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Salt-independent adsorption of human serum proteins on cyanocarbon gels[1]

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Abstract

Electron donor acceptor gels based on cyanocarbons have been tested for human serum protein adsorption in the absence of salt-promotion by water-structuring salt. This phenomenon was compared with a normal adsorption process in the presence of salt. The tricyanoaminopropene–divinyl sulfone–agarose displayed unusual protein adsorption properties as binding could occur both independently or dependently of the salt-promotion. The absence of hydrophobic or ionic character of the salt-independent interaction suggests an electron donor acceptor adsorption mechanism which is shown, for the first time, to occur independently of salt-promotion in aqueous solution. Study of the protein adsorption specificity showed similar protein selectivity for the fractions adsorbed in both conditions.

Keywords: Electron donor acceptor gels; Adsorption chromatography, salt-promoted; Proteins

1. Introduction

In 1990, Porath coined the term salt-promoted adsorption chromatography (SPAC) to regroup the chromatographic techniques which require a high salt concentration for protein adsorption to occur [1]. Indeed hydrophobic interaction chromatography (HIC), thiophilic adsorption chromatography (TAC) and electron donor acceptor chromatography need a high concentration of water-structuring salts to promote the adsorption of proteins to the gel. Although the underlying molecular recognition events are supposed to differ, in each case the salt effect on protein adsorption is explained as resulting from the unfavourable increase of the free energy for unbound proteins in the presence of high concentrations of salt. Protein elution from the matrix is, therefore, simply achieved by deleting the salt from the adsorption buffer.

Recently, we applied the effective electron withdrawing capacity of the $-C\equiv N$ group to potentiate the electron acceptor properties of unsaturated alkenes for increasing protein adsorption in electron donor acceptor chromatography [2]. In this study, human serum proteins were adsorbed to various extents on the cyanocarbon–divinyl sulfone (DVS)–

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agarose gels when salt-promoted adsorption was used. Among the different gels tested, the tricyanoaminopropene coupled with DVS to agarose (TCP gel) was found to be the most potent protein adsorbent also displaying a selectivity for immunoglobulins. Except for HIC, where proteins often are adsorbed irreversibly [3], the total protein recovery in SPAC is usually over 95% of the injected proteins. As expected, most of the proteins are eluted by deleting the salt and only a few per cent of the proteins further require an amphophile such as ethylene glycol or the addition of a denaturing agent to be removed from the matrix. We observed for the TCP gel that a surprisingly large amount of proteins (10%) remained bound to the gel after deleting the salts. The kind of interaction involved remained unclear to us, as neither ionic nor hydrophobic interactions were likely to be involved. With this background, we decided to further study if an alternate mechanism of interaction also may be involved in the affinity of proteins for the TCP gel.

2. Experimental

2.1. Reagent and chemicals

Sepharose 6B was obtained from Pharmacia (Uppsala, Sweden). 2-Amino-1,1,3-tricyanopropene (TCP) was purchased from Fluka (Buchs, Switzerland) and propylamine, aminocrotonitrile (ACN), diaminomaleonitrile (DAMN) and DVS were from Aldrich (Milwaukee, WI, USA). All other reagents were of analytical grade. Normal human serum was supplied by the Blood Bank at the University Hospital (Uppsala, Sweden).

2.2. Gel synthesis

The ligand reagents propylamine, ACN, DAMN and TCP were coupled to Sepharose 6B via a spacer arm generated by DVS as follows. The agarose was equilibrated with 0.2 *M* sodium carbonate (pH 11). Fifty grams of suction-drained agarose were added to 50 ml of a mixture of DVS 15% v/v (2.2% v/v for activation of the TCP gel) and 0.2 *M* sodium carbonate (pH 11). After shaking for 4 h at room temperature, the slurry was washed with deionized water on a sintered-glass filter until the filtrate was neutral. In the second step, 50 g of DVS-activated-agarose was equilibrated with 0.2 *M* sodium hydrogen carbonate (pH 10), suction-drained and added to 50 ml of a mixture of 0.2 *M* sodium hydrogen carbonate (pH 10) and 0.1 *M* of the desired ligand. The pH of the mixture was adjusted to pH 10. After shaking for 24 h at room temperature, the slurry was transferred to a glass filter and washed with water, dimethyl sulfoxide and again with water. Three different batches of the TCP gels were found to contain 453, 572 and 671 μmol of ligand/g of dry weight as determined by nitrogen elementary analysis.

