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Amphiphilic agarose-based adsorbents for chromatography Comparative study of adsorption capacities and desorption efficiencies

Sven Oscarsson[a,b,*], Dalila Angulo-Tatis[b], Grigoriy Chaga[a], Jerker Porath[c]

[a]*Biochemical Separation Centre, Uppsala University, Box 577, S-751 23 Uppsala, Sweden*
[b]*Department of Chemical Engineering, Mälardalen University, Box 325, S-631 05 Eskilstuna, Sweden*
[c]*Department of Biochemistry, Life Sciences West, University of Arizona, Tucson, AZ 85721, USA*

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Abstract

A number of hydrophobic derivatives attached to cross-linked agarose were studied as protein adsorbents. Differences in the adsorption and desorption behaviour were determined as functions of type and concentration of selected salts. Whereas octyl- and phenyl-Sepharose adsorb serum albumin preferentially, pyridyl-S-agarose shows a much stronger preferential affinity for IgG in the presence of high concentrations of lyotropic salts, such as sulphates. In contrast to pyridyl-S-agarose, a large portion of proteins remained fixed to octyl- and phenyl-Sepharose after extensive washing with 1 *M* NaOH.

1. Introduction

Hydrophobic adsorbents have several applications in the field of biology and medicine for the isolation of proteins. The factors involved in hydrophobic interaction and hydrophobic affinity chromatography have been extensively studied [1–8]. Not only the nature of the adsorbent, but also the concentration and chemical nature of the salt solutions employed for salt-promoted adsorption or elution affect the results.

Two different theories have been proposed to explain the effect of solvent additives on the binding of proteins in hydrophobic affinity chromatography: the preferential protein solvent interaction theory by Arakawa [9] and the surface tension theory proposed by Melander and Horváth [10]. Measurement of the preferential protein solvent interactions gives a measure of the change in the chemical potential of the proteins induced by the addition of co-solvents [11,12], while the surface tension effect studied by Melander and Horváth applies to the cavity theory developed by Sinanoğlu and co-workers [13,14].

With the introduction of thiophilic interaction chromatography [15], our interest has been refocused on the parameters governing protein adsorption. Electrically neutral ligands containing delocalized π-electrons interact selectively with surface-located amino acid side-groups that are

* Corresponding author. Address for correspondence: Biochemical Separation Centre, Uppsala University, Box 577, S-751 23 Uppsala, Sweden.

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rich in π-electrons (indolyl, etc.) according to our working hypothesis.

Are π,π-complexation and hydrophobic interactions synergistic in their adsorption effects? In an attempt to clarify some of these effects, we have measured the adsorption capacities and desorption efficiencies for serum proteins in the presence of different salts and at different salt concentrations.

2. Experimental

2.1. Chemicals and materials

NaOAc, Na_2SO_4, K_2SO_4, NH_4OAc and butylglycidyl ether were purchased from Fluka (Buchs, Switzerland) and NaCl and $NaBH_4$ from Merck (Darmstadt, Germany). Trisma base was obtained from Sigma (St. Louis, MO, USA). Sepharose 4B, octyl-Sepharose, phenyl-Sepharose and octylglycidyl ether were gifts from Pharmacia BioProcess Technology (Uppsala, Sweden). NaOH was purchased from EKA Nobel (Surte, Sweden), mercaptopyridine from Aldrich (Steinheim, Germany) and ethanol from Svensk Sprit (Stockholm, Sweden). All deuterated solvents used for NMR analyses were purchased from Glaser (Germany). Serum samples mixed from 50 different patients to obtain a serum pool were purchased from Uppsala University Hospital. Gels for electrophoresis were from Pharmacia Biotech (Uppsala), Sweden.

2.2. Determination of dynamic capacity

The serum sample was adjusted to 0.5 *M* K_2SO_4 by adding solid salt and 0.1 *M* Tris–HCl buffer (pH 7.5), resulting in 11.5 mg/ml of protein. A column (3 × 1 cm I.D.) filled with the respective adsorbent was equilibrated with 0.5 *M* K_2SO_4–0.1 *M* Tris buffer (pH 7.5). The protein solution was loaded on the column at a flow-rate of 0.64 ml/min until the protein concentration in the eluate was the same as that in the applied sample. Dynamic capacity was determined by the method of Winzor [16].

