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Thiophilic nature of divinylsulphone cross-linked agarose

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Abstract

We present data to suggest that divinylsulphone-activated matrix (DVS-gel) purifies immunoglobulins with the same protein–ligand interaction as the thiophilic ligand, T-gel. Divinylsulphone-activated matrix purifies IgG (>90% pure) from blood serum with a binding capacity of 6.3 mg/ml, compared to 6.9 mg/ml with T-gel. It also separates IgG and sIgA from cheese whey, with the same dependency on water-structuring salt concentration as T-gel. We offer an explanation for these results in terms of the polymerisation of divinylsulphone and, therefore, offer a new structure for the so-called thiophilic ligand.

Keywords: Thiophilic interactions; Stationary phases, LC; Agarose; Immunoglobulins; Divinylsulfone

1. Introduction

Porath et al. [1,2] have described a class of chromatography ligand as 'thiophilic'. The currently proposed, general structure of a thiophilic ligand is: P–O–$CH_2CH_2SO_2CH_2CH_2$–R, where P=solid-phase polymer and R, the end ligand, is either a thioether, secondary amine or ether linkage with an electron donating group attached [3]. The ligand structure is derived from the reaction of divinylsulphone (DVS) with polymer, and subsequently the reaction of a hydroxyl, primary amine or sulphydryl containing compound with the DVS-activated polymer. Thiophilic chromatography has been shown to purify immunoglobulins directly from blood serum [1], ascites fluid [4], tissue culture supernatant [5], colostral whey [6] and cheese whey [7,8]. Thiophilic chromatography binds immunoglobulins and other proteins [9,10] in the presence of water-structuring salts like $(NH_4)_2SO_4$ and K_2SO_4 . The proteins are eluted using buffers containing NaCl, thereby distinguishing it from hydrophobic interaction chromatography (HIC). The binding interaction is not sensitive to temperature [11], again unlike HIC.

It is not known how the ligand interacts with immunoglobulins, but Porath and Belew [2] have postulated that the sulphur of the sulphone group accepts electrons from the π-orbitals of aromatic amino acids, whereas the thioether (or ether or secondary amine) electrons interact with the edge of the aromatic ring. The commercially available T-gel ligand (DVS/2-mercaptoethanol) is called the 2S thiophilic structure. Further derivatives of the 2S ligand have been made, 3S up to 6S, by additions of one other sulphone group and/or further thioether groups [5,12]. Higher binding affinities for IgG and also for single-chain antibody fragments [13] are claimed for some of these longer ligands.

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Divinylsulphone is a well known cross-linking and ligand immobilisation agent [14] for agarose gels. The cross-linking of cotton fibres by divinylsulphone had been studied and the chemical nature of some cross-links determined, after acid hydrolysis, to be an ether linkage joining two hydroxyethylsulphone monomers [15]. Such poly(hydroxyethylsulphone) structures may also be present in agarose-based gels. According to the proposed mechanism of thiophilic binding this vinylsulphone derived structure should purify immunoglobulins without the need for an end ligand of the type described above. We therefore conducted a series of experiments to test the requirement of the thiophilic end ligand for immunoglobulin binding and purification.

2. Materials and methods

Defatted, pasteurised cheddar cheese whey was supplied by Appleby Cheese (Appleby-in-Westmoreland, UK). Human polyclonal IgG (hIgG) and sheep blood serum was kindly supplied by the Scottish National Blood Transfusion Service (Edinburgh, UK) and Affiniti Research Products (Exeter, UK) respectively. PROSEP-THIOSORB was a gift from Bioprocessing (Co. Durham, UK). Octyl-Sepaharose was obtained from Pharmacia Biotechnology (Milton Keynes, UK).

Divinylsulphone (DVS), L-tryptophan, 2-hydroxypyridine, 8-hydroxyquinoline, pyridine and 2-mercaptoethanol were purchased from Sigma Chemical (Dorset, UK). Ammonium sulphate, sodium chloride, ethanol, sodium thiosulphate and sodium carbonate were purchased from BDH Chemicals (Dorset, UK).

