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Comparison of the protein adsorption selectivity of salt-promoted agarose-based adsorbents
Hydrophobic, thiophilic and electron donor–acceptor adsorbents

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Abstract

Protein adsorption of human serum onto six different agarose-based chromatographic gels that were representative of the salt-promoted adsorbent family [octyl- and phenyl-Sepharose, mercaptoethanol–divinyl sulfone agarose (T gel), mercaptomethylene pyridine-derivatized agarose gel (MP gel), tricyanoaminopropene–divinyl sulfone agarose (DVS–TCP gel), tricyanoamino-propene–bisoxirane agarose (bisoxirane–TCP gel)] was studied in the presence of moderate or high concentrations of the water structuring salt, sodium sulfate. Study of the protein adsorption selectivity by two-dimensional gel electrophoresis revealed an opposed selectivity for hydrophobic interaction adsorbents and electron donor–acceptor adsorbents. The T gel, MP gel and TCP gels belonged to the electron donor–acceptor adsorbents, displaying a main selectivity for immunoglobulins, whereas octyl-Sepharose belonged to the hydrophobic adsorbents, displaying a main selectivity for 'hydrophobic' proteins. Phenyl-Sepharose for its part was described as an example of a composite selectivity of both families. The conclusion of this work is two-fold: (1) hydrophobic interaction chromatography (HIC) and electron donor–acceptor chromatography (EDAC) have opposed protein selectivities and are both salt-promoted. As a main consequence, it means that high concentrations of a water-structuring salt can promote different types of weak molecular interactions, resulting in different protein adsorption selectivities: (2) thiophilic adsorption chromatography (TAC) should be renamed EDAC as similar protein selectivity is demonstrated for electron donor–acceptor ligand devoid of sulfur atoms.

Keywords: Adsorbents; Adsorption; Hydrophobic interaction chromatography; Electron donor–acceptor chromatography; Stationary phases, LC; Proteins; Immunoglobulins

1. Introduction

Adsorption of protein in hydrophobic interaction chromatography (HIC) is promoted by a high concentration of water-structuring salt [1]. Equivalently, salt promotion has been applied for other classes of

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amphiphilic adsorbents that are not directly analogous to HIC [2,3]. In 1990, Porath [4] coined the term salt-promoted adsorption chromatography (SPAC) to regroup these chromatographic techniques that require a high salt concentration to promote protein adsorption. Thiophilic adsorption chromatography (TAC), electron donor–acceptor chromatography (EDAC) and HIC were therefore included in this family. The salt effect on protein adsorption was explained as resulting from the unfavourable increase of the free energy, ΔG, for unbound proteins in the presence of high concentrations of salt [5,6]. The thermodynamic consequence of this is the promotion of protein binding to a ligand because of the smaller surface area of the complex exposed to the solvent and co-solvent, i.e., the bound form of the protein is thermodynamically more stable. Protein elution from the matrix is then achieved simply by deletion of the salt from the adsorption buffer.

In HIC, the hydrophobic interactions occur between hydrophobic patches on the surface of the protein and the hydrophobic ligands. The first gels of practical use for HIC were of a mixed hydrophobic–ionic character [7–9]. Neutral adsorbents (alkyl and aryl ethers) were later prepared by Porath et al. [1] and Hjertén et al. [10], the latter leading to the introduction of octyl- and phenyl-Sepharose. About ten years later, thiophilic adsorption on mercaptoethanol–divinyl sulfone agarose (T gel) was discovered by Porath et al. [11]. The adsorption mechanism was then interpreted as involving a two-point attachment of the protein to the β-mercaptoethanol and the divinyl sulphone spacer arm through a possible electron donor–acceptor thiophilic interaction [12]. On further investigation, similar adsorption behaviour was observed on epichlorhydrin-activated gels to which 2-mercaptopyridine had been coupled [13]. More recently, Berna and Porath [14,15] showed that salt-promotion was also efficient in promoting protein adsorption onto a cyanocarbon substituted gel, the tricyanoaminopropene–divinyl sulfone agarose (DVS–TCP gel) through electron donor–acceptor interactions.

