Thiophilic adsorption – a new method for protein fractionation

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Divinylsulphone-activated agarose to which mercaptoethanol is coupled showed very selective group adsorption of human serum proteins, in particular the immunoglobulins The adsorption increases markedly in the presence of high concentrations of neutral water-structure forming salts and is distinct from adsorptions based on hydrophobic interaction A characteristic feature of this new type of adsorbent is the structure of the groups attached to the polymer, Ⓟ, i e , R-S-CH_2-CH_2-SO_2-CH_2-CH_2-O-Ⓟ, where R is a small aliphatic residue Our results indicate that the thioether sulphur and the adjacent sulphone group act cooperatively and are apparently necessary to maintain the distinct behaviour of such absorbents

Divinylsulfone *Immunoglobulin* *Affinity chromatography* *Hydrophobic interaction (salting-out)* *Serum fractionation*

1 INTRODUCTION

Divinylsulphone (DVS) is a versatile reagent for coupling ligands for affinity chromatography [1] and for cross-linking agarose [2]. After coupling, residual vinyl groups are routinely blocked by coupling some inert ligand of low M_r One such ligand we used was mercaptoethanol. But we observed that the control gel to which only mercaptoethanol has been coupled showed very distinct group adsorption properties toward specific serum proteins, in particular the immunoglobins and α_2-macroglobulins Further investigations revealed that the adsorption obtained is salt dependent and increases significantly in the presence of high concentrations of neutral salts This salting-out chromatography is reminiscent of one of the conditions set forth for hydrophobic interaction chromatography [3] and, therefore, a comparative study using a commercially available hydrophobic adsorbent was made. Our results indicate that the adsorption characteristics of the new adsorbent are distinct from those based on hydrophobic interaction.

The general structure of the new type of adsorbent can be represented as· Ⓟ -O-CH_2 - CH_2-SO_2-CH_2-CH_2-S-R- where Ⓟ is the polymer matrix and R is a small aliphatic residue (R-S- or analogous group will be referred to as ligand) A series of adsorbents were prepared using structurally similar ligands and their adsorption characteristics were investigated. The results obtained, which form the basis for this report, are consistent with our hypothesis that the sulphur in the form of a thioether and the adjacent sulphone group act cooperatively to bind specific types of proteins We have thus tentatively called this hitherto unknown type of interaction 'thiophilic' and its chromatographic applications 'thiophilic interaction chromatography' (TIC). Here, the synthesis and adsorption behaviour toward human serum proteins of our new series of thiophilic adsorbents is presented.

2. EXPERIMENTAL

The thiophilic adsorbent (T-gel) was synthesized as follows: the polymer matrix (6% agarose gel in

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the form of spherical beads) was activated essentially as described [4] except that the amount of DVS used was reduced by 50% and the coupling time was extended to 18 h. To 700 g of the activated gel in a 3 l round-bottomed reaction vessel was added 700 ml of 0.1 M $NaHCO_3$ solution (pH 9) and 70 ml mercaptoethanol. The suspension was stirred gently for 20 h at room temperature and then washed thoroughly with distilled water until the washings were neutral. The ligand concentration was estimated at around 910 $\mu mol \cdot g^{-1}$ dry gel calculated from elemental S analysis of the gel before and after coupling with mercaptoethanol.

The synthesis of the amino-substituted agarose (A-gel) was identical to that of the T-gel except that ethanolamine was used instead of mercaptoethanol to form the ligand. The degree of substitution was estimated at around 920 $\mu mol \cdot g^{-1}$ dry gel (1.28% $N \cdot g^{-1}$ dry gel). The hydrophobic adsorbent (H-gel) is the commercially available octyl-Sepharose CL-4B produced by Pharmacia, Uppsala.

To illustrate the adsorption behaviour of the T-gel and compare it with a hydrophobic gel (H-gel) the chromatographic arrangement shown in fig.1 was set up. Details related to the chromatographic experiments are outlined in the legend to fig.1.