2.3. Calculation methods for determination of dynamic capacity

The dynamic capacities were calculated by using the equation

$$\text{Dynamic capacity} = [(V_x - V_0)/V_t)]P \quad (1)$$

where V_0 is the volume of the voids between the particles in the bed, V_t the total bed volume and V_x is the volume of protein solution added to the column to achieve an eluate protein concentration corresponding to 50% P, where P is the protein concentration of the aplied sample, V_t = 2.35 ml and P = 11.5 mg/ml, as determined by amino acid analysis.

2.4. Determination of salt-dependent adsorption capacity

In order to achieve high reproducibility, an optimized programme for adsorption, desorption and rinsing of the adsorbent was executed by means of a programmable fast protein liquid chromatography (FPLC) system. Serum was diluted with 0.1 *M* Tris-HCl buffer (pH 7.5) to a protein concentration of 13.6 mg/ml in the presence of the salt to be studied. A column (3 × 1 cm I.D.) packed with the respective adsorbent was equilibrated with the salt dissolved in the 0.1 *M* Tris buffer. A 1-ml volume of sample was loaded on the column at a flow-rate of 0.64 ml/min, followed by 15 ml of the equilibration buffer. A 10-ml volume of 0.1 *M* Tris buffer was passed through the column at a flow-rate of 0.64 ml/min. Finally, the gel was rinsed with 0.1 *M* NaOH when pyridine-S-agarose was used or with 20% (v/v) ethanol in deionized water for the other adsorbents. The procedure described above was repeated at 75% and 50% of the original salt concentrations in addition to the 100% concentration.

2.5. Recovery studies

These studies were performed in the same manner as the salt-dependent adsorption capacity measurements. However, following the elution with 0.1 *M* Tris buffer, the adsorbents were

eluted with 30% ethylene glycol, then rinsed with 0.1 *M* NaOH or 1 *M* NaOH. The amount of protein eluted from the column on excluding salt from the elution buffer was determined by amino acid analysis. The content of human serum albumin (HSA) in the fractions collected during recovery studies was determined by a conventional radioimmunodiagnostic technique. The content of immunoglobulin G (IgG) was determined by a nephelometric technique using the array protein system (Beckman Instruments, Fullerton, CA, USA) at the Department of Clinical Immunology, Uppsala University Hospital. The amount of protein that remained on the adsorbent after rinsing with sodium hydroxide was measured by amino acid analysis on hydrolysed samples. Sepharose 6B was used to investigate the influence of the matrix without any ligands.

2.6. Preparation methods for gels

Pyridyl-S-derivatized gels were prepared according to Oscarsson [17]. Different degrees of substitution of butyl, phenyl and octyl groups on agarose were obtained by varying the concentrations of the respective substances, the reaction time and the reaction temperature.

Preparation of phenylagarose

Sepharose 4B was cross-linked and activated by adding 25 ml of 4 *M* NaOH to 50 g of suction-dried gel (prewashed with deionized water) under stirring followed by 0.35 g of $NaBH_4$; 25 ml of epichlorohydrin were added continuously over 1 h to a final volume of 50 ml. After 20 h at room temperature, the gel was rinsed with deionized water on a sintered-glass filter followed by addition of x g of phenol, 0.34 g of $NaBH_4$ and y ml of NaOH of a certain concentration for a specified reaction time and temperature according to Table 1 (phenylagarose).

Preparation of butylagarose

Sepharose 6B was crosslinked and activated by adding 25 ml of 1 *M* NaOH under stirring to 25 g of suction-dried gel (prewashed with deionized water) followed by 0.35 g of $NaBH_4$ and 4 ml of epichlorohydrin. After reaction for 30 min at room temperature, the gel was rinsed with deionized water on a sintered-glass filter followed by addition of x ml of butyl glycidyl ether, 0.17 g of $NaBH_4$ and y ml of NaOH of a certain concentration and during a specified reaction time and temperature according to Table 1 (butylagarose).

Preparation of octylagarose

Octyl glycidyl ether was used as reagent and x ml were added to the reaction mixture followed by 0.17 g $NaBH_4$ and y ml of NaOH according to Table 1. Otherwise the conditions were the same as for butylagarose.

2.7. Hydrolysis of gels for determination of the degree of substitution by NMR

A 20-mg amount of the respective freeze-dried adsorbent was hydrolysed by adding approximately 2 ml of 20% (v/v) deuterated hydrochloric acid (^2HCl) to an NMR tube and heating the tube at 70°C for 2–3 min. After cooling, the solvent was evaporated and deuterated dimethyl sulphoxide (DMSO) was added to the tube for analysis.

