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High-performance liquid chromatography of amino acids, peptides and proteins

CXI[a]. Retention behaviour of proteins with macroporous tentacle-type anion exchangers

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ABSTRACT

The chromatographic behaviour of various proteins with Fractogel-TMAE and LiChrospher-TMAE tentacle-type strong anion-exchange sorbents has been investigated. In particular, the log $\bar{k}$ *versus* log $1/\bar{c}$ (where $\bar{k}$ is the median capacity factor and $\bar{c}$ is the corresponding salt concentration) dependencies for two salt systems (NaCl and KBr) have been documented. The Fractogel-TMAE sorbent exhibited retention characteristics in terms of relative protein charge dependencies (slope Z_c) and affinity values (log K_c, which is log $\bar{k}$ at $c = 10^{-6}$ M, where c is the concentration of the displacer ion) which were similar to the more conventional anion-exchange sorbents such as the Mono-Q sorbent. However, the Z_c and log K_c values obtained with the LiChrospher-TMAE sorbent were significantly decreased for most proteins. Furthermore, proteins such as carbonic anhydrase and myoglobin were not retained at pH values up to 4 and 2 units above their pI values, respectively. These results illustrate the different adsorptive properties of the tentacle-type sorbents compared to other monolayer or polymer layer ion exchangers in terms of accessibility of the charged ligand and the underlying retention mechanism.

INTRODUCTION

Over the past several years, research interest in the development of specially designed macroporous ion-exchangers with enhanced features for the analysis and purification of proteins has been stimulated by two conceptual considerations. The first consideration, epitomised by the work of Afeyan and co-workers [1,2], has lead to considerable interest with micro- and mesoparticulate porous support materials of very large nominal pore diameters, *e.g.* with pore diameters typically in the range 250–500 nm. Ion-exchange sorbents, developed from such macroporous support ma-

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terials, exhibit adsorption characteristics with proteins which are typified by convective rather than diffusive control processes. With this class of high-performance ion-exchangers, high rates of protein adsorption have been reported at very high linear flow velocities [2].

Advancements in the second consideration, namely the evaluation of new criteria for the design and localisation of ligands in particular chemical environments at the surface of macroporous support materials, has lead to significant improvements in biomimetic affinity chromatography, immobilised metal chelate chromatography, and ion-exchange chromatography. Arising from research in this area has been the emergence of the so-called tentacle-type sorbents [3,4]. With this class of sorbent, linear polyelectrolyte chains are initially introduced to the surface of a hydrophilic support material, followed where necessary by subsequent chemical derivatisation reactions. In a previous study, we examined the protein adsorption characteristics of pretreated glass beads and LiChrospher Si 1000 derivatised with N-trimethylaminomethylacrylamide (TMAE-acrylamide) according to the three-step procedure of Müller *et al.* [3,4]. With these tentacle type sorbents, the adsorption data corresponded to Hill-type isotherms, with the Hill coefficients indicating an approximately Gaussian affinity distribution of the binding sites for the protein. The present study extends these investigations through evaluation of the retention dependencies of several proteins chromatographed under gradient conditions on TMAE-Fractogel HW65 (Fractogel-TMAE) and TMAE-LiChrosper Si 1000 (LiChrospher-TMAE) sorbents with two different displacing salt systems.

MATERIALS AND METHODS

Chemicals and reagents

Bovine erythrocyte carbonic anhydrase, sperm whale muscle myoglobin (type III), hen egg white lysozyme (grade 1), bovine ribonuclease A (type III A), bovine insulin and piperazine were obtained from Sigma (St. Louis, MO, USA). The recombinant pig growth hormone (pGH) was available in highly purified form from associated studies carried out in these laboratories. The proteins were purified and characterised as described previously [5]. Sodium chloride and potassium bromide (AnalaR grade) were obtained from BDH (Port Fairy, Australia).

Quartz-distilled water was further purified on a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Buffers were adjusted to pH 9.60 using either hydrochloric acid (specific gravity 1.16, AnalaR grade) or hydrogen bromide (specific gravity 1.46–1.49, AnalaR grade) all of which were purchased from BDH.

Apparatus

All chromatographic procedures were performed with a Pharmacia (Uppsala, Sweden) FPLC system consisting of two P-500 pumps, a V-7 injector and 278-nm fixed wavelength UV monitor coupled to a Perkin Elmer LC1-100 integrator via a Norton digital processor. Isocratic and gradient elution were controlled with a Pharmacia GP-250 solvent programmer. The Fractogel-TMAE and the LiChrospher-TMAE sorbents were obtained as prepacked columns (150 × 10 mm I.D. and 50 × 10 mm I.D., respectively) from E. Merck, Darmstadt, Germany. The Mono-Q sorbent, as prepacked columns, (50 × 5 mm I.D.) was obtained from Pharmacia. The

prepacked LiChrospher- and Fractogel-TMAE and Mono-Q columns were interfaced with the UV detector by zero dead volume connectors. Samples