ELSEVIER

Journal of Chromatography A, 764 (1997) 193–200

JOURNAL OF CHROMATOGRAPHY A

Polyol-promoted adsorption of serum proteins to amphiphilic agarose-based adsorbents

Nathalie Berna[a,b], Patrick Berna[a,b,*], Sven Oscarsson[a,b]

[a]*Biochemical Separation Centre, University of Uppsala, BMC, Box 577, S-751 23 Uppsala, Sweden*

[b]*Department of Chemical Engineering, Mälardalen University, Box 325, S-631 05 Eskilstuna, Sweden*

Received 26 July 1996; revised 23 October 1996; accepted 23 October 1996

Abstract

We tested the promotion of protein adsorption onto amphiphilic agarose-based adsorbents by addition of high concentrations of polyols during the adsorption phase. C_3- to C_5-polyols were inefficient in promoting protein adsorption, whereas some of the C_6-polyols studied (sorbitol, dulcitol and mannitol) could promote serum protein adsorption onto mercaptomethylene pyridine-derivatized agarose, octyl- and phenyl-Sepharose. Sorbitol was the most potent protein adsorption promoter, with a direct relation between the amount of protein adsorbed and the concentration of sorbitol. For each chromatographic gel, the effects of increasing concentrations of sorbitol or sodium sulfate on protein adsorption were similar and two-dimensional electrophoresis revealed the preservation of the protein adsorption specificity whether sorbitol or sodium sulfate was used. These results show that a water-structuring salt or a polyol can promote protein adsorption in the same manner, presumably by a related mechanism.

Keywords: Adsorption; Stationary phases, LC; Proteins; Polyols

1. Introduction

Cosolvents are usually added to the mobile phase to alter the chromatographic behaviour of proteins. The water-structuring salts have been known for a long time to promote protein binding to hydrophobic [1] and other more amphiphilic adsorbents [2,3], elution being performed by omission of the salt. These salts, when present in solution with proteins, are known to be excluded from the protein surface [4], and this property has been applied to rationalize the promotion of protein binding to an amphiphilic matrix by salts. Polyols also belong to the category of cosolvents that are preferentially excluded from the protein surface [4]. In an earlier study we showed that sorbitol could also promote the adsorption of proteins onto an amphiphilic matrix, the mercaptomethylene pyridine-derivatized agarose gel (MP-gel), whereas glycerol, another polyol, could not [5].

Therefore, we decided to investigate systematically the effect of polyols on the promotion of serum protein adsorption onto the MP-gel and also onto hydrophobic gels (octyl- and phenyl-Sepharose). The protein adsorption promoted upon addition of sorbitol or the water-structuring salt sodium sulfate at various concentrations has been compared, and their

*Corresponding author: Present address: Laboratories Jouveinal, Department of Biochemistry and Biotechnology, 3-9 Rue des Loges, B.P. 100, F-94265 Fresnes Cedex, France

PII S0021-9673(96)00933-8

possible influence on protein adsorption selectivity was studied by two-dimensional (2-D) electrophoresis.

2. Experimental

2.1. Chemicals

Glycerol, sodium sulfate, NaOH and pentaerythritol were from Merck (Darmstadt, Germany); Trizma base (Tris), MOPS (3-morpholinopropanesulfonic acid), bovine serum albumin, adonitol, xylitol, *meso*-erythritol, dulcitol and threitol were from Sigma (St. Louis, MO, USA); *meso*-inositol was from Fluka (Buchs, Switzerland); D-mannitol was from DIFCO (Detroit, MI, USA); sorbitol was from Carl Roth (Karlslruhe, Germany); and phenyl-Sepharose and octyl-Sepharose were from Pharmacia (Uppsala, Sweden).

2.2. Samples

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

2.3. Preparation methods

3-(2-Pyridylmethylenethio) - 2 - hydroxypropylagarose (mercaptomethylene pyridine-derivatized agarose) was prepared according to Berna et al. [5]. The ligand concentration was found to be 950 and 885 μmol g^{-1} of dried product, as calculated from the sulfur and nitrogen content, respectively (see below).