2.8. Other methods

Standard proton NMR spectra were obtained by use of a 200-MHz NMR spectrometer (Varian, Palo Alto, CA, USA). Amino acid analyses were performed according to Spackman et al. [18]. All analyses of elements were performed with an NA 1500 automatic nitrogen, carbon and sulphur analyser (Carlo Erba, Milan, Italy).

3. Results

3.1. Dynamic adsorption capacities

The total amount of protein loaded on to the column was approximately two thirds of the maximum dynamic capacity for the respective adsorbent. The maximum dynamic capacities in K_2SO_4 solutions for phenyl-, octyl- and pyridyl-

Table 1
Conditions for the preparation of phenyl-, octyl- and butylagarose with different degrees of substitution

Adsorbent	Degree of substitution (μmol/g)	Amount of phenol (g)	NaOH (ml)	Reaction time (h)	Temperature (°C)
Phenylagarose	220	6	50 (1 *M*)	24	22–25
	366	10	50 (1 *M*)	24	22–25
	923	4	50 (2 *M*)	6	40
	1066	10	50 (2 *M*)	10	22–25
		Octylglycidyl ether (ml)			
Octylagarose	9	2	10	20	22–25
	20	6	25	20	22–25
	48	10	25	20	22–25
	65	10	25	20	40
	93	10	25	20	60
		Butylglycidyl ether (ml)			
Butylagarose	67	5	25 (4 *M*)	20	22–25
	134	20	25 (4 *M*)	20	22–25
	300	30	50 (4 *M*)	6	40
	546	25	50 (1 *M*)	5	70

S-agarose were found to be 28.4, 23.5 and 21.00 mg of protein per ml of wet gel, respectively. This corresponds to a respective degrees of substitution of 1400, 48 and 1028 μmol/g freeze-dried adsorbent.

3.2. Salt-dependent adsorption capacities

To facilitate our discussion we introduce a parameter that we call the "salt-dependent adsorption capacity" (SAC). SAC is defined as the percentage of the protein adsorbed that can be released from the adsorbent by omitting the salt from the elution buffer.

The effect of the type of salt on the capacity to adsorb serum proteins was studied for four different adsorbents in the presence of five types of salts at one or more salt concentrations. The selected degree of substitution for each gel was 1400 μmol/g freeze-dried adsorbent for phenyl-Sepharose, 48 μmol/g for octyl-Sepharose, 923 μmol/g for pyridyl-S-agarose and 300 μmol/g for butylagarose.

As demonstrated in Fig. 1, pyridyl-S-agarose has the same or a higher SAC for adsorbing proteins than most of the other hydrophobic adsorbents for all the salts studied. When sodium acetate was used in the 1–2 *M* concentration range, SAC ranged from 26.8 to 42.8% of the total applied protein. The corresponding values for phenyl-Sepharose were in the range 13.5–22.9%. Octyl-Sepharose adsorbed smaller amounts of serum proteins compared with butylagarose (see Fig. 1a).

In 0.2–0.6 *M* Na_2SO_4 the SAC values for pyridyl-*S*-agarose were found to be in the range 10.6–40.8% whereas these values for butyl-, octyl- and phenyl-Sepharose were 1.9–16, 0.3–28.8 and 8.7–31.8%, respectively (see Fig. 1b). In 0.75–3 *M* ammonium acetate the SAC values