Although all of these gels require high concentrations of water-structuring salts to provide protein binding, no study has been carried out for direct comparison of the protein adsorption specificity of hydrophobic, thiophilic and electron donor–acceptor agarose-based gels. Here, we determined the similarities and differences in the protein adsorption selectivities of the different agarose-based chromatographic gels representative of the salt-promoted adsorbent family; namely, T gel, mercaptomethylene pyridine-derivatized agarose gel (MP gel), DVS–TCP gel, tricyanoaminopropene–bisoxirane agarose (bisoxirane–TCP gel) and two hydrophobic gels, octyl- and phenyl-Sepharose. The adsorption was tested using human serum as a model sample that contained proteins of a wide range of physico-chemical properties and in the presence of the water-structuring salt, sodium sulfate, at various concentrations. Comparison of the protein adsorption selectivity by two-dimensional gel electrophoresis allowed classification of the different adsorbents and demonstrated the inadequacy of the term thiophilic interaction to define protein adsorption on the T gel.

2. Experimental

2.1. Chemicals

Na_2SO_4 and NaOH were obtained from Merck (Darmstadt, Germany), Trizma base (Tris), 3-(*N*-morpholino)propanesulfonic acid (MOPS) and bovine serum albumin were from Sigma (St. Louis, MO, USA) and urea was from Riedel-de Haën (Seelze, Germany).

2.2. Samples

Human serum samples (samples from 50 patients were mixed together to obtain a serum pool) were purchased from the University Hospital (Uppsala, Sweden).

2.3. Preparation methods for adsorbents

3 - (2 - Pyridylmethylenethio) - 2 - hydroxypropyl-agarose (MP gel), TCP gels (DVS–TCP and bisoxirane–TCP gels) and T gel were prepared according to the methods of Berna et al. [16], Berna and Porath [14] and Porath et al. [11], respectively. Their structures are described in Fig. 1. The ligand concentrations were found to be 885, 760, 367 and 910 μmol/g of dried product, respectively, as calculated