2.4. Chromatography studies

A 3×1 cm I.D. column was packed with the gel to be tested. The same MP-gel was used during the whole study, whereas a new phenyl- or octyl-Sepharose gel was used for every chromatographic run to avoid effects of irreversible adsorption [2]. Adsorption, desorption and regeneration of the adsorbent was programmed by means of a fast protein liquid chromatography (FPLC) system from Pharmacia. A flow-rate of 0.64 ml/min was used with all buffers except those containing sorbitol at 2 *M*, 3 *M* and 3.5 *M* (0.2 ml/min), 4 *M*, 4.25 *M* and 4.5 *M* (0.1 ml/min), and at 5 *M* (0.05 ml/min), to avoid back pressure problems. A solution of cosolvent of defined concentration in 0.1 *M* Tris (pH 7.5), was used for equilibration of the column. Serum (1 ml) containing the cosolvent at the defined concentration was loaded onto the column and then washed with the equilibration buffer, followed by 0.1 *M* Tris buffer (pH 7.5), and finally, by 0.1 *M* NaOH. Sodium sulfate was tested from 0 to 0.55 *M*, sorbitol from 0 to 5 *M*, and the other polyols were tested at the following concentrations: 3.5 *M* and 7.6 *M* glycerol, 3.5 *M* erythritol, 4.1 *M* threitol, 3.5 *M* xylitol, 0.4 *M* pentaerythritol, 2.6 *M* adonitol, 0.5 *M* inositol, 0.8 *M* mannitol and 0.2 *M* dulcitol. These polyol concentrations were about 80–90% of the maximum concentration soluble in 0.1 *M* Tris.

2.5. Analytical methods

The gel elementary analyses 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 with bovine serum albumin as a standard [6]. The results were expressed either in percentage of the total proteins recovered in the flow-through fraction and the elution step, or in mg of proteins retained. The proteins eluted with 0.1 *M* NaOH were not taken into account in this value, since they represented less than 3% of the total applied protein. The total protein recovery in each chromatographic run always exceeded 90% of the total applied protein. Each unit of data used in the tables and figures represents the average of at least two analyses. The results did not vary more than ±5%.

Two-dimensional electrophoretic analysis was done on the fractions adsorbed on the MP-gel by using sodium sulfate at 0.35 *M* and sorbitol at 5 *M*, on the octyl-Sepharose by using sodium sulfate at 0.5 *M* and sorbitol at 4.25 *M*, and on the phenyl-Sepharose gel by using sodium sulfate at 0.2 *M* and sorbitol at 3 *M*. The level of adsorbed proteins was approximately the same for the six fractions (1.7–2 mg protein). The samples were first dialysed against 5 m*M* MOPS–acetate buffer (pH 7.6), then freeze-

dried and redissolved in 50 m*M* MOPS–acetate buffer (pH 7.6). Electrophoresis of 2 μg of protein were accomplished using the Immobiline drystrip kit pH 3.5–10L, 11 cm and ExcelGel sodium dodecyl sulfate (SDS) gradient 8–18% from Pharmacia following the manufacturer's instructions. The electrophoretic gels were silver-stained at 22°C for 6 min exactly and scanned on a Bio-Rad GS-700 imaging densitometer (Hercules, CA, USA) using the Molecular analyst 2-D polyacrylamide gel electrophoresis (PAGE) software version 1.0 from Bio-Rad for image analysis. Image analysis allowed quantification of the protein relative percentages. 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) and radioimmunodiagnostic (Pharmacia diagnostics) techniques, respectively.

The surface tension was determined for the different solutions at 23°C according to the Wilhelmy plate technique [7].