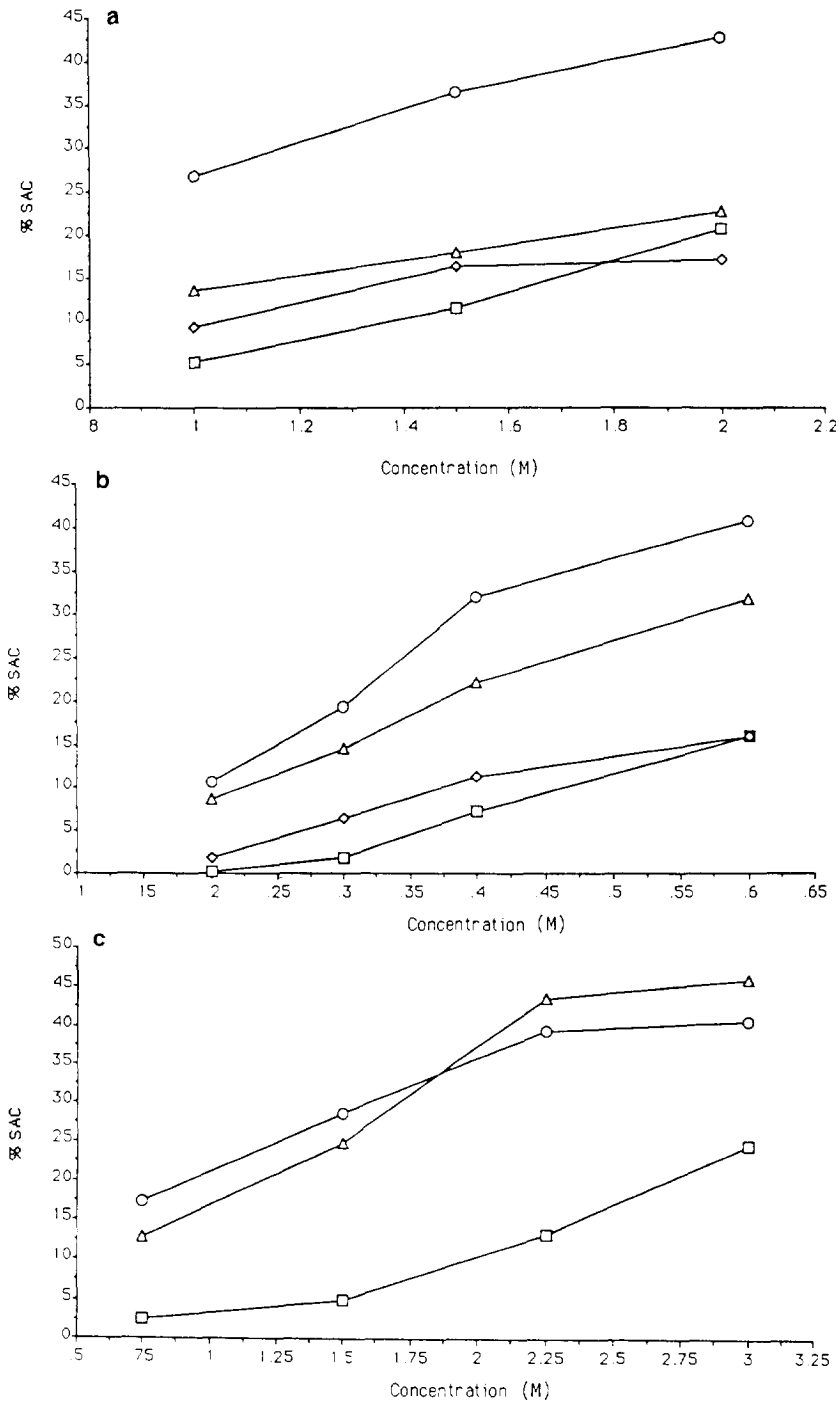

Fig. 1. (a) Percentage of material eluted from a column (3 × 1 cm I.D.) on changing the concentration of NaOAc in the elution buffer (SAC). The protein solution was loaded on the column in the presence of respective salt and salt concentration with a flow-rate of 0.64 ml/min. The protein was eluted at various concentrations of NaOAc in 0.1 *M* Tris buffer (pH 7.5). □ = octyl-Sepharose; ◇ = butylagarose; ○ = pyridyl-S-agarose; △ = phenyl-Sepharose. (b) As (a) except that Na_2SO_4 was used instead of NaOAc. (c) As (a) except that NH_4Cl was used instead of NaOAc.