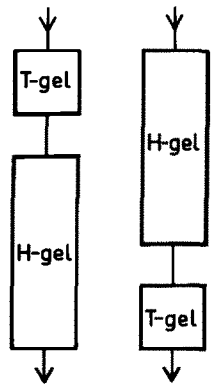

Fig 1 Arrangement of the tandem T-gel/H-gel columns for the chromatographic experiments reported here The total bed volume of the T-gel was 8 ml and of the H-gel 23 ml The tandem column was equilibrated with 0.1 M Tris-HCl buffer, pH 7 6 (buffer A), containing 0 5 M K_2SO_4 Normal human serum was exhaustively dialysed against this equilibrating buffer and 4 8 ml (≙ 350 mg protein) was applied to the column at a flow rate of 16 ml cm⁻² h⁻¹ Elution was continued with the equilibrating buffer until all non-adsorbed material had been eluted The column was then dismantled and the adsorbed material from each section was desorbed by stepwise elution with (1) buffer A, (2) ethylene glycol (40%, v/v) in buffer A, and (3) isopropanol (30%, v/v) in buffer A The eluted fractions from the T-gel (T, T_E and T_p) and from the H-gel (H, H_E and H_p) were concentrated and analysed by gradient gel electrophoresis

3. RESULTS AND DISCUSSION

The results obtained after chromatography of human serum on a tandem column of T-gel and H-gel are shown in table 1 and fig 2. The group separation of serum proteins on the tandem column is distinct and reproducible Irrespective of the arrangement of the tandem column, the T-gel invariably adsorbed IgG, IgM and IgA (table 1) including some other minor serum components TIC is thus an effective adsorbent for serum immunoglobulins. Contaminating α_2-macroglobulin and transferrin can subsequently be removed easily by IMA chromatography [5].

The hydrophobic gel adsorbs mainly albumin including several other serum components as shown in table 1 and fig 2. It also adsorbs α_2-macroglobulin when it is the first column in the tandem arrangement (fig 1) The results indicate that adsorption of proteins to the T-gel is not due to hydrophobic interaction although high concentrations of structure-forming salts are necessary for efficient adsorption in both cases.

A similar set of experiments to the above were performed using a T-gel/A-gel tandem column. The results showed that about 31% of the serum proteins (identical in composition and pattern to those shown in table 1 and fig 2, respectively) were adsorbed on the T-gel and less than 1% on the A-gel. Ion-exchange effects are excluded in view of the high salt concentration used in the adsorption step. It is thus apparent that the thioether sulphur is essential for the characteristic adsorption properties of the T-gel.

In another series of experiments, rat serum was chromatographed on the T-gel alone to test its specificity. The rat serum was pre-fractionated on an Ni-TED Sepharose column mainly to remove α_2-macroglobulin, hemopexin and transferrin [5] About 20% of the applied material was adsorbed

Table 1

Analysis of the fractions obtained after adsorption of serum on a T-gel/H-gel tandem column

Pooled fractions	Recovery (%)[a]		Main components identified
	Whole serum	Delipidized serum	
T-gel			
T[b]	19 7	22 7	IgG + α_2-macroglobulin (>95% of the T-fraction), IgM, IgA
T_E[c]	6 0	8.9	
T_P[d]	3 2	<0 2	
H-gel			
H[b]	7 2	9	albumin + haptoglobin
H_E[c]	7 9	15 5	
H_P[d]	11 5	5 4	
Unadsorbed material from the tandem column	19 7	20	α_1-antitrypsin, haptoglobin, orosomucoid, albumin
Total recovery (%)	75 2	81 5	

[a] Estimated from absorbance measurements at 280 nm The total recovery is low due to incomplete desorption from the H-gel by the eluents used. As indicated earlier (see text) total recovery from the T-gel alone is well over 90% The relatively higher recovery obtained when delipidized serum was used indicates that the lipids in serum are strongly bound to the tandem column They can be desorbed from both gels by elution with 95% ethanol

[b] Material eluted by buffer A

[c] Material eluted by buffer A and 40% (v/v) ethylene glycol

[d] Material eluted by buffer A and 30% (v/v) isopropanol

Reversal of the tandem column to a H-gel/T-gel gave almost identical results The main components in the various fractions were identified and quantitatively determined by immunoelectrophoresis