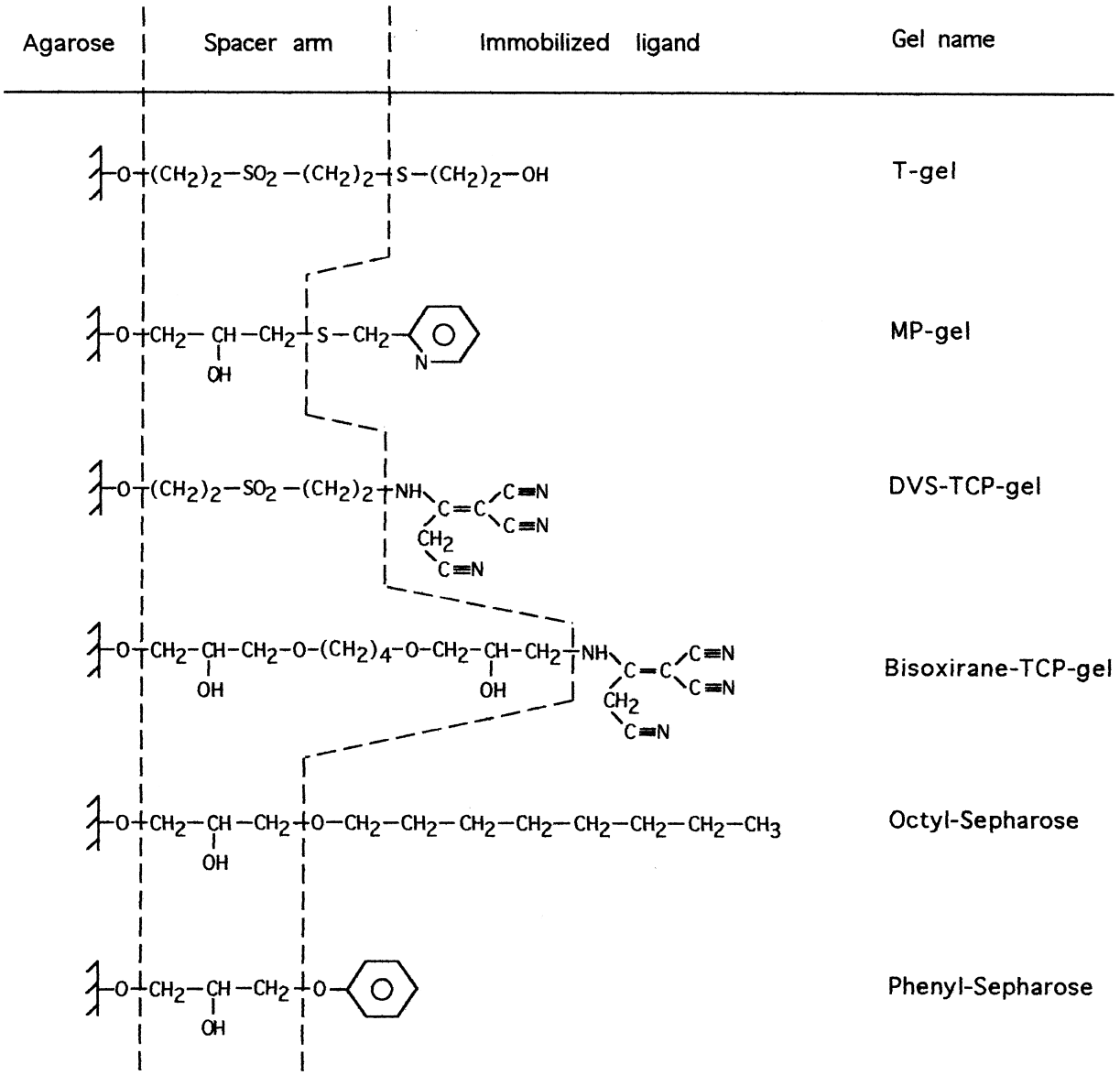

Fig. 1. Structures of the ligands in the different agarose gels studied.

from the sulfur and nitrogen contents. Octyl- and phenyl-Sepharose CL 4B were purchased from Pharmacia (Uppsala, Sweden). According to the manufacturer's product information, their ligand concentrations were 40 μmol/ml of gel bed and 40 μmol/ml of drained gel, respectively. On a basis of 40 mg of dry weight per ml of drained gel, these were equivalent to ca. 770 and 1000 μmol/g of dried product, respectively.

2.4. Chromatographic studies

A 3×1 cm I.D. column was packed with the gel to be tested, except for T gel and TCP gels, where a

7×0.5 cm I.D. column was used. The studies on T gel, MP gel and TCP gels were performed using the same column throughout the work, whereas new phenyl- or octyl-Sepharose gels were packed for every chromatographic analysis, to avoid possible disturbance by irreversible protein adsorption. Adsorption, desorption and regeneration of the adsorbent were programmed by means of a fast protein liquid chromatography (FPLC) system from Pharmacia. The gel was equilibrated at a flow-rate of 0.64 ml/min (1 ml/min for T gel and TCP gels) using the adsorption buffer [100 m*M* Tris, pH 7.5 (50 m*M* MOPS acetate, pH 7.5, for T gel and TCP gels)] containing sodium sulfate at the desired concentration. Solid sodium sulfate was added to the serum sample to the desired final concentration. A 1-ml volume of sample (0.5 ml for T gel and TCP gels to keep the equivalent ratio of sample loading per unit of gel volume) containing approximately 60 mg protein/ml of centrifuged (5000 *g*, 5 min) human serum was applied to the column and the gel was washed with the equilibration buffer for at least fifteen bed volumes, which allowed the absorbance to return to the baseline, followed by the equilibration buffer without Na_2SO_4 and, finally, using 100 m*M* NaOH (6 *M* urea in 50 m*M* MOPS acetate, pH 7.5, for T gel and TCP gels) as a cleaning step. The eluate from one step was recovered as one fraction. The influence of the Na_2SO_4 concentration was tested from 0 to 0.7 *M*.

2.5. Analytical methods

Elementary analyses of the gels were performed with an NA 1500 automatic nitrogen and sulfur analyser (Carlo Erba, Milan, Italy).