2.3. Chromatographic conditions

For chromatography studies, a 7.4×0.5 cm I.D. column was packed with the test gel. A program for equilibration, adsorption, desorption and regeneration of the adsorbent was executed by means of a programmable FPLC system from Pharmacia. Solid salt (sodium sulphate or sodium chloride) was added to the serum sample to the desired final concentration. The gel was equilibrated at a flow-rate of 60 ml/h with the adsorption buffer: 50 m*M* Mops–acetate buffer (pH 7.6), containing the studied salt at the desired concentration. A 0.5 ml sample containing approximately 68 mg protein/ml of centrifuged (3000 *g*, 5 min) human serum was applied to the column and the gel was washed for 40 min with the adsorption buffer. The adsorbed material was first eluted by deleting the salt from the adsorption buffer (50 m*M* Mops–acetate buffer, pH 7.6) and then by adding 1 *M* NaCl in 50 m*M* Mops–acetate buffer, pH 7.6. The gel was finally rinsed for 30 min with 50 m*M* Mops–acetate buffer (pH 7.6) containing 6 *M* urea as a final cleaning step. The eluate from one step was recovered as one fraction.

2.4. Analytical

The protein content of the chromatographic fractions was measured using the micro-Bradford assay with bovine serum albumin as a standard. Nitrogen and sulphur analyses of the coupled gels were performed by Microkemi (Uppsala, Sweden).

2.5. Two-dimensional electrophoresis

Two-dimensional electrophoretic analysis was performed on eluted fractions presenting equivalent percentage of desorbed proteins (9–10%). Before electrophoresis, the chromatographic samples were dialysed against 5 m*M* Mops buffer, pH 7.6, freeze dried, and solubilized in 50 m*M* Mops buffer, pH 7.6. Electrophoresis of 2 μg protein samples was accomplished using the Immobiline® drystrip kit (pH 3.5–10 linear, 11 cm) and ExcelGel™ [sodium dodecyl sulfate (SDS) gradient 8–18% from Pharmacia following the manufacturer's instructions]. The electrophoresis gels were silver-stained for 6 min exactly at 22°C using the silver staining kit from Pharmacia and scanned on a Bio-Rad GS-700 imaging densitometer (Hercules, CA, USA) using the Molecular analyst 2-D PAGE software version 1.0 from Bio-Rad for image analysis. Image analysis allowed for quantification of the protein relative percentages. For saturated spots of known protein; namely, immunoglobulin G and albumin, relative percentages were confirmed by nephelometric (Department of Clinical Immunology, Uppsala University Hospital) and radioimmunodiagnostic (Pharmacia Diagnostics) techniques, respectively.

3. Results

The injection of human serum onto cyanocarbon gels in the presence of 0.5 *M* Na_2SO_4 (Fig. 1A) resulted in various degrees of protein adsorption (3.2 to 23.8%) (Table 1). The percentage of protein eluted in each fraction increased with the number of cyano-groups on the ligands. The "salt-dependent fraction" (SDF) eluted by removing the salt from the adsorption buffer represented at least 60% of the total protein adsorbed. The use of 1 *M* NaCl allowed us to desorb most of the remaining bound proteins, and this fraction was labelled "salt-independent fraction" (SIF 0.5 *M* Na_2SO_4). The final cleaning step eluted at the maximum a few per cents of proteins. These results were reproducible for more than ten runs on the same gel or different gel batches provided that the ligand density was comparable.