3. Results and discussion

In an earlier study, we showed that sorbitol could promote the adsorption of proteins onto a MP-gel, an electron-donor–acceptor adsorbent which is normally used under salt-promoted conditions, whereas other polyols tested, such as glycerol and pentaerythritol, could not [5]. Here, we systematically investigated the effect of this cosolvent family of polyols on several amphiphilic adsorbents. The first study was carried out on the MP-gel. The effect of the polyol chain length on protein binding was tested using linear polyols of increasing numbers of carbon atoms carrying hydroxyl functions: glycerol ($C_3H_8O_3$), erythritol and threitol ($C_4H_{10}O_4$), xylitol and adonitol ($C_5H_{12}O_5$), mannitol, dulcitol and sorbitol ($C_6H_{14}O_6$), and also a ramified polyol, pentaerythritol ($C_5H_{12}O_4$) and a cyclic polyol, inositol ($C_6H_{12}O_6$). To maximise their effect, the polyols were tested at concentrations close to the maximal concentration soluble in 0.1 *M* Tris. The serum sample containing one of the polyols to be studied was injected onto a column equilibrated with the buffer containing the polyol. The proteins adsorbed on the MP-gel were then eluted by omitting the cosolvent from the buffer. The amount of protein adsorbed was expressed as a percentage of the total proteins recovered in the flow-through fraction and in the elution step (Table 1). None of the C_3- to C_5- or cyclic C_6-polyols could promote protein adsorption onto this gel, whereas 0.1–5.2% of the injected proteins were adsorbed when linear C_6-polyols were used. Among the C_6-polyols, very small quantities of proteins could be adsorbed with dulcitol or mannitol but sorbitol had a clear potency for protein adsorption promotion. These results confirm that not all of the polyols are equally effective in promoting protein adsorption onto the MP-gel. Although the limited solubility of some of the polyols precluded a fair comparison of all of them, study of the protein adsorption for the polyols tested at 3.5 *M* (Table 1) suggested a possible requirement for at least 6 carbon atoms bearing hydroxyl functions to promote protein adsorption. Polyols, like water-structuring salts, are excluded from native proteins [4]. The thermodynamic consequence is an unfavourable increase of the free energy of a protein in the presence of these cosolvents. This promotes the binding of a protein to a ligand because of the smaller surface area of the complex exposed to them; i.e., the bound form of the protein is thermodynamically more

Table 1
Promotion of protein adsorption by different C_3- to C_6- polyols on the MP-gel

C_n	Polyol	Polyol concentration (*M*)	Proteins adsorbed (%)
C_3	Glycerol	3.5	0
		7.6	0
C_4	Erythritol	3.5	0
	Threitol	4.1	0
C_5	Xylitol	3.5	0
	Pentaerythritol[a]	0.4	0
	Adonitol	2.6	0
C_6	Inositol[b]	0.5	0
	Mannitol	0.8	0.1
	Dulcitol	0.2	0.2
	Sorbitol	3.0	0
		3.5	0.6
		5.0	5.2

[a] Pentaerythritol is ramified.
[b] Inositol is cyclic.

stable. As it is known that the more carbon atoms bearing a hydroxyl function that a polyol has, the more it is excluded from native proteins, probably due to an increased hydrophilic character [8], this could explain why C_6-polyols could promote more protein adsorption than shorter ones. Unfortunately, polyols with more than 6 carbon atoms bearing hydroxyl functions are not commercially available and this impeded the verification of the requirement of at least 6 carbon atoms bearing hydroxyl function to promote protein adsorption. Among the C_6-polyols, sorbitol promoted at least 25-fold more protein adsorption to the gel than did dulcitol or mannitol. The higher potency for protein adsorption promotion of sorbitol could be explained by its greater solubility which allowed a higher concentration to be tested. Indeed, sorbitol at concentrations lower than 3 *M* was unable to promote protein adsorption. A quantitative effect is therefore demonstrated for sorbitol as has also been described for other families of protein adsorption promoters.