The protein content of each chromatographic peak was measured using the Bio-Rad protein assay and bovine serum albumin as a standard. The results were expressed as a percentage of the total protein recovered in the flow-through fraction, the elution step and the cleaning step. The total protein recovery in each chromatographic run always exceeded 90% of the total protein applied. Each chromatographic run was performed in duplicate. The results did not vary by more than ±5%.

Two-dimensional electrophoretic analysis was carried out on the fractions adsorbed upon use of Na_2SO_4 at the following concentrations: 0.35 *M* on the T gel, 0.35 *M* on the MP gel, 0.35 *M* on the DVS–TCP gel, 0.6 *M* on the bisoxirane–TCP gel, 0.5 *M* on the octyl-Sepharose and 0.2 *M* on the phenyl-Sepharose. The percentage of adsorbed proteins was approximately the same for the five fractions (3 to 4% of the protein injected). The samples were first dialysed against 5 m*M* MOPS acetate buffer, pH 7.6, then freeze-dried and resolubilized in 50 m*M* MOPS acetate buffer, pH 7.6. Electrophoresis of a 2-μg protein sample 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 precisely at 22°C using the silver-staining kit from Pharmacia and the gels were scanned on a Bio-Rad GS-700 imaging densitometer (Hercules, CA, USA) using the molecular analyst two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) software, version 1.0, from Bio-Rad for image analysis. Image analysis allowed quantification of the relative percentages of protein. For saturated spots of known protein, namely, immunoglobulin G (IgG) and albumin, relative percentages were confirmed by nephelometric (Department of Clinical Immunology, Uppsala University Hospital, Uppsala, Sweden) and radioimmunodiagnostic (Pharmacia Diagnostics) techniques, respectively.

3. Results

The adsorption of human serum proteins onto six different adsorbents, whose ligand structures are described in Fig. 1, has been compared for increasing concentrations of sodium sulfate (Fig. 2). Proteins could be adsorbed on all of the gels and the percentage of adsorbed proteins augmented for increasing concentrations of Na_2SO_4. At all of the concentrations tested, the percentages of proteins adsorbed were similar for the T gel, MP gel and DVS–TCP gel. The bisoxirane–TCP gel showed a similar response to increasing concentrations of sodium sulfate, but shifted towards higher concentrations. In comparison, the percentages of proteins adsorbed on the phenyl-Sepharose gel were the

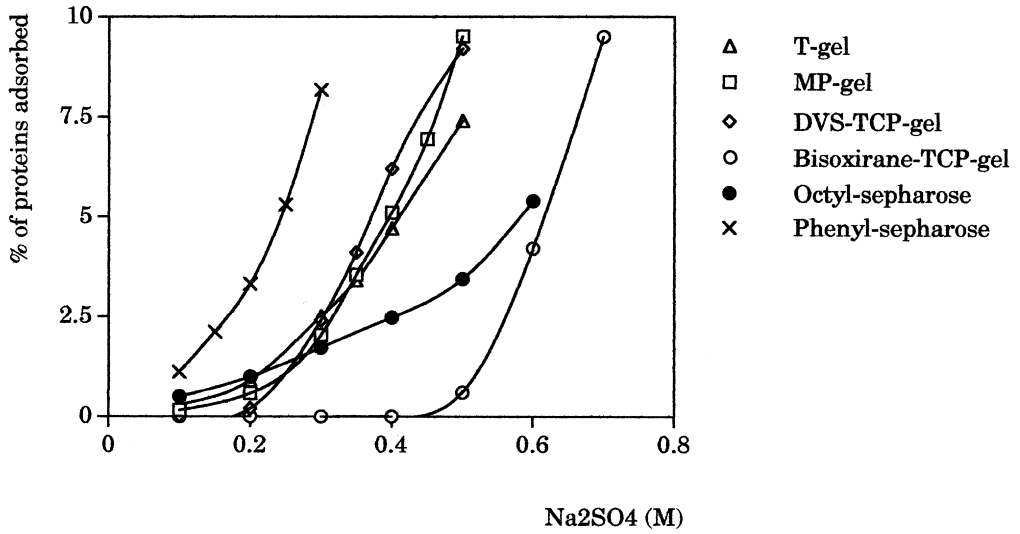

Fig. 2. Percentages of serum protein adsorbed onto the T gel, MP gel, DVS–TCP gel, bisoxirane–TCP gel, octyl-Sepharose and phenyl-Sepharose gels versus the concentration of Na_2SO_4 used in the adsorption buffer.

highest, whereas the slope of the curve obtained with the octyl-Sepharose gel was the lowest.