2.1. Ligand manufacturing protocols

Ten grams of suction-dried 4B-CL agarose (Pharmacia Biotechnology) was added to 5 ml 50 m*M* sodium carbonate buffer pH 10.5–11.5. To this 1 ml of DVS was immediately added and shaken overnight at room temperature. The DVS-activated matrix (DVS-gel) was then washed with 500 ml distilled water. Ten grams of DVS-gel was added to 5 ml carbonate buffer pH 10.5–11.5 containing either 1 ml 2-mercaptoethanol, 1 ml pyridine, 0.5 g 8-hydroxyquinoline, 0.5–1.0 g 2-hydroxypyridine, or 0.5–1.0 g L-tryptophan and shaken overnight at room temperature. The thiophilic gel was then washed thoroughly as before. DVS/thiosulphate was produced by reacting an equal volume of DVS-gel with 3 *M* sodium thiosulphate for 48 h. The quantity of sodium hydroxide produced was used to determine the end ligand concentration.

2.2. Ligand analysis

S,C,H,N chemical analysis was performed by Medac (Brunel University, Middlesex, UK). Samples sent for S,C,H,N analysis were washed consecutively with 15% ethanol, 50% ethanol, distilled water, 1 *M* NaCl, distilled water and dried at 100–120°C overnight. Under these conditions 1 g of dried matrix is equal to 11.7 ml of matrix in solution. DVS-gel was washed only with distilled water before being dried for ligand analysis.

2.3. Human IgG binding isotherms

Equilibrium binding isotherms were constructed in a manner similar to the recycling partition equilibrium techniques [15–17], by recirculating the human IgG solution through 1–2 ml columns until equilibrium had been established. The quantity of protein bound was determined by the decrease in absorbance at 280 nm of the recirculating solution. Once equilibrium was reached further protein was added to the solution and recirculation continued until a new equilibrium point was established. All gels were washed with 0.8 *M* $(NH_4)_2SO_4$ and the protein eluted with distilled water.

2.4. Cheese whey separations

Cheese whey was adjusted to pH 6.8 with sodium hydroxide and solid $(NH_4)_2SO_4$ added to a final concentration of 0.8 *M*. Twenty bed volumes (bv) of cheese whey were applied to 1–2 ml columns of gel which were subsequently washed with 4 bv of 0.8 *M* $(NH_4)_2SO_4$. Immunoglobulins were eluted with distilled water and quantified by the Lowry [18] and Bradford [19] total protein assays and radial immunodiffusion assay against bovine IgG and bovine IgA (The Binding Site, Birmingham, UK). High-

performance filtration chromatography (HPFC) was performed on a BioSep-Sec-S2000 300×7.8 mm column (Phenomenex, Cheshire, UK), run isocratically with 50 m*M* sodium phosphate pH 6.8 at 0.5 ml/min and loading 10-μl samples per analysis. The eluate was monitored at 280 nm.

During the temperature dependence experiments, cheese whey separations were performed in a 4°C refrigerator, at room temperature, and in a 37°C, 42°C and a 56°C incubator.

2.5. Blood plasma separation

Blood serum was diluted with an equal volume of 1.6 *M* $(NH_4)_2SO_4$ and a 2-ml sample was applied to 2-ml columns of DVS-gel and T-gel, followed by washing with 10 ml 0.8 *M* $(NH_4)_2SO_4$. Distilled water was used to elute protein from the columns.

3. Results

3.1. Langmuir binding isotherms

Six chromatographic gels were produced. These were DVS-gel (7.1% S, <0.1% N), DVS/2-mercaptoethanol (10% S), DVS/L-tryptophan (1.68% N), DVS/thiosulphate, DVS/pyridine (0.18% N) and DVS/2-hydroxypyridine (1.15% N).

Four gels were analysed for the Langmuir binding isotherm they produced with human polyclonal IgG in 0.8 *M* $(NH_4)_2SO_4$. The results presented in Fig. 1 agree with previously published isotherm data for DVS/2-mercaptoethanol (T-gel) [11]. T-gel produced a binding capacity of 43 mg/ml with a dissociation constant of 0.49 μM. DVS-gel gave a lower dissociation constant of 0.29 μM and a binding capacity of 38 mg/ml. 4B-CL agarose and Octyl-Sepharose act as controls for non-specific binding and hydrophobic interactions, respectively. Octyl-Sepharose possessed one fifth of the binding capacity of the thiophilic gels under the same binding conditions. The recovery of hIgG from T-gel and DVS-gel was consistently 70–80% of the protein applied. Freshly prepared DVS-gel was stored in 0.8 *M* $(NH_4)_2SO_4$ for 48 h to determine their reactivity. Chemical analysis of the DVS-gel showed the nitrogen content to be <0.1%. The absence or low level of amine groups in DVS-gel stored in $(NH_4)_2SO_4$, procludes their involvement in hIgG binding.