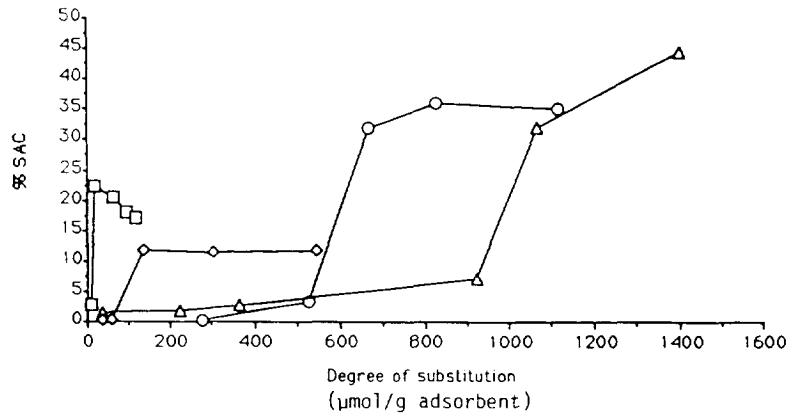

Fig. 2. SAC as a function of the degree of substitution of the ligand (μmol/g adsorbent). Column dimensions, flow-rate and symbols as in Fig. 1. Sample: 0.2 ml of serum diluted with 0.1 *M* Tris buffer (pH 7.5) to a total volume of 1.0 ml. K_2SO_4 was added to a final concentration of 0.5 *M*. Solvent: 0.1 *M* Tris–HCl (pH 7.5) containing 0.5 *M* K_2SO_4. Elution: 0.1 *M* Tris–HCl (pH 7.5).

for pyridyl-S-agarose were found to be in the range 17.2–40.9% whereas for octyl-Sepharose SAC was 1.02–24.7% and for phenyl-Sepharose 12.7–46.3% (see Fig. 1c).

3.3. SAC as a function of degree of substitution

As shown in Fig. 2, maximum capacity was reached at a lower degree of substitution for long aliphatic apolar groups than for a short group. Thus, the degrees of substitution for reaching saturation values were 150 and 20 μmol/g dry gel for butylagarose and octyl-Sepharose, respectively. More than 650 μmol of mercaptopyridine per gram of carbohydrate were required to attain the plateau value for pyridyl-S-agarose, whereas for phenyl-Sepharose a plateau value was not reached even at a degree of substitution as high as 1400 μmol/g carbohydrate.

3.4. Desorption efficiency as a function of the degree of substitution

Fig. 3 illustrates the fraction of protein remaining on the adsorbent after omitting salt from the buffer, as determined by amino acid

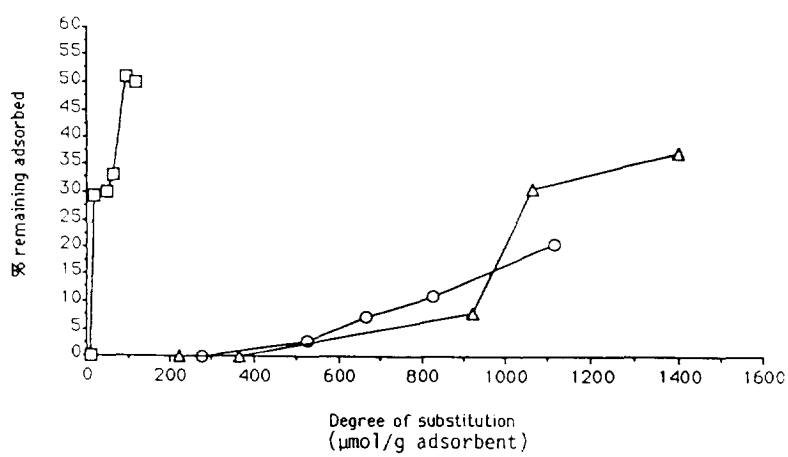

Fig. 3. Percentage of applied protein remaining on the adsorbents after omission of salt (0.5 *M* K_2SO_4) from the solvent. Conditions as in Fig. 2; symbols as in Fig. 1.

analysis. It was found that 2.7–20.7% of the proteins applied on the pyridine-S-adsorbent were virtually irreversibly adsorbed when the degree of substitution was in the range 500–1115 μmol/g adsorbent. The corresponding values for phenyl-Sepharose were 8.1–34.6% at a degree of substitution between 923 and 1400 μmol/g and 29.3–50.0% for octyl-Sepharose when the degree of substitution was 20–116 μmol octyl groups/g dry adsorbent.

3.5. Recovery studies

To obtain more complete information about the recoveries from the different adsorbents, we determined (1) the percentage of the applied proteins that passed through the column, (2) those which were eluted with omission of salt from the elution buffer and (3) those which were eluted with ethylene glycol included in the buffer. Only K_2SO_4 and NaCl as adsorption promoters were studied (see Table 2). Yields between 93 and 110% were obtained as determined by measuring A_{280} and amino acid analyses. The efficiency of 0.1 and 1 *M* NaOH solution in removing proteins adsorbed from the pyridine-S-gel was determined by amino acid analyses. For pyridine-S-agarose, 0.5–1% of the total applied protein remained adsorbed after rinsing the adsorbent with ethylene glycol followed by 0.1 *M* NaOH. The corresponding values were 25% for octyl-Sepharose, 8.3% for phenyl-Sepharose and <1% for butylagarose.