The protein adsorption specificity for the different adsorbed fractions was studied by two-dimensional gel electrophoresis (Fig. 3). All protein maps displayed proteins presenting broad ranges of isoelectric points and molecular masses. Study of the adsorbed fractions that contained comparable percentages of protein showed similar protein patterns for the T gel, MP gel, DVS–TCP gel and bisoxirane–TCP gel, whereas the patterns obtained for the two hydrophobic gels were clearly different (as well as being different from each other). The different serum proteins were identified by comparison to a standard protein map of the human plasma [17] and their relative percentages were quantified by image analysis (Table 1). The immunoglobulins were the major proteins retained on the T gel, MP gel and the two TCP gels, representing between 76 and 88% of the total adsorbed protein. With the exception of fibrinogen (between 2 and 4.8%), all of the other proteins represented less than 2% of the total protein. For these four gels, the relative percentages of each protein adsorbed were very close, with a few minor proteins having significant changes. On the octyl-Sepharose gel, the main proteins retained were hydrophobic proteins, namely albumin (56%), haptoglobins and apolipoproteins, but immunoglobulins were also present (24.5%). The proteins retained on the phenyl-Sepharose gel showed a more complex pattern, which corresponded to a mixture of the proteins retained on the T gel, MP gel and TCP gels on one hand and on the octyl-Sepharose gel on the other hand. Immunoglobulins represented 54% of the proteins adsorbed on this gel, and albumin made up 23%.

4. Discussion

As expected, chromatographic study of the different salt-promoted adsorbents indicated the clear dependence of protein adsorption on sodium sulfate concentration. Comparisons of this dependence were made possible for the different ligands due to the similar matrix, similar ligand density (except for the bisoxirane–TCP gel) and equivalent quantity of proteins injected per volume unit of chromatographic gel. The similar salt-dependencies observed for increasing concentrations of sodium sulfate for the T gel, MP gel and TCP gels contrasted with the salt-dependence adsorption displayed by the octyl- and phenyl-Sepharose gels. On investigation of the protein adsorption selectivities, octyl- and phenyl-

