHY

Edited by

Andrew Kenney and Susan Fowell



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Practical Protein Chromatography

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Practical Protein Chromatography

Methods in Molecular Biology

John M. Walker, SERIES EDITOR

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Preface

One of the many impacts of recombinant DNA technology over the last 15 years has been a strongly refreshed interest in methods for the separation and purification of proteins. This interest has encompassed not only analytical separations, but also small- and large-scale preparative methods directed to both pure and applied research throughout biology and biomedicine.

Many of the new or substantially modified techniques developed have been reported in the literature, but a sufficiency of *detailed* practical help in establishing these methods for the first time in a new laboratory has often been difficult to find. With these problems in mind, we expect that *Practical Protein Chromatography*, designed as a key volume in the *Methods in Molecular Biology* series, will provide concise practical help to those carrying out new techniques for the first time.

Each chapter has been written by expert authors known to have direct and regular practical experience with their chosen techniques. The structure of each chapter is designed to make it easy for a worker new to the method to follow it to an effective conclusion. An Introduction treats the theory behind the method being described. The Materials and Methods sections allow the reader to prepare for, and then perform techniques in a rational stepwise manner. The Notes sections provide the sort of background 'hints' and 'tricks' that are so often essential for success, but are rarely reported in the literature. They also contain information about modifications to the basic methods that may help the reader to apply the technique in novel ways to new problems.

It is anticipated that *Practical Protein Chromatography* will be of use to research workers at all levels wishing to use a method for the first time. It is also hoped that the wide variety and range of methods and procedures covered here will encourage the reader to innovate with them in their own research, thereby extending and developing still more fully the inherent capabilities of the techniques described.

Andrew Kenney
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Contents

Chapter 1

Thiophilic Adsorption Chromatography

T. William Hutchens

1. Introduction

Thiophilic adsorption is useful for the purification of immunoglobulins under mild conditions (e.g., *see* ref. *1*). Although there are several established procedures for the purification of immunoglobulins *(2–5)*, thiophilic adsorption appears thus far to be unique in its capacity to adsorb three major classes of immunoglobulins (and their subclasses) *(6–8)*. Furthermore, in contrast to other affinity purification methods (e.g., *see* refs. *3,4*), recovery of the adsorbed (purified) immunoglobulins from the thiophilic adsorption matrix is accomplished efficiently at neutral pH, without the need for perturbation of protein structure *(1)*. The most important utility of thiophilic adsorption is perhaps its use for the selective depletion of immunoglobulins from complex biological fluids (e.g., calf serum and hybridoma culture media, colostrum and milk) *(6,7,9)*. This latter development has been particularly useful with hybridoma cell culture applications *(9,10)*, and in the investigation of milk-immunoglobulin function during early periods of human infant nutrition *(6,7)*.

Thiophilic adsorption actually describes the affinity of proteins for a specific set of sulfur-containing (i.e., thioether-sulfone) immobilized ligands, which is observed in the presence of certain salts. Since the first demonstration of this adsorption phenomenon *(11)*, it has been further developed and used primarily for the selective adsorption of

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immunoglobulins (e.g., *see* refs. *1,6–8*). Even though thiophilic adsorption chromatography, like hydrophobic interaction chromatography, is a salt-promoted adsorption process, hydrophobic proteins, such as serum albumin, are not thiophilic *(10)*. The mechanism of thiophilic adsorption is not understood thoroughly at the present time *(10,12)*. In practice, we do know that the selectivity of thiophilic adsorption is dependent upon the type and density of immobilized thiophilic ligand, as well as the concentration and type of water-structure promoting (i.e., antichaotropic) salt used to promote adsorption. The F_c, F_{ab}, and $F_{(ab')2}$ immunoglobulin fragments are each thiophilic, although different concentrations of antichaotropic salt are required to promote adsorption. Only those procedures for the thiophilic adsorption of intact immunoglobulins are presented here.