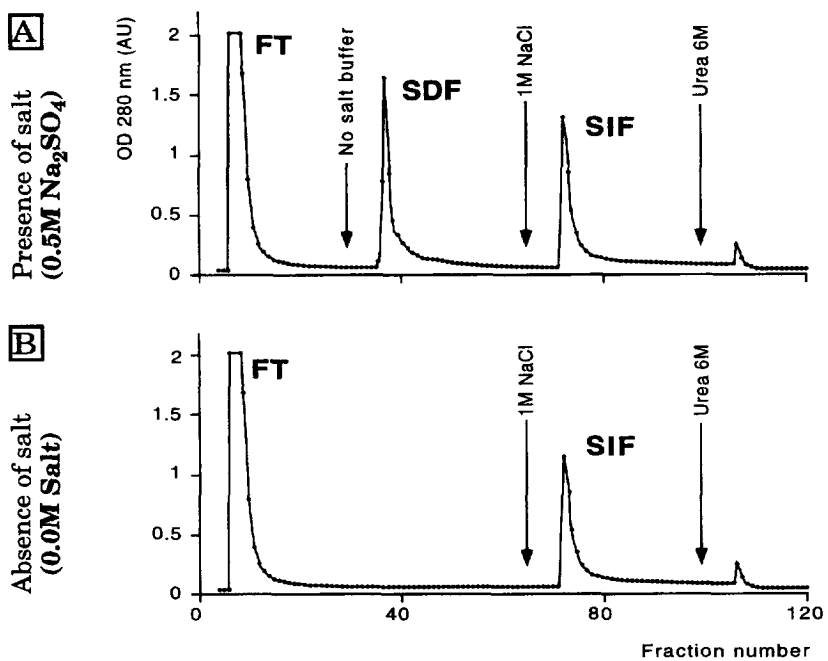
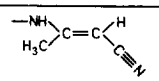
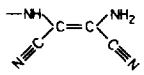
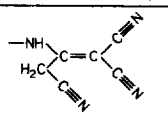
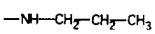
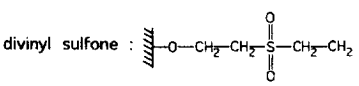


Fig. 1. Normal chromatographic profile of human serum onto the tricyanoaminopropene–DVS–agarose (TCP gel) in the presence (A) or in the absence (B) of protein adsorption promotion by Na_2SO_4. SDF=salt-dependent fraction; SIF=salt-independent fraction; FT=flow-through fraction.

Table 1
Structure of the different ligands coupled to agarose gels via divinyl sulfone (DVS) spacer arm

Ligand	Degree of substitution (μmol/g)	% of protein desorbed in the SDF	% of protein desorbed in the SIF	% of protein desorbed in 6M Urea	Total % of protein adsorbed	Total % of protein recovery
 Amino Crotonitrile (ACN)	303	2.0±0.3	0.7±0.1	0.5±0.2	3.2±0.6	91.7±2.2
 Diamino Maleonitrile (DAMN)	312	6.8±0.3	1.0±0.1	1.0±0.5	8.8±0.9	97.0±2.1
 Tricyano Amino Propene (TCP)	453	14.0±0.4	7.0±0.4	2.8±0.5	23.8±0.9	95.4±0.4
 Propylamine	456	0.0	0.0	0.0	0.0	96.2±0.7



Degree of substitution (μmol of ligand/g of dried gel) and percentage of human serum proteins desorbed by salt deletion (SDF), desorbed by addition of 1 *M* NaCl (SIF) and desorbed by 6 *M* urea for the different cyanocarbon gels. Adsorption buffer: 50 m*M* Mops–acetate (pH 7.6), containing 0.5 *M* Na_2SO_4.

The TCP gel, the most potent adsorbent, was selected for further study. The maximum protein adsorption capacities reached during our study for this gel were 11 mg of protein per ml of packed gel for the SDF and 13.7 mg of protein per ml of packed gel for the SIF (Fig. 2). For a defined concentration of salt used, the ratio between the SDF and SIF protein fractions was constant for the gel tested, independent