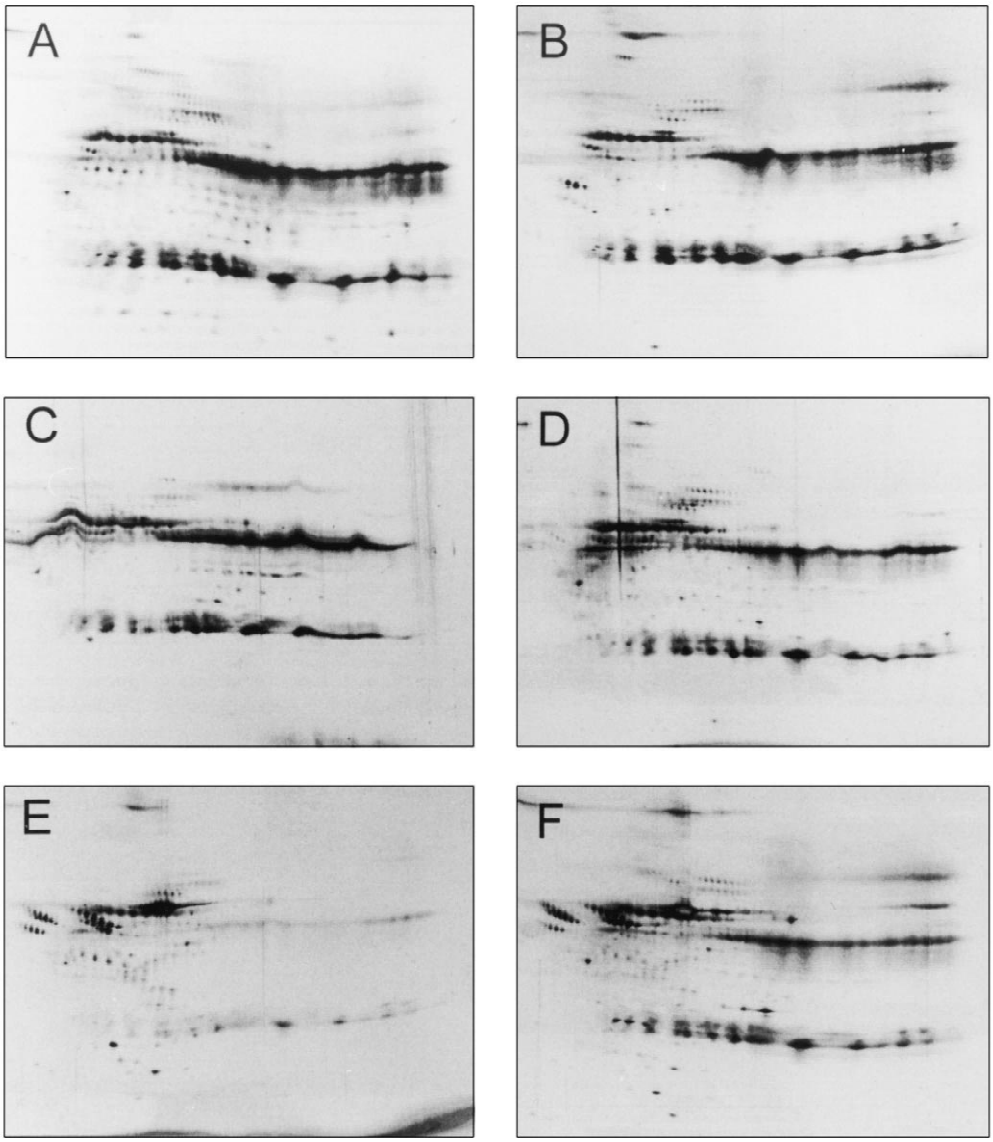

Fig. 3. Two-dimensional electrophoresis patterns of proteins adsorbed onto the T gel (A), MP gel (B), DVS–TCP gel (C), bisoxirane–TCP gel (D), octyl-Sepharose (E) and phenyl-Sepharose (F) gels. The gels are shown with the acidic isoelectric point to the left and with low molecular mass at the bottom.

Sepharose were again opposed to the T gel, MP gel and TCP gels, having preferential adsorption of 'hydrophobic' proteins rather than immunoglobulins. From these differences, we noticed the emergence of two categories of salt-promoted adsorbents; one that we define as adsorbents based on the electron donor–acceptor (EDA) concept and the other category that was originally defined as hydrophobic interaction adsorbents.

Indeed, the octyl- and phenyl-Sepharose, which were originally defined as hydrophobic interaction adsorbents, have different behaviours in terms of response to salt-promoted protein adsorption and protein selectivity when compared to EDA adsor-

Table 1
Relative percentages[a] of human serum proteins adsorbed upon the addition of Na_2SO_4 to the T gel, MP gel, DVS–TCP gel, bisoxirane–TCP gel, octyl-Sepharose and phenyl-Sepharose

Proteins	T gel	MP gel	DVS–TCP gel	Bisoxirane–TCP gel	Octyl-Sepharose	Phenyl-Sepharose
IgG[b]	68.5	72.9	60.1	62.0	12.3	40.0
IgA	9.5	11.9	18.5	12.0	11.1	12.2
IgM	3.8	2.7	1.2	2.5	1.1	1.6
Total immunoglobulins	81.8	87.5	79.8	76.5	24.5	53.8
Albumin[c]	0.6	0.8	1.1	1.1	56.2	23.2
α_2-Macroglobulin	0.4	1.2	0.4	0.7	1.4	
Fibrinogen	3.3	2.0	4.3	4.8		2.9
C_3-complement	0.6	1.0	1.6	0.7		0.5
C_4-complement	0.3	0.2	1.5			1.0
α_1-Antitrypsin	1.6	0.7	1.5	1.1	0.5	2.2
α_1-Antichymotrypsin			1.0		1.8	2.9
α_2HS-glycoprotein					1.0	1.0
Haptoglobulins	1.1				3.2	2.4
Gc-globulin					0.9	
Hemopexin					1.1	
Transferrin	0.8					
Plasminogen					1.3	
Apolipoproteins					1.0	
Unidentified proteins[d]	9.5	6.6	8.8	15.1	7.1	10.1