Stationary phases for thiophilic adsorption chromatography are not presently commercially available. Preparation and use of the thiophilic adsorbent is, however, relatively simple even for the nonorganic chemist. This chapter presents a detailed description of the synthesis of both polymeric- and silica-based thiophilic adsorbents and of the use of these thiophilic adsorbents for the selective and reversible adsorption of multiple immunoglobulin classes.

2. Materials

2.1. Materials for the Synthesis of Thiophilic (T-Gel) Adsorbents

2.1.1. Agarose-Based T-Gel

1. Agarose (6%) or Pharmacia Sepharose 6B.
2. Divinyl sulfone.
3. 2-Mercaptoethanol.
4. Ethanolamine.

2.1.2. Silica-Based T-Gel

1. Porous silica (e.g., LiChrosorb 60).
2. γ-glycidoxypropyltrimethoxysilane.
3. Sodium hydrosulfide.
4. Divinyl sulfone.
5. 2-Mercaptoethanol.
6. Dithiothreitol.
7. 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB: Ellman's reagent).

8. Methanol.
9. Acetone.
10. Toluene.
11. Diethyl ether.

2.1.3. Buffers and Eluents

1. 2, 4-Hydroxyethyl-1-piperazine ethanesulfonic acid (HEPES), sodium phosphate, tris [hydroxymethyl] aminomethane (Tris) (*see* Section 2.3.1.).
2. Ethylene glycol (*see* Section 2.3.2.).
3. Isopropanol (*see* Section 2.3.3.).

2.2. Chromatographic Columns and Equipment

The thiophilic adsorbents described here, if used under the conditions specified, are quite selective and have a high capacity for immunoglobulins *(1)*. Conventional open-column procedures with an agarose-based T-gel have been used in my laboratory with column bed volumes ranging from 1 to 2000 mL. High flow rates can be used *(1)*: the stability of the agarose beads is increased substantially because of the crosslinking with divinyl sulfone. Since this is an adsorption—desorption procedure (no resolution required), specific column dimensions are not critical to the success of the operation. Note, however, that the adsorption of other proteins can be induced with higher concentrations of ammonium sulfate (or other water-structure-forming salts) *(12)*; isocratic elution (e.g., milk lactoferrin) and gradient elution of proteins are also possible. Under these conditions column dimensions (length vs diameter) may be much more important.

Monitor protein elution with any type of flow-through UV (280 nm) detector. Different types of peristaltic pumps may be required to deliver the appropriate buffer flow rate for the various column diameters used. The step-wise elution protocol described here does not require a fraction collector, although the use of a fraction collector may help eliminate dilution during sample recovery.

Dialysis (or diafiltration) of the immunoglobulin-depleted sample (e.g., bovine calf serum or milk) may be required if subsequent utilization is desired. This requires dialysis membranes or some type of diafiltration apparatus (e.g., Amicon, Beverly, MA). Alternatively, size-exclusion chromatography may be used. Isolated immunoglobulins can be recovered in various buffers at low salt concentrations (even water) so that dialysis is not required.

2.3. Thiophilic Adsorption and Elution Buffer

The thiophilic adsorption of proteins to the T-gel is a salt-promoted process at neutral pH. Protein desorption simply requires removal of the adsorption-promoting salt, also at neutral pH. The salts best suited for the promotion of thiophilic adsorption include combinations of anions and cations of the Hoffmeister series that are counter the chaotropic ions. Ammonium sulfate and potassium sulfate are excellent choices for work with immunoglobulins. The inclusion of sodium chloride (e.g., 0.5*M*) does not have a negative influence on either adsorption or desorption. We normally include sodium chloride to improve the solubility of the purified and concentrated immunoglobulins.

2.3.1. Sample Preparation and T-Gel Column Equilibration Buffer

Sample: Add solid ammonium sulfate to the sample to a final concentration of between 5% (e.g., milk) and 10% (e.g., serum or cell culture media) (w/v) and adjust the pH to 7.0 if neccssary (pH 7–8 is optimal). Filter (e.g., 0.45-μm Millipore HAWP filters) or centrifuge (e.g., 10,000*g* for 10 min) the sample to remove any insoluble material. Column equilibration buffer: 20 m*M* HEPES (pH 8.0), 0.5*M* NaCl, 10% (w/v) ammonium sulfate. Sodium phosphate (20–50 m*M*) or Tris-HCl (50 m*M*) may be substituted for the HEPES buffer.