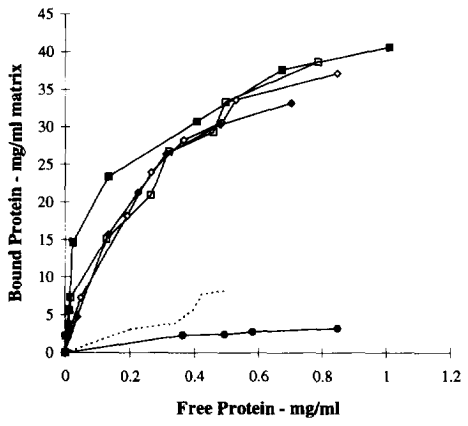

Fig. 1. Human polyclonal IgG binding isotherms for DVS/2-mercaptoethanol (◇), DVS/thiosulphate (□), DVS/tryptophan (◇), DVS-gel (■), Octyl-Sepharose (- - -) and underivatised 4B-CL Sepharose (●).

3.2. Purification of IgG/sIgA from cheese whey

Cheese whey was applied to columns of prepared thiophilic matrices, DVS-gel and underivatised 4B-CL agarose. The column eluates were analysed by both the Lowry and the Bradford total protein assays and radial immunodiffusion assay (RID). The immunoglobulin purity of the eluates was estimated by

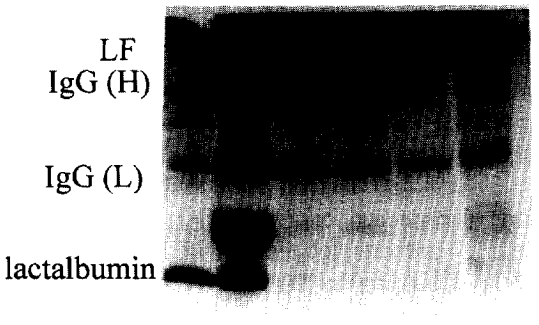

Fig. 2. The purification of immunoglobulins from cheese whey. 15% SDS-PAGE run under reducing conditions and stained with Coomassie Brilliant Blue R-250: (from left to right) lane 1, marker proteins (top to bottom), lactoferin (75 Kd), IgG heavy chain (55 Kd), IgG light chain (30 Kd) and α-lactalbumin (14–15 Kd); lane 2, cheese whey; lane 3, DVS-gel eluate; lane 4, T-gel eluate; lane 5, DVS/thiosulphate eluate; lane 6, DVS/2-hydroxypyridine gel eluate.

Table 1
The binding capacities of thiophilic ligands for IgG and sIgA containing different end ligands

Thiophilic end ligand	End ligand density (μmol/ml)	Total protein capacity (mg/ml)		Ig capacity (RID) (mg/ml)		Total Ig purity (% protein)
		Lowry	Bradford	IgG	IgA	
Tryptophan	50	3	3.5	1.1	0.15	36
Hydroxypyridine	69	4.5	4.8	2.6	0.3	61
Hydroxyquinoline	10	3.35	3	2	0.2	73
Pyridine	10.8	3.3	2.9	2	0.25	77
Thiosulphate	15	2.9	2.6	2.1	0.22	86
2-Mercaptoethanol	76	4	4.3	2.5	0.3	66
Controls						
DVS activated 4B-CL	None	2.8	2.5	1.5	0.2	68
4B-CL		0.1	0.3	0	0	0

A measure of the purity of immunoglobulin obtained is calculated from the immunoglobulin content (RID assay) as a proportion of the total protein content (Bradford).

SDS-PAGE (Fig. 2). From Table 1, DVS/2-hydroxypyridine possessed the highest capacity for IgG/IgA: 2.9 mg/ml. DVS-gel had a lower binding capacity of 1.7 mg/ml but yielded an immunoglobulin product of similar purity to T-gel.