[a]Quantification by image analysis of a 2D electrophoretogram.
[b]Quantification confirmed by nephelometric assay.
[c]Quantified by a radioimmunodiagnostic technique.
[d]Each unidentified protein represented less than 0.5% of the total protein content.

bents. Increasing the concentration of sodium sulfate had a lesser effect on protein adsorption for the octyl-Sepharose and a stronger effect for the phenyl-Sepharose. Protein selectivity was mainly directed towards 'hydrophobic' proteins such as albumin, apolipoproteins and haptoglobins for the octyl-Sepharose gel, but represented a composite selectivity of the octyl gel and an EDA gel for the phenyl-Sepharose. From this study, we therefore define the octyl-Sepharose as a representative of the hydrophobic interaction adsorbents category and the phenyl-Sepharose as a mixed-mode HIC–EDAC adsorbent. This mixed-mode interaction was, however, not surprising as it had previously been suggested by several authors who studied the retention of small organic compounds [18], the retention of model proteins [19] or adsorption of IgG subclasses [20]. Moreover, this is in agreement with previous descriptions of the phenyl group as a hydrophobic ligand having a potential for $\pi-\pi^*$ [21] or weakly polar (n–σ^*) [22] EDA interactions.

In contrast, the second category of salt-promoted adsorbents, namely the EDA adsorbents, displayed a main characteristic adsorption specificity for the immunoglobulin molecules. This correlates with previous observations of immunoglobulin adsorption and non-retention of hydrophobic proteins for the individual gels [11–14]. In these studies, the decrease in protein adsorption capacity at increasing temperatures of these gels confirmed that hydrophobic interaction was not the main cause of protein adsorption. The demonstration of the absence of ionic or covalent interactions confirmed the possibility that these gels [11–14] had of a 'new' type of adsorption mechanism based on electron donor–acceptor interactions.

The common protein adsorption selectivity observed here for the T gel, MP gel, DVS–TCP gel and bisoxirane–TCP gel confirms their common mechanism of adsorption and, therefore, indicates that they belong to the category of EDA adsorbents. Indeed, although defined separately, their individual mecha-

nisms are all based on EDA interactions. For the T gel, the thioether sulfur was said to cooperate with the sulfone dipole to achieve an electron donor–acceptor thiophilic interaction mechanism [12]. In the case of the MP gel, the pyridine nucleus, together with the exocyclic sulfur, was supposed to act predominantly as an electron donor and produce an adsorbent with properties similar to that of the T gel [13]. For the TCP gels, 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 [14].

However, the surprising versatility of the ligand structures compared to the unique protein adsorption specificity for the tested EDA adsorbents still addresses the question of what is the common denominator on the adsorbent for these short range weak interactions? Sulfur is not a prerequisite to EDA interactions, as the bisoxirane–TCP gel, which is devoid of sulfur atom, gave a similar protein adsorption specificity to both T gel or DVS–TCP gel. However, this sulfur may potentiate the tendency of the matrix to engage in electron donor–acceptor interactions, as, although the shape of the adsorption curve for the sulfur-free gel was identical to that of other EDA gels, it was markedly shifted towards higher salt concentrations. From this, it can be seen that the protein adsorption specificity of T gel is not different from that of other ligands that act based on EDA interactions and the thiophilic interaction mechanism would be better described under the wider heading of EDA interactions. We propose here that the name thiophilic adsorption chromatography (TAC) should be updated to electron donor–acceptor chromatography (EDAC).