2.3.2. Immunoglobulin Elution Buffer

Immunoglobulin elution buffer is the same as column equilibration buffer except that the ammonium sulfate is eliminated. A secondary elution buffer of 50% (v/v) ethylene glycol in 20 m*M* HEPES (pH 7–8) is sometimes useful to elute immunoglobulins of higher affinity.

2.3.3. T-Gel Column-Regeneration Buffers

The agarose- or silica-based T-gel columns may be regenerated and used (100–300 times) over extended periods. Wash the column with 30% isopropanol and water before reequilibration. We have washed columns with 6*M* guanidine HCl, 8*M* urea, and even 0.1% sodium dodecylsulfate (SDS) without noticeable changes in performance (capacity or selectivity). Wash the silica-based columns with 0.5*N* HCl (e.g., 1 h at room temperature). There should be no loss of performance.

2.4. Reagents and Material Required for Analysis of Immunoglobulin Content, Purity, and Class

Estimate the total protein in your starting samples and isolated fractions using the protein–dye binding assay of Bradford *(13)* or the bicinchoninic acid method as described by Smith et al. *(14)* and modified by Redinbaugh and Turley *(15)* for use with microtiter plates. The reagents for these assays are commercially available from BioRad (Richmond, CA) and Pierce (Rockford, IL), respectively (*see* Chapter 20).

Immunoglobulins are a heterogenous population of proteins. Even monoclonal antibodies can exhibit microheterogeneity. Thus, the methods and criteria used to verify immunoglobulin purity are more subjective than usual.

SDS polyacrylamide gel electrophoresis (*see* Chapter 20) is used frequently for this purpose *(16)*. The silver-staining method of Morrissey (17) works well to reveal impurities. An alternate method, Coomassie blue staining, is easier to use, but is much less sensitive. Because of the presence of disulfide–linked heavy and light chains immunoglobulin sample preparation (denaturation) in the presence and absence of reducing agents, such as 2-mercaptoethanol, will affect the electrophoretic profile significantly.

The quantitive determination of isolated immunoglobulin class and subtypes may be accomplished by the use of enzyme-linked immunosorbent assay (ELISA) methods. A variety of species- and class-specific antisera are available commercially. It is beyond the scope of this presentation to review these methods.

3. Methods

3.1. Synthesis of the Agarose-Based Thiophilic Adsorbent or T-Gel

Synthesize the agarose-based sulfone-thioether stationary phase or T-gel ligand,

$$\text{agarose–OCH}_2\,\text{CH(SO}_2\text{)CHCH}_2\,\text{SCH}_2\text{CH}_2\text{OH}$$

as follows:

1. Suspend suction-dried Sepharose 6B (1 kg/100 mL) in 0.5*M* sodium carbonate (pH 11.0) and incubate with divinyl sulfone (DVS) for 20 h at room temperature (21–24°C) on a rotary shaker.

2. Wash the divinylsulfone-crosslinked and activated gel exhaustively with water. After suction-drying on a sintered-glass funnel, suspend it in 1 L of 0.1*M* sodium bicarbonate (pH 9.0) with 100 mL of 2-mercaptoethanol and incubate overnight at room temperature on a rotary shaker.
3. Wash away excess 2-mercaptoethanol with distilled water and store the gel as a suction-dried or semidry material at 4°C (stable for several months).
4. Elemental (C, H, N, and S) analyses should be performed after each step in the T-gel synthesis (we use Galbraith Laboratories in Knoxville, TN) to estimate immobilized ligand density. The T-gel adsorbents used in most of the investigations cited here contained from 7.6 to 9.4% S and were calculated to have between 320 and 890 μmol of the sulfone-thioether ligand per g of dried gel. As an independent verification of terminal ligand density, test aliquots of the DVS-crosslinked agarose were reacted with ethanolamine instead of 2-meraptoethanol to produce "N-gel." The "N-gel" was evaluated for %N and calculated to contain the same terminal ligand density (e.g., 320 mol N vs 319 mol S/g dried gel). The agarose T-gel ligand density for the procedures described here was 750 μmol/g dried gel.