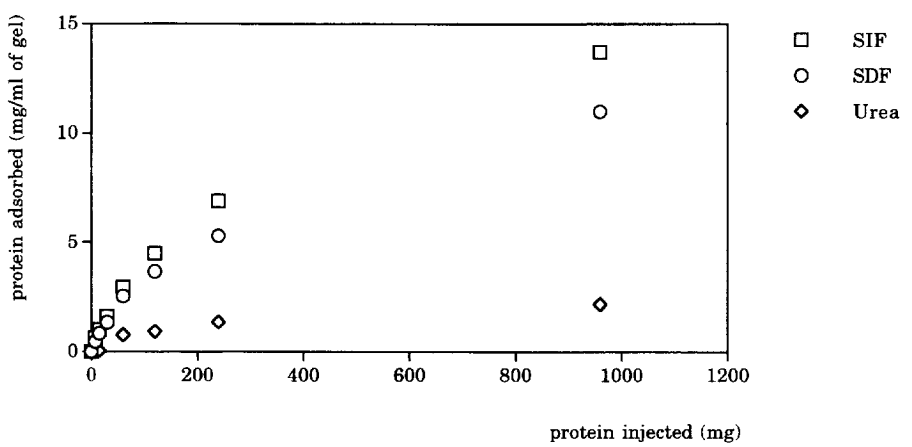


Fig. 2. Adsorption of human serum proteins onto the tricyanoaminopropene–DVS–agarose (TCP gel, 671 μmol of ligand/g of dry weight) in the presence of 0.5 *M* Na_2SO_4 as a function of the amount of protein loaded. SDF=salt-dependent fraction; SIF=salt-independent fraction; FT=flow-through fraction.

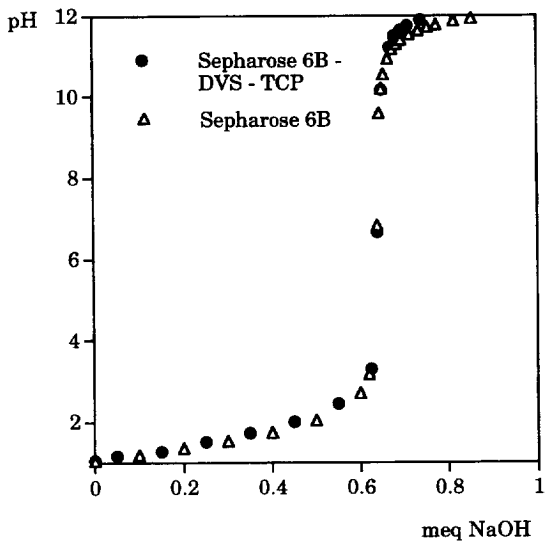


Fig. 3. Titration curves for the tricyanoaminopropene–DVS–agarose (TCP gel) and the nude Sepharose 6B.

of protein loading and human serum batches. However this ratio varied from gel batch to gel batch depending on their degree of substitution.

When the adsorption of human serum was tested in the absence of salt-promotion (Fig. 1B), the addition of 1 *M* NaCl also eluted a SIF fraction (named SIF 0.0 *M* salt). Its percentage of protein adsorption was equivalent to the percentage of protein adsorption observed in the peak eluted in the same conditions (SIF 0.5 *M* Na_2SO_4) when salt-promotion was used. Titration of the TCP gel and of Sepharose 6B (Fig. 3) revealed that the derivatized and nude matrices carry no net charge in the chromatographic conditions used, which preclude therefore the occurrence of ion-exchange.

Differences in protein adsorption selectivity between SDF, SIF 0.5 *M* Na_2SO_4 and SIF 0.0 *M* salt were looked for by image analysis of two-dimensional electrophoresis gels (Fig. 4 and Table 2). Except for the flow-through fraction, all protein maps dis-