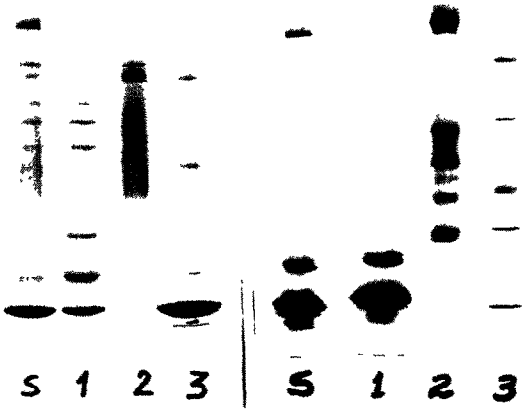

Fig 2 Gradient gel electrophoretograms of human serum (left) and rat serum (right) fractions obtained after thiophilic interaction chromatography performed as outlined in the legend to fig 1 In each case, a 4–30% polyacrylamide gradient gel (PAA 4/30 obtained from Pharmacia, Uppsala, Sweden) was used The duration of electrophoresis was 16 h at 20°C (Left) S, unfractionated human serum 1, the fraction that passed the tandem T-gel/H-gel unadsorbed; 2, adsorbed material on the T-gel that was eluted by buffer A, 3, adsorbed material on the H-gel that was eluted by buffer A. (Right) Fractions obtained from chromatography of rat serum on T-gel S, starting sample of rat serum that was prefractionated on an Ni^{2+}-TED-agarose adsorbent [5] 1, material that passed the T-gel unretarded, 2, adsorbed material that was eluted from T-gel by buffer A; 3, standard calibration proteins (top to bottom thyroglobulin, 669 kDa, ferritin, 440 kDa; catalase, 232 kDa, lactate dehydrogenase, 140 kDa, albumin, 67 kDa)

whose gel electrophoretic pattern is shown in fig.2. All the adsorbed proteins have apparent M_r values in excess of 140000. The results indicate that the same types of proteins are adsorbed by the T-gel from the rat and human serum and that no detectable amounts of albumin are adsorbed by the T-gel.

A variety of ligands other than mercaptoethanol have also been coupled to the same batch of DVS-activated agarose gel and their adsorption capacity towards human serum proteins was determined as in the previous cases. The ligand concentration was not determined but the same molar excess of reagents was used during coupling. The results obtained are summarized in table 2 The results indicate the necessity of the thioether sulphur as part of the structure of a thiophilic adsorbent, as pointed out above Despite small variations in the adsorption capacity of the various thiol-containing ligands, they showed similar specificities towards the serum proteins they adsorbed.

Table 2

Comparison of the adsorption capacities of different ligands coupled to DVS-activated gels

Ligand	Amount applied	Total amount adsorbed (%)
-S-CH_2-CH_2-OH	9	36
-S-CH_2-CH(OH)-CH_2-OH	7	32
S-CH_2-CH(SH)-CH_2-OH	10	25
-S-CH_2-CH(OH)-CH(OH)-CH_2-SH	10	25
-S-CH_2-CH_2-SH	11	25
-NH-CH_2-CH_2-OH	6	0
-NH-CH_2-COOH	8	10
-OH	10	0

The general structure of the adsorbent is. Ⓟ-O-CH_2-CH_2-SO_2-CH_2-CH_2-R where the ligand R is a small aliphatic group For experimental details cf legend to fig.1. The amount of applied material refers to the total A_{280} of the dialysed human serum taken in each case The total recovery of applied material is about 90% or higher

Since agarose gels cross-linked by DVS withstand very high flow rates [2], the T-gel is very well suited for large-scale purification of immunoglobulins from serum and applications in the HPLC field. We have further found that the characteristic adsorption properties of the T-gel are more or less preserved when the sulphone group is replaced by sulphoxide or even sulphur. Selenium could also be exchanged for the sulphur but with a resulting marked decrease in adsorption capacity

Thiophilic adsorbents have also been produced successfully using matrices other than agarose, e.g., polyacrylamide (Eupergit C) and silica gels, thus extending the usefulness of the T-gel for various other applications.

The reorganization of water molecules around the interaction sites apparently plays an important role, since antichaotropic salts such as K_2SO_4 significantly influence the extent and strength of the adsorption, as is the case in hydrophobic interaction However, the adsorption capacity of the T-gel for proteins was found to decrease with increasing temperature. Also, different chaotropic salts affect differently the adsorption behaviour of serum proteins on the T-gel and H-gel. These findings thus exclude possible hydrophobic interactions as a major principle underlying the observed phenomenon and give further support to the concept of a different mechanism to account for adsorptions due to thiophilic interaction.

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