3.2. Synthesis of the Silica-Based Thiophilic Adsorbent or T-Gel

The silica-based thiophilic adsorbent or T-gel may be synthesized by the procedure described above for the agarose-based adsorbent. Alternatively, a modified synthetic route has recently been described by Nopper et al. *(8)*. This procedure, summarized below, produces the following thiophilic adsorbent:

silica–$OSiCH_2CH_2CH_2OCH_2CHOHCH_2SCH_2CH_2SO_2CH_2CH_2SCH_2CH_2OH$

1. Silanize the silica gel under anhydrous conditions according to the description of Larsson et al. *(18)*. Wash 20 g of LiChrospher™ silica gel with 20% HNO_3, water, 0.5*M* NaCl, water, acetone, and diethyl ether. Dry in a 500-mL 3-neck reaction flask for 4 h at 150°C under vacuum. Cool the flask and suck 300 mL of sodium-dried toluene into the flask. Add 5 ml of γ–glycidoxypropyltrimethoxysilane (Dow Corning Z 6040) and 0.1 mL triethanolamine to the reaction mixture, stir (overhead), and reflux for 16 h under a slow stream of dry (H_2SO_4) nitrogen gas to maintain anhydrous conditions.
2. Wash the silanized epoxy-silica gel sequentially with toluene, acetone, and diethyl ether. Dry the washed silica gel under vacuum.

3. The number of epoxide groups introduced may be assayed by titration according to the method of Axen et al. (19). This step is optional.
4. Cleave the epoxide groups with sodium hydrosulfide (tenfold molar excess) in 0.2*M* Tris-HCl buffer (pH 8.5) for 1 h at room temperature.
5. Wash the hydrosulfide modified silica gel with water, methanol, acetone, and diethyl ether.
6. Assay the number of thiol groups introduced into the gel by the Ellman DTNB method *(20)*. This step is optional.
7. Stir the gel with divinyl sulfone (1.5 mL/g gel) in 0.2*M* Tris-HCl buffer, pH 8.8, for 1 h at room temperature.
8. Wash the DVS-activated gel an a sintered-glass funnel with water, acetone, and diethyl ether. Dry under vacuum.
9. The quantity of double bonds available for reaction with 2-mercaptoethanol (next step) can be estimated after reaction of a portion of the activated gel with dithiothreitol followed by the assay of thiol groups by the method of Ellman *(20)*.
10. Incubate the DVS-activated silica gel with excess 2-mercaptoethanol in 0.2*M* Tris-HCl, pH 8.5.
11. Finally, wash the thiophilic silica gel product with water, acetone, and diethyl ether, and dry under vacuum.

The ligand density of this product should approach 800 mol/g dry silica.

3.3. Column Packing and Equilibration

Suspend T-gel in column-equilibration buffer (*see* Section 2.3.1.), pour into a column of desired dimensions, adjust the flow rate to maximum (<60 cm/h), and equilibrate with column-equilibration buffer (monitor elution pH and conductivity). High-performance (i.e., HPLC) stationary phases (i.e., silica-based) for thiophilic adsorption should be packed into columns specifically designed for HPLC applications. Use packing pressures recommended by the manufacturer or supplier of the silica particles.

3.4. Preparation of Sample for Thiophilic Adsorption

Add solid ammonium sulfate to the sample (up to 10–12% w/v) and adjust the pH to 7.0 if necessary (pH 7–8 is optimal). The final concentration of ammonium sulfate in the sample varies with (1) sample type (e.g., 5–8% ammonium sulfate for colostrum or milk; 10–12% ammonium sulfate for bovine or human serum, ascites fluid or