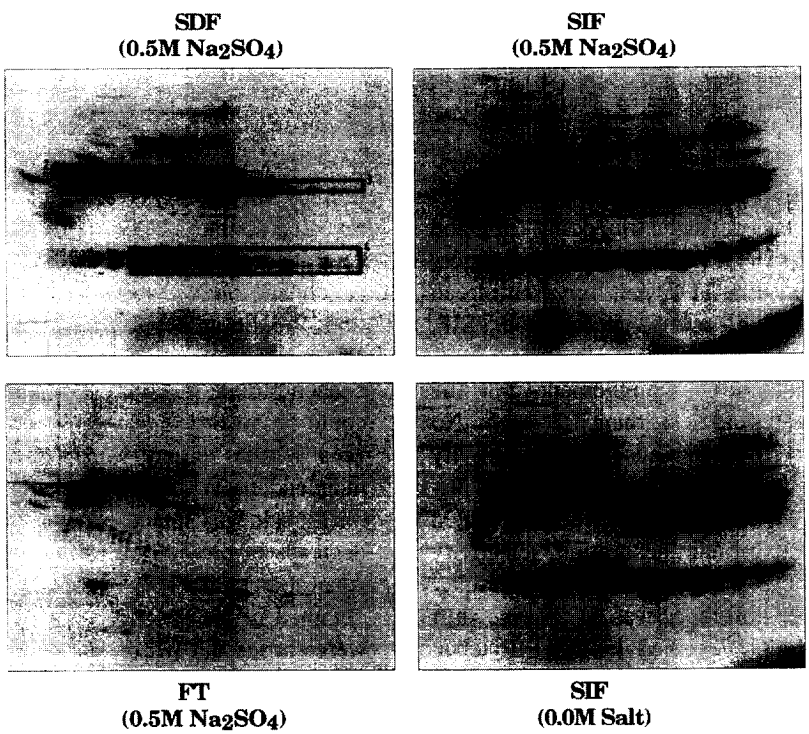


Fig. 4. Two-dimensional electrophoresis patterns of different fractions coming from chromatographies onto the tricyanoaminopropene–DVS–agarose (TCP gel). The gels are shown with the acidic p*I* on the left and the low molecular mass at the bottom. SDF=salt-dependent fraction; SIF=salt-independent fraction; FT=flow-through fraction; 1=IgM μ chain; 2=IgA α chain; 3=IgG γ chain; 4=Ig light chain λ, κ; 5=albumin.

Table 2
Relative percentages[a] of human serum proteins present in different fractions coming from chromatographies onto the tricyanoaminopropene–DVS–agarose (TCP gel)

Protein	SDF (%) (0.5 *M* Na_2SO_4)	SIF (%) (0.5 *M* Na_2SO_4)	SIF (%) (0.0 *M* Salt)	Flow through (%) (0.5 *M* Na_2SO_4)
IgG[b]	52.0	68.5	66.5	2.0
IgA	21.7	13.4	13.1	1.7
IgM	1.4	3.7	5.6	–
Total immunoglobulins	75.1	85.6	85.2	3.7
Fibrinogen	4.7	2.1	2.9	0.7
Plasminogen	–	0.6	1.4	–
C3 complement	2.0	–	–	–
C4 complement	–	0.6	1.4	–
α_1-Antitrypsin	–	0.6	0.9	1.8
α_1-Antichymotrypsin	2.0	–	–	0.9
α_2HS-glycoprotein	–	–	–	0.7
Haptoglobulins	–	–	–	2.6
Gc-globulin	–	–	–	0.6
Hemopexin	–	–	–	0.7
Transferrin	–	–	–	1.5
Apo-lipoproteins	–	–	–	1.5
Transthyretin	–	–	–	0.5
Albumin[c]	1.4	0.9	0.7	82.0
Non-identified proteins[d]	14.8	9.6	7.5	2.8

[a] Quantification by image analysis of a two-dimensional electrophoretogram.
[b] Quantification confirmed by nephelometric assay.
[c] Quantification by radioimmunoassay.
[d] Each non-identified protein represented less than 0.5%.

played a similar general pattern with proteins presenting broad ranges of isoelectric point (p*I*) and molecular mass. The Ig G, A and M were the majors proteins retained on the TCP gel, representing in total 75% of the SDF and 85% of both SIF. Excluding fibrinogen, plasminogen, albumin, α_1-antichymotrypsin, C3 and C4 complements, all the other proteins represented less than 1% each. No significant difference in protein adsorption selectivity could be observed between the two SIF. This justifies our identical labelling of both fractions eluted by 1 *M* NaCl. The protein adsorption selectivity between the SIF and the SDF was also very similar. The most remarkable difference was an alkaline shift in the p*I* of the Ig G desorbed in the SIF when compared to SDF. Image analysis of a two-dimensional gel electrophoresis of the flow-through fraction confirmed that hydrophobic proteins such as albumin, haptoglobulins and apo-lipoproteins went through the gel.