From these results it can be seen that DVS-gel purifies immunoglobulins from cheese whey. The capacity of DVS-gel towards immunoglobulins is increased by 27–70% if end ligands are attached to the DVS-gel. The only exception was DVS/L-tryptophan which produced a low-purity product probably due to interactions of the tryptophan end ligand with other whey proteins.

A characteristic of the thiophilic interaction is the requirement for water-structuring salts such as $(NH_4)_2SO_4$ [2]. DVS-gel and T-gel were used to bind immunoglobulins from 6 bv of cheese whey containing varying concentrations of ammonium sulphate. The eluted products were analysed for total protein and IgG content. T-gel and DVS-gel had the same dependence on $(NH_4)_2SO_4$ concentration in binding immunoglobulins, with overall reduced levels of binding by DVS-gel (Fig. 3). The sudden decrease in purity of IgG eluted from the 1.0 *M* $(NH_4)_2SO_4$ cheese whey sample was due to competition from other whey proteins; analysis of eluates from each separation by SDS-PAGE and HPFC showed a marked increase in the quantity of β-lactoglobulin, α-lactalbumin and bovine serum albumin binding to the columns at this salt concentration.

DVS-gel gave no increase in binding capacity with

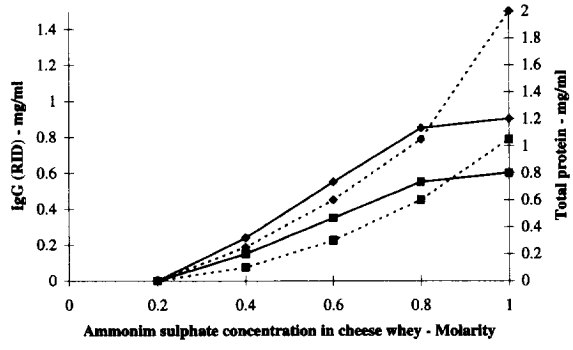

Fig. 3. Comparison between DVS-gel (■) and T-gel (◇) in their immunoglobulin separations from cheese whey containing increasing concentrations of $(NH_4)_2SO_4$. Six bv of cheese whey sample were applied to the columns. The solid lines represent the IgG capacity, as determined by RID. The dotted lines represent the total protein capacity (Lowry).

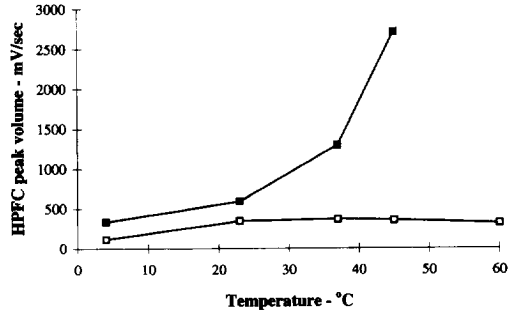

Fig. 4. Effect of increased temperature on the quantity of immunoglobulins purified from cheese whey by DVS-gel (□) and on α-lactalbumin purified by Octyl-Sepharose (■), as analysed by HPFC on column eluates.