To test whether C_6-polyols were also potent in promoting protein adsorption onto chromatographic matrices for hydrophobic interactions, promotion of protein adsorption onto octyl- and phenyl-Sepharose was studied. The percentage of proteins adsorbed onto these gels and onto the MP-gel is compared in Table 2. A constant ratio of injected protein per unit of gel volume and similar ligand density for all the studied gels, made this comparison possible. Except for the cyclic C_6-polyol on the MP-gel, all other combinations resulted in protein adsorption. The most significant amount of protein was adsorbed on the phenyl-Sepharose gel, followed by the octyl-Sepharose gel, and the lowest adsorption occurred on the MP-gel. The greater effect of polyols on the promotion of protein adsorption onto the "hydrophobic" gels phenyl-Sepharose and octyl-Sepharose gels than on the MP-gel was expected. Indeed, as polyols are preferentially excluded from hydrophobic patches of the protein [4], they also should be expelled from the hydrophobic ligands, which in turn favors an easier binding of proteins to the ligand [9]. As observed previously for the MP-gel, sorbitol was the C_6-polyol which gave the highest protein adsorption onto the two hydrophobic gels at the concentration tested. Again the limited solubility of the other C_6-polyols did not allow a valuable comparison of all of the C_6-polyols. But the higher protein adsorption obtained on the hydrophobic gels confirmed that C_6-polyols were potent promoters, whereas xylitol and glycerol were not (results not shown). As sorbitol was the only polyol which showed a possible application to protein adsorption promotion, we selected it for further studies.

To study the influence of the cosolvent concentration on promotion of protein adsorption, and to gather a better understanding of its mechanism of action, we compared the adsorption of proteins onto the three gels using sorbitol and sodium sulfate at different concentrations during the adsorption phase (Fig. 1). The water-structuring salt sodium sulfate was selected as a reference, due to its frequent use in salt-promoted adsorption chromatography. Protein adsorption on the three gels was augmented by increasing the concentration of sorbitol, as observed with sodium sulfate. But, compared to sodium sulfate, sorbitol was less potent in promoting protein adsorption by at least one order of magnitude. Nevertheless, the responses to increasing concentrations of sodium sulfate or sorbitol in protein adsorption onto the MP-gel were comparable, as was

Table 2
C_6-polyol promotion of protein adsorption on the MP-gel, octyl-Sepharose and phenyl-Sepharose gels

Promoter	Surface tension value (mN/m)	Proteins adsorbed (%)		
		MP-gel	Octyl-Sepharose	Phenyl-Sepharose
Inositol (0.5 *M*)	67.0	0	0.6	1.3
Mannitol (0.8 *M*)	67.9	0.1	0.6	0.7
Dulcitol (0.2 *M*)	73.1	0.2	0.3	0.4
Sorbitol (4 *M*)	74.1	1.5	5.8	8.0[a]
Tris (0.1 *M*)	72.7			
Water	71.9			

[a] With sorbitol at 3.5 *M*.

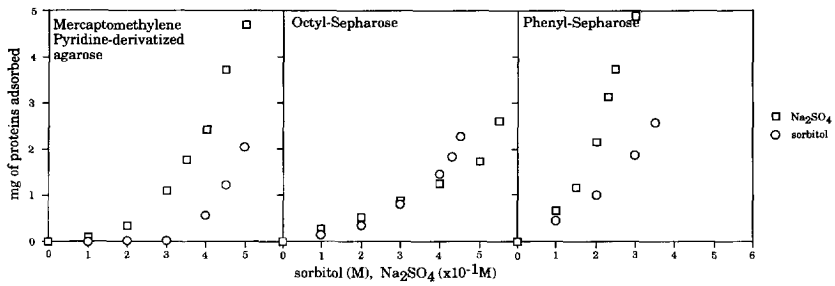

Fig. 1. Amount of proteins adsorbed onto the MP-gel (mercaptomethylene pyridine-derivatized agarose), octyl-Sepharose and phenyl-Sepharose gels versus the concentration of sorbitol or sodium sulfate used in the adsorption buffer.