Comparison of the promotion of protein adsorption by increasing concentration of sodium sulfate revealed distinct behaviors for each fraction (Fig. 5). The adsorption of proteins in the SDF was clearly dependent on the water-structuring salt concentration. Protein adsorption only started to occur for 0.2 *M* sodium sulfate and then increased with salt concentration. In contrast, for the SIF the proteins could be adsorbed in the absence of salt-promotion. This is the reason we named this fraction salt-independent. When the concentration of water-structuring salt was increased, we first observed a small decrease in protein adsorption but the percentage of adsorption quickly returned to its original value obtained in the absence of salt. Similar experiments were carried out with NaCl at various concentrations and gave different results (Fig. 6). In the range of concentration tested, the SDF was very small and negligible. This time the SIF showed a decrease in protein adsorption with increasing NaCl concentration. Protein adsorption was completely abolished for a concentration of 1 *M* NaCl.

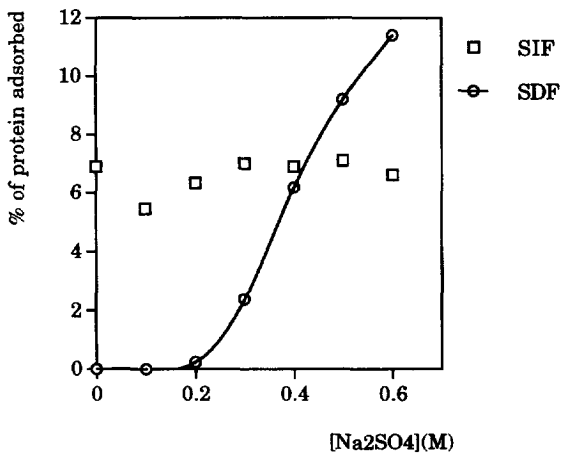


Fig. 5. Influence of Na_2SO_4 concentration on the percentage of proteins adsorbed in the salt-dependent fraction (SDF) and the salt-independent fraction (SIF) onto the tricyanoaminopropene–DVS–agarose (TCP gel, 572 μmoles of ligand/g of dry weight).

4. Discussion

The proteins adsorbed on the TCP gel were chromatographically resolved into two parts, SDF and SIF. Whereas the elution of the SDF by deletion of the salt was expected from a salt-promoted adsorption chromatography, the elution of the SIF by addition of 1 *M* NaCl was a totally new outcome. Surprisingly, study of the amino acid composition or analysis of the affinity for Concanavalin A–agarose of these two fractions (data not shown) indicated that they were very similar in their composition. This assumption was further corroborated by the image analysis of the protein maps obtained by two-dimensional gel electrophoresis. The similar protein specificity of SDF and SIF is rather intriguing, since the two TCP fractions are distinctly different in their behavior towards salt-promotion. How can these results be accounted for?

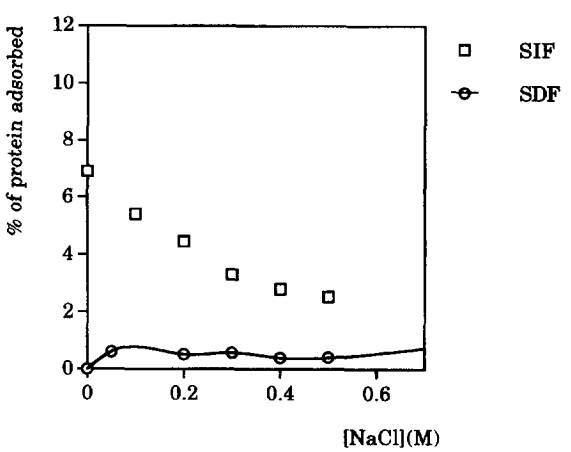


Fig. 6. Influence of NaCl concentration on the percentage of proteins adsorbed in the salt-dependent fraction (SDF) and the salt-independent fraction (SIF) onto the tricyanoaminopropene–DVS–agarose (TCP gel, 572 μmol of ligand/g of dry weight).