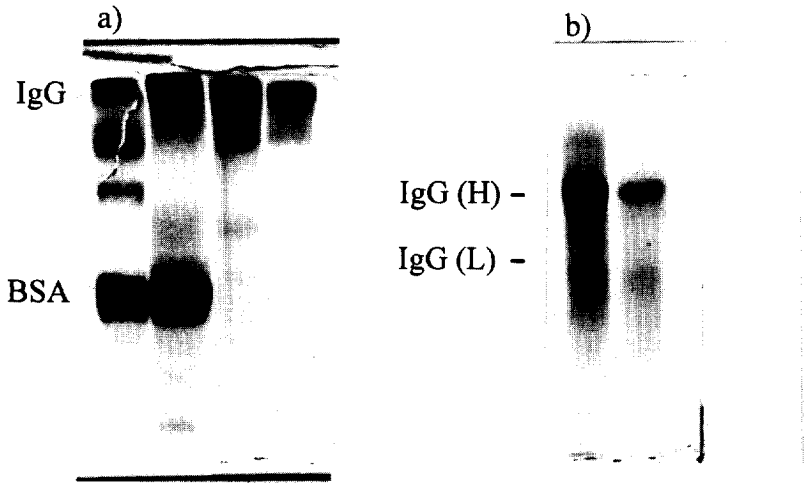

Fig. 5. (a) The purification of IgG from blood plasma. 10% SDS-PAGE run under non-reducing conditions (from left to right): lane 1, blood serum; lane 2, BSA and human IgG markers; lane 3, T-gel eluate; lane 4, DVS-gel eluate. (b) 15% SDS-PAGE run under reducing conditions comparing the eluates of DVS-gel and T-gel (from left to right): lane 1, T-gel eluate; lane 2, DVS-gel eluate.

increase in temperature (Fig. 4). Under identical conditions, Octyl-Sepharose was shown by HPFC to bind only α-lactalbumin from cheese whey and to have increased binding capacity with increased temperature. This shows the IgG binding interaction of DVS-gel is not entropy driven as would be expected with hydrophobic interactions and is consistent with previous results with T-gel [11].

3.3. Purification of IgG from blood serum

DVS-gel and DVS/2-mercaptoethanol (T-gel) were used to purify immunoglobulins from blood plasma. DVS-gel eluted 6.3 mg protein/ml matrix, whereas T-gel eluted 6.9 mg protein/ml matrix. Each eluate was shown by SDS-PAGE to contain one major band corresponding to IgG (Fig. 5). A commercial thiophilic matrix (PROSEP-THIOSORB) gave comparable results (7 mg/ml matrix capacity), under identical conditions. DVS-gel therefore performed an immunoglobulin separation from blood plasma with the same purification factor and recovery of product as T-gel under identical conditions.

4. Discussion

Immunoglobulin binding studies suggest that DVS-gel could bind pure IgG with a greater affinity and similar capacity to T-gel. Additional end ligands were attached to DVS-gel and analysed for IgG binding. All showed a similar Langmuir binding interaction to T-gel, irrespective of the chemical nature of the end ligand.

DVS-gel purified IgG from blood plasma with the same quantity of product recovered as T-gel and with the same purity. This illustrates the non-covalent interaction and specific binding nature of the DVS-gel for immunoglobulins. Evidence of the thiophilic nature of DVS-gel was obtained in the cheese whey studies. Firstly, DVS-gel not only purified IgG but also sIgA, exactly as performed by T-gel in previous studies [4]. Secondly, DVS-gel showed the same dependence on water structuring salts as T-gel for immunoglobulin binding. It is also notable that both gels had the same optimum $(NH_4)_2SO_4$ concentration for IgG purification namely 0.8 *M*. Thirdly, the binding of immunoglobulins by DVS-gel was also shown to be independent of temperature, as T-gel.

During studies conducted to identify the nature of cross-links formed by divinylsulphone in textile fibres [20], it was shown that divinylsulphone can form dimers connected by an ether linkage. We suggest that similar linkages occur during thiophilic ligand formation and that the following ligand structure would be produced:

$$P-O-CH_2CH_2SO_2CH_2CH_2-O-$$
$$CH_2CH_2SO_2CH_2CH_2-OH$$

where P = solid-phase polymer.

This ligand structure would explain why DVS-gel falls into the class of ligands described as thiophilic. Even without the end ligand, the sulphone and adjacent ether is present forming the proposed thiophilic site. There is much evidence to conclude that the nature of the thiophilic end ligand can affect the capacity of an immunoglobulin separation [3]. This is supported by our studies using cheese whey. The increase in binding capacity by the addition of an end ligand in the cheese whey studies, could be due to either a contribution to the thiophilic interaction by the end ligand or to an increase in the accessibility of the DVS dimer by acting as an end cap. The thiophilic effect may then depend on the presence of two sulphone groups, rather than one sulphone and one ether or thioether. It is also possible that the free vinyl groups of DVS-gel covalently bind protein which itself acts as a thiophilic ligand.

In conclusion, the immunoglobulin binding region of thiophilic matrices is found in the divinylsulphone derived portion of the ligand. Further experiments will now be carried out to determine whether the thiophilic effect is found exclusively within this region of the ligand and whether any protein–protein interactions in the DVS-gel account for immunoglobulin binding.

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