also true for the hydrophobic gels. On the contrary, the effect on protein adsorption of increasing concentrations of one cosolvent was different from one gel to another. Indeed, no protein adsorption onto the MP-gel occurred at low concentrations of cosolvent (sorbitol or sodium sulfate) but did at higher concentrations, whereas on the two hydrophobic gels the adsorption started to increase linearly with the cosolvent concentration, with an increase in the slope after a certain concentration, the average slope being much higher on the phenyl-Sepharose gel. These results show that for one gel, increasing concentrations of sorbitol or sodium sulfate created the same response in promoting protein adsorption and suggest a similar mechanism of protein adsorption promotion for both of them.

A related mechanism for cosolvent promotion of protein adsorption would suggest unchanged protein adsorption specificity whether sodium sulfate or sorbitol are used. We therefore studied the proteins adsorbed onto the chromatographic gels by two-dimensional electrophoresis of fractions containing a comparable quantity of proteins. The patterns of the proteins adsorbed onto the MP-gel, octyl-Sepharose and phenyl-Sepharose gels upon using sodium sulfate or sorbitol to promote the adsorption are represented in Fig. 2. Although the protein patterns are very different from one chromatographic gel to another, for each gel the patterns are very similar, whether sorbitol or sodium sulfate was used to promote the adsorption, i.e., the same proteins became adsorbed whichever cosolvent was used. The different serum proteins present in these fractions were characterized by image analysis of the gels and comparison with a standard 2-D protein map of human serum and quantified. The relative percentages of the different serum proteins are shown in the Table 3. The immunoglobulins were the major proteins retained on the MP-gel, representing 88% of the total retained proteins. Apart from albumin (1%), fibrinogen (2%), α2-macroglobulin (1%), and C3-complement (1%), all other proteins represented less than 0.5% each. On the octyl-Sepharose gel, as expected, the main proteins retained, such as albumin, haptoglobins and apolipoproteins, were hydrophobic. The proteins retained on the phenyl-Sepharose gel showed a more complex pattern and represented, in fact, a mixture of the proteins retained on the MP-gel and on the octyl-Sepharose gel. This might also be expected of a gel with a mixed-mode mechanism of hydrophobic and electron-donor–acceptor interactions as phenyl-Sepharose has been described. But for our study, the most striking point was that for any purification chromatographic gel, the relative percentages of each protein adsorbed were similar whether sorbitol or sodium sulfate was used to promote adsorption. Few minor proteins showed significant changes. Therefore, the equivalent protein adsorption selectivity for each gel, whether we used sodium sulfate or sorbitol, taken together with the concentration dependence of the adsorption promotion by sorbitol and the similar effects on protein adsorption of increasing concentrations of sodium sulfate and sorbitol, strongly suggested that sodium sulfate and sorbitol promote protein adsorption by a similar mechanism. The effects of water-structuring salts on the protein binding in hydrophobic interaction chromatography are explained by their surface tension increment of water [10]. According to a generalisation proposed