One possibility may be the presence of two non-equivalent adsorption sites, a result of partial hydrolysis or some other chemical conversion of the ligand before or after immobilisation. Another explanation may be found in assuming a ligand behaving differently when localised in two different microenvironments in the matrix. A third possibility is obvious, although improbable, the proteins belong to two classes differing in their sterical structure. A last and quite elegant explanation would have been that the SIF proteins represent a group of proteins interacting with the TCP ligand and that in turn SDF would represent proteins interacting with the proteins already adsorbed on the TCP gel. However, study of the protein adsorption function of the TCP substitution degree (data not shown) eliminated this hypothesis as it showed that adsorption of the SIF fraction was not a prerequisite for SDF adsorption. Whatever the explanation for this intriguing dichotomic adsorption behavior, we find the observed phenomena worthwhile for further study. The characteristic group separation obtained is highly reproducible. The results have been obtained by using different batches of adsorbent and, for a same batch of gel and a defined concentration of salt used, the ratio between the protein adsorbed in the SDF and the protein adsorbed in the SIF was found to be constant for one gel, for different protein loadings and different human serum samples. This last fact argued for a constant composition of the serum and precluded the hypothesis of an eventual separation based on complexation with the complement or dimerisation of the immunoglobulins. From a practical point of view, the method offers a useful group separation of the plasma proteins into one part consisting of the immunoglobulins, complement and blood clotting factors and another, the non-adsorbed, break-through fraction containing over 99.5% of all

albumin. The adsorbent has a potential for rapid and sharp micro-bench or macro-scale group fractionation of plasma proteins.

Although hydrophobic and ionic interactions may play some minor roles, they certainly cannot account for the observed adsorption behavior. Indeed, hydrophobic proteins such as albumin are not retarded on the gel and although proteins adsorbed in the absence of salt could be eluted by increasing sodium chloride concentration, titration of the TCP gel confirmed the neutral character of the matrix. If any more convincing evidence was needed to eliminate the possibility of ionic interaction, the existence of protein adsorption in the presence of sodium sulfate would account for it. Therefore, we conclude that there must be another molecular interaction operating and significantly contributing to the results.

In a previous study on cyanocarbon ligands, we suggested for the SDF that electron acceptor interactions were the most probable mode of interaction [2]. We are again arguing here in favor of electron donor acceptor transfer behind the mechanism of adsorption for the SDF and the SIF. The dichotomic protein distribution under the experimental conditions between the combined fractions SDF and SIF on one hand, and the non-adsorbed fraction on the other hand, is strikingly similar to that obtainable by TAC on T gel [4]. However, the hypothetical electron donor acceptor concept is more easily defended in the case of TCP adsorption. The ligand reagent, TCP (and presumably, the gel-fixed ligand) has a structure that favors a strong electron accepting capacity due to its double bond and the adjacent three electron withdrawing groups. If we compare the ligand structures and protein distribution in Table 1, it becomes immediately clear that the number of cyano-groups has a very strong influence on the adsorption. The C≡N group is slightly hydrophobic but it is the electron structure that governs the aromatic affinity. Indeed the lack of π-electron for the propylamine ligand (Table 1) resulted in the absence of protein adsorption.

For complexation depending on partial electron transfer to occur, the proteins must have available counter-parts to the ligand and, apart from hydrogen bond donors and acceptors, the only reasonable candidates, in our view, are the aromatic side chains. Here they would form weakly polar interactions with the adsorbent such as those discussed by Burley and Petsko in their study on protein intramolecular interactions [5]. Local differences in the dielectric constant of the gel surface and screening effect of water (protein hydration) towards electron donor acceptor interactions would then explain the differential behavior of the two TCP-fractions towards salt-promotion. Whatever the explanation of the salt-independent adsorption can be, this work shows that electron donor acceptor interactions can appear in an aqueous environment without the need of salt-promotion. This particularity of the TCP gel as revealed by its selectivity for immunoglobulins and the mild conditions for their desorption can be strong assets for its application in the fractionation of immunoglobulins where other gels such as the T gel [6] or the histidine gel [7] are already competitors to the costly and less stable protein A adsorbent.

From this work, we are coming to the following rather important conclusions:

– Electron donor acceptor chromatography of proteins may occur in aqueous solvent in the absence of salt-promotion.

– Immunoglobulins can be adsorbed onto the TCP gel without the need of salt-promotion and desorbed in mild conditions by 1 *M* NaCl.

– Finally, in salt-promoted adsorption chromatography, protein mixtures can be fractionated according to two distinctly different adsorption mechanisms of opposed selectivity: (1) hydrophobic interaction and (2) electron donor acceptor interaction. Hydrophobic interactions are chiefly directed towards hydrophobic patches represented by aromatic and aliphatic side chains on the protein surface, whereas electron donor acceptor affinity presumably depends primarily on the presence of aromatic residues.

Acknowledgments

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