Although the interaction site(s) on the ligand is (are) not known, the interaction counter-site present on the protein may be represented by the surface-accessible tryptophan residue. Indeed, as early as 1959, a study by Porath and Gelotte on the temperature dependence of the interaction between EDA gels and Trp–Trp indicated that this amino-acid residue was one of the most plausible candidates. This has to be confirmed and more work is required, especially on the protein adsorption counter-site, as well as on the influence of the chromatographic matrix in the salt-promoted adsorption mechanism itself, to determine the minimum ligand requirement for protein adsorption in EDAC.

Based on the protein adsorption specificity, we have shown that the six different adsorbents can be regrouped into two main families displaying generic molecular recognition characteristics when sodium sulfate is used. T gel, MP gel, DVS–TCP gel and bisoxirane–TCP gel pertain to the EDA adsorbent family, displaying a main selectivity for immunoglobulins. Octyl-Sepharose belongs to the ‘hydrophobic adsorbents’ family, displaying a main selectivity for hydrophobic proteins, whereas phenyl-Sepharose represents an adsorbent defined by its mixed-mode hydrophobic and EDA interactions. Therefore, when selecting various media for purification or fractionation of proteins, one should keep in mind that different selectivities can be achieved by using ‘hydrophobic’ or EDA matrices.

Finally, since all of these gels require sodium sulfate for protein binding, we suggest that sodium sulfate may promote various types of molecular interactions, all of which adsorb by weak forces and act over short distances, by some indistinctive general phenomenon. This has to be verified for other water-structuring salts in similar series of experiments.

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References

[1] J. Porath, L. Sundberg, N. Fornstedt, I. Olsson, Nature 245 (1973) 465.
[2] S. Oscarsson, D. Angulo-Tatis, G. Chaga, J. Porath, J Chromatogr. A 689 (1995) 3.
[3] J. Porath, Biotechnol. Prog. 3 (1987) 14.
[4] J. Porath, J. Chromatogr. 510 (1990) 47.
[5] W. Melander, C. Horváth, Arch. Biochem. Biophys. 183 (1977) 200.
[6] S.N. Timasheff, Ann. Rev. Biophys. Biomol. Struct. 22 (1993) 67.
[7] R.J. Yon, Biochem. J. 126 (1972) 765.
[8] Z. Er-El, Y. Zaidenzaig, S. Shaltiel, Biochem. Biophys. Res. Commun. 49 (1972) 383.

[9] B.H.J. Hofstee, Biochem. Biophys. Res. Commun. 50 (1973) 751.
[10] S. Hjertén, J. Rosengren, S. Påhlman, J. Chromatogr. 101 (1974) 281.
[11] J. Porath, F. Maisano, M. Belew, FEBS Lett. 185 (1985) 306.
[12] J. Porath, W. Hutchens, Int. J. Quant. Chem. 14 (1987) 297.
[13] J. Porath, S. Oscarsson, Makromol. Chem., Macromol. Symp. 17 (1988) 359.
[14] P.P. Berna, J. Porath, J. Chromatogr. A 753 (1996) 57.
[15] P.P. Berna, J. Porath, J. Chromatogr. B 693 (1997) 277.
[16] N. Berna, P.P. Berna, S. Oscarsson, Arch. Biochem. Biophys. 330 (1996) 188.
[17] O. Golaz, G.J. Hughes, S. Frutiger, N. Paquet, A. Bairoch, C. Pasquali, J.C. Sanchez, J.D. Tissot, R.D. Appel, C. Walzer, L. Balant, D.F. Hochstrasser, Electrophoresis 14 (1993) 1223.
[18] W.J. Gelsema, C.L. De Ligny, J. Chromatogr. 498 (1990) 237.
[19] P. Kårsnäs, T. Lindblom, J. Chromatogr. 599 (1992) 131.
[20] P. Bridonneau, F. Lederer, J. Chromatogr. 616 (1993) 197.
[21] K.O. Eriksson, in J.C. Janson, L. Rydén (Editors), Protein Purification: Principles, High Resolution Methods and Applications, VCH, New York, 1989, p. 207.
[22] S.K. Burley, G.A. Petsko, Adv. Prot. Chem. 39 (1988) 125.