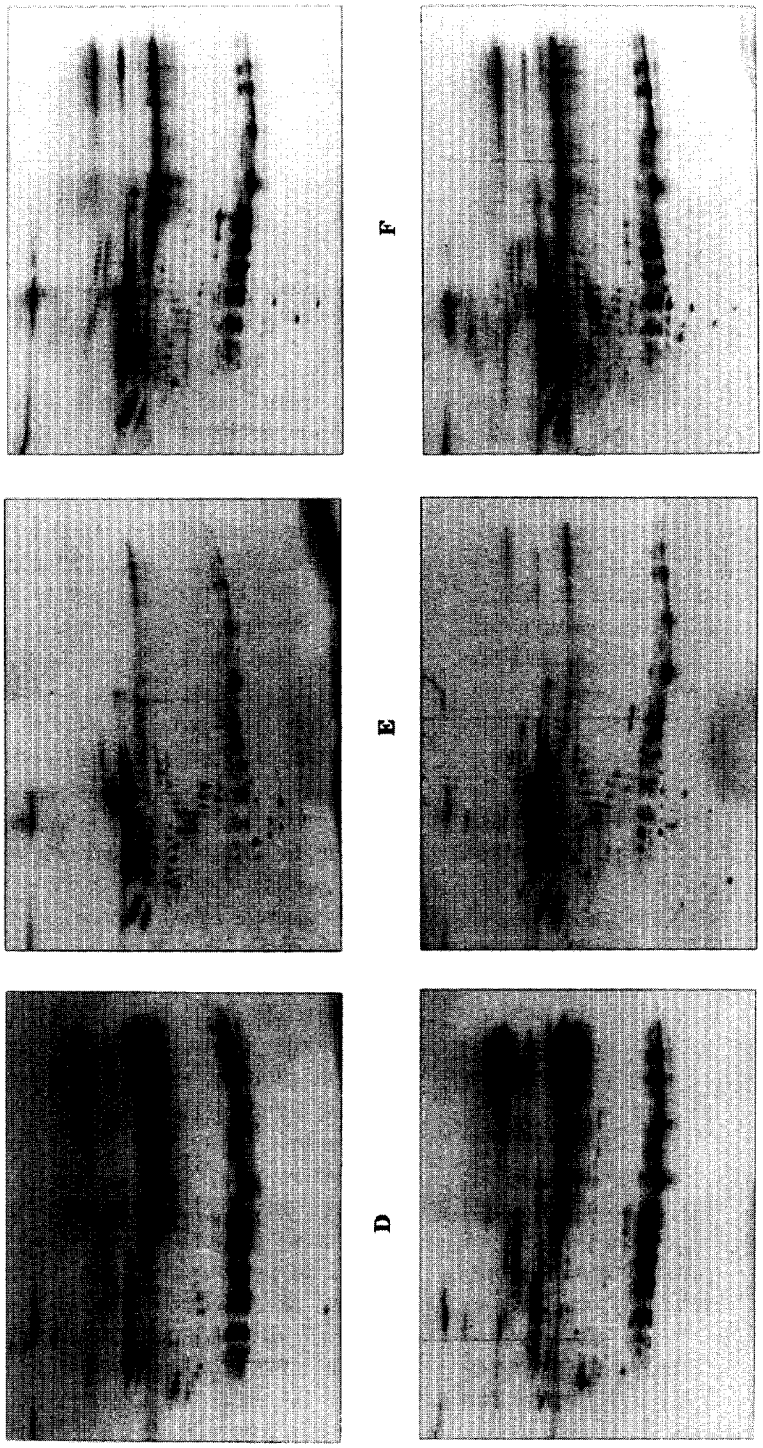

Fig. 2. Two-dimensional electrophoretic patterns of protein adsorbed onto the MP-gel (mercaptomethylene pyridine-derivatized agarose) (A and D), octyl-Sepharose (B and E) and phenyl-Sepharose gels (C and F) by using sodium sulfate (A, B and C) or sorbitol (D, E and F) for the promotion of protein adsorption. The gels are shown with the acidic p*I* to the left and with low molecular mass at the bottom.

Table 3
Relative percentages[a] of human serum proteins adsorbed on the MP-gel and octyl-Sepharose and phenyl-Sepharose gels by using sodium sulfate or sorbitol to promote the adsorption