As much as 4.7–5.3% of the applied proteins remained adsorbed on the gel even after treatment of phenyl-Sepharose with 1 *M* NaOH; the corresponding level for octyl-Sepharose was 19.2–21.1%. Pyridyl-S-agarose stands out as a far superior adsorbent; only 0.3–0.4% of applied proteins remained adsorbed after treatment with 1 *M* NaOH.

3.6. Adsorption capacities for IgG and HSA on respective adsorbent

The amounts of HSA and IgG were determined in the fractions obtained after omission of salt from the buffer (operation 2, Table 3). The results clearly show the differences in specificity for HSA and IgG.

Table 2
Recovery studies on serum proteins

Operation[a]	Octyl-Sepharose (48 μmol/g)[b]		Butylagarose (300 μmol/g)[b]		Sepharose 6B		Phenyl-Sepharose (1400 μmol/g)[b]		Pyridyl-S-agarose (1028 μmol/g)[b]	
	K_2SO_4	NaCl	K_2SO_4	NaCl	K_2SO_4	NaCl	K_2SO_4	NaCl	K_2SO_4	NaCl
1	50.2	44.1	87.2	83.4	96.7	92.0	26.8	21.2	56.4	48.0
2	11.6	27.2	6.5	9.6	0.7	3.3	47.3	59.4	31.5	35.0
3	11.7	6.2	1.3	0.8	<1	1.6	12.6	10.0	7.9	5.2
4	0.8	7.1	1.0	0.6	<1	<1	1.9	0.5	3.0	7.2
5	25.0	25.7	0.4	0.7			8.3	6.1	1.4	0.9
Total adsorbed[c]	49.1	66.3	9.2	10.4	0.7	4.9	70.1	76	43.8	48.3
Yield (%)	99.3	110.4	96.4	93.8	97.4	96.9	96.9	97.2	100.2	96.3

Recoveries are given as a percentage of the amount of protein applied.

[a] 1 = Proteins non-adsorbed from 1 ml of serum [diluted 1:5 with 0.1 *M* Tris buffer (pH 7.5)] in the presence of 0.5 *M* K_2SO_4 or 3 *M* NaCl. 2 = Desorption of proteins from the respective adsorbent by omitting salt from the elution buffer. 3 = Desorption of proteins by elution with 30% (v/v) ethylene glycol. 4 = Proteins remaining after rinsing of the adsorbent with 0.1 *M* NaOH. 5 = Amino acid analysis of proteins left on the gel.

[b] Degree of substitution of adsorbent with ligand.

[c] Total adsorbed is the sum of the figures from operations 2, 3, 4 and 5 and gives information about the total amount of protein which was adsorbed on the respective adsorbent. The amount of proteins passing through in operations 1–4 was determined from A_{280} values.

Table 3
Amounts of human IgG and HSA in the fractions obtained when proteins were desorbed from the adsorbents by omitting salt from the elution buffer

Material	Octyl-Sepharose (48 μmol/g)[a]		Butylagarose (300 μmol/g)[a]		Phenyl-Sepharose (1400 μmol/g)[a]		Pyridyl-S-agarose (1028 μmol/g)[a]	
	K_2SO_4	NaCl	K_2SO_4	NaCl	K_2SO_4	NaCl	K_2SO_4	NaCl
Human IgG (μg)	–[b]	360	200	–[b]	1900	1800	1570	1380
HSA	1200	1650	330	30	6000	6050	15	24

[a] Degree of substitution of adsorbent with ligand.
[b] Below the detection limit.

4. Discussion

This study was carried out to evaluate the effect of different salts on protein adsorption on amphiphilic agarose-based adsorbents and to test the effectiveness of different methods of protein desorption. The adsorbents compared are not purely hydrophobic, which is why we use the term "amphiphilic" to indicate the complexity of the adsorption involved in the chromatographic process. Serum was chosen as a model for both capacity and selectivity studies. This selection makes the evaluation more complicated, but at the same time more informative, since serum contains proteins with a wide range of physicochemical characteristics.