Proteins	MP-gel		Octyl-Sepharose		Phenyl-Sepharose	
	Na_2SO_4	Sorbitol	Na_2SO_4	Sorbitol	Na_2SO_4	Sorbitol
IgG	72.9	74.3	12.3	13.9	40.0	41.0
IgA	11.9	8.6	11.1	11.9	12.2	10.2
IgM	2.7	6.1	1.1	1.1	1.6	1.6
Total inmunoglobulins	87.5	89.0	24.5	26.9	53.8	52.8
Albumin[b]	0.8	1.2	56.2	54.8	23.2	24.5
Fibrinogen	2.0	2.3	0.3	1.1	2.9	1.9
α_2-Macroglobulin	1.2	0.8	1.4	0.5		1.1
C_3-complement	1.0	1.1			0.5	0.5
α_1-Antitrypsin			0.5	1.6	2.2	0.7
Plasminogen			1.3			
α_1-Antichymotrypsin			1.8	1.5	2.9	2.1
α_2HS-glycoprotein			1.0	1.7	1.0	2.0
Haptoglobins			3.2	2.5	2.4	2.0
Gc-globulin			0.9	0.6		
Hemopexin			1.1	0.5		
Apo-lipoproteins			1.0	1.0		
Non-identified proteins[c]	7.5	5.6	6.8	7.3	11.1	12.4

[a] Quantification by image analysis of a 2-D electrophoregram.
[b] Quantifed by a radioimmunodiagnostic technique.
[c] Each non-identified protein represented less than 0.5%.

by Arakawa and Narhi, the compounds that facilitate protein binding to hydrophobic ligands should both be excluded from the native proteins and increase the surface tension of water [9]. Our results suggest that polyols do not fit into this theory, as inositol and mannitol were protein adsorption promoters on all three gels studied (except inositol on MP-gel), however they decrease the surface tension of water (Table 2). Although the incremental surface tension effect could not rationalize these results, the common exclusion of sodium sulfate and sorbitol from the protein surface as the mechanism of protein adsorption promotion is still valid. In the specific case of polyols, the source of exclusion would be as defined by Timasheff [4], their exclusion from the hydrophobic patches of the protein resulting from the high affinity of the polyols for the water-lattice structure. Other theories of exclusion, such as the exclusion volume defined for PEG do not seem to us to apply to low molecular weight components such as polyols.

In conclusion, we have shown that the C_6-polyols could promote protein adsorption onto different amphiphilic chromatographic gels, although on the other side of this chemical family of polyols, small molecules like ethylene glycol and glycerol are known to facilitate elution [9]. Furthermore, the preservation of the protein adsorption specificity whether sodium sulfate or sorbitol was used is a striking fact. This suggests that the type of cosolvent does not influence the protein adsorption mechanism in itself and implies that cosolvents do not take part in the adsorption mechanism, although they promote it. Unfortunately, sorbitol, the most effective polyol promoter, was less effective than sodium sulfate, and its application in protein purification seems limited by its high viscosity. To solve this problem we are now investigating the promotion of protein adsorption by other chemical families which are known to be also excluded from the protein surface.

Acknowledgments

We thank Dr. D. Eaker for linguistic revision of this paper. This work was supported by the Swedish Ministry of Education.

References

[1] J. Porath, L. Sundberg, N. Fornstedt and I. Olsson, Nature, 245 (1973) 465.
[2] S. Oscarsson, D. Angulo-Tatis, G. Chaga and J. Porath, J. Chromatogr. A, 689 (1995) 3.
[3] J. Porath, Biotechnol. Progress, 245 (1987) 14.
[4] S.N. Timasheff, Ann. Rev. Biophys. Biomol. Struct., 22 (1993) 67.
[5] N. Berna, P. Berna and S. Oscarsson, Arch. Biochem. Biophys., 330 (1996) 188.
[6] M.M. Bradford, Anal. Biochem., 72 (1976) 248.
[7] J.D. Andrade, L.M. Smith and D.E. Gregonis, in J.D. Andrade (Editor), Surface and Interfacial Aspects of Biomedical Polymers, Vol. 1, Surface Chemistry and Physics, Plenum Press, New York, NY, 1985, p. 262.
[8] K. Gekko, J. Biochem., 90 (1981) 1643.
[9] T. Arakawa and L.O. Narhi, Biotechnol. Appl. Biochem., 13 (1991) 151.
[10] W. Melander and C. Horvath, Arch. Biochem. Biophys., 183 (1977) 200.