We determined the salt-dependent adsorption capacities, i.e. how much of the adsorbed proteins becomes released from the adsorbent on omitting salt from the buffer solution. The determination of dynamic capacity gave valuable, but not complete, information about the efficiency of the chromatographic process, as illustrated by our results. The dynamic capacity was nearly the same for octyl-, phenyl- and pyridyl-S-agarose, but the salt-dependent adsorption capacity differed among these gels. These results reflect differences in desorption efficiencies and selectivity characteristics of the adsorbents. The salt-dependent adsorption capacity was high for pyridyl-S-agarose. The results of the studies on the effect of different salts show that, for most of the salts investigated, the proteins remain on the phenyl and octyl gels after omitting salt from the elution buffer. Effective rinsing of the adsorbent is extremely important, particularly when the adsorbent will be reused. Therefore, we investigated use of ethylene glycol followed by 0.1 or 1 *M* NaOH for elution. Even after treatment with 1 *M* NaOH, a large amount of protein remained adsorbed on octyl-Sepharose and phenyl-Sepharose. In contrast, the pyridyl-S-agarose adsorbent under the same conditions eluted with high efficiency.

These observations reveal a serious weakness in the commercially available hydrophobic adsorbents for bioprocessing where washing with 1 *M* NaOH is a standardized stripping method. There is substantial risk of contamination of fractions when reusing the adsorbent. In addition, the adsorption behaviour can be drastically changed when a protein-covered hydrophobic adsorbent is reused and drastic reductions in adsorption capacity and protein selectivity can occur.

We know from earlier investigations [17,19] that HSA is preferably adsorbed on alkyl ether adsorbents and IgG on pyridyl-S-agarose in the presence of K_2SO_4 or NaCl. This difference was quantified by determination of the total amount of IgG and HSA in those fractions that contained proteins released on deleting salt from the elution buffer. According to these results (see Table 3), a large amount of HSA was adsorbed on both phenyl- and octyl-Sepharose compared with pyridyl-S-agarose. The difference in selec-

tive behavior for IgG and HSA of the different gels strongly indicates that specific interactions occur, especially in the case of pyridyl-S-agarose. However, the side-groups in the proteins involved in the interactions remain unknown.

In order to optimize the downstream processes, the relationship between the salt-dependent adsorption capacity and the efficiency of the desorption of proteins as a function of the degree of substitution with ligands has to be known. From the data in Figs. 2 and 3, we can conclude that, for the pyridyl-S-adsorbent, in the presence of potassium sulphate, the optimum relationship between high salt-dependent adsorption capacity and good desorption efficiency will be obtained at a substitution degree of 650–700 μmol/g of pyridine thioether groups. Under these conditions, the salt-dependent adsorption capacity for serum proteins is 32–35% and only 5–7% remains on the gel after omission of the salt. For octyl-Sepharose the optimum degree of substitution is 20 μmol of alkyl groups, which gives 23% SAC with 29–30% of protein remaining on the gel. The corresponding values for phenyl-Sepharose at a ligand concentration of 1400 μmol/g dried adsorbent are 45% SAC and 36–38% of serum proteins remaining on the gel on omitting salt from the buffer.

The values obtained for butylagarose were too few to be included in Fig. 3. However, from studies performed on butylagarose with a degree of substitution corresponding to 300 μmol/g adsorbent, the SAC value was 11% with 2–3% of the protein remaining on the gel. To compensate for the low capacity for butylagarose, the length of the carbon chain or the bed size must be increased. Increasing the chain length of the ligands will decrease the desorption efficiency.

5. Conclusions

The commercially available hydrophobic interaction chromatography (HIC) adsorbents octyl- and phenyl-Sepharose, are too hydrophobic to be ideal for downstream processes, as too much protein remains on the column after omitting salt from the elution buffer. Even after rinsing the adsorbents with sodium hydroxide the amount of protein remaining on the gel is unacceptable. Use of the less hydrophobic butylagarose will not improve the situation because the capacity of this adsorbent is too low. It is surprising that these undesirable properties have not previously been brought to the general attention of users. New types of less hydrophobic adsorbents need to be developed that can fulfil the demand for a high salt-dependent adsorption capacity and at the same time maintain a high desorption efficiency. Pyridyl-S-agarose is superior in several respects to the conventional adsorbents used for hydrophobic interaction chromatography, but it differs in some respects in its affinity properties. The influence of different salts on the type of proteins adsorbed to respective adsorbents is under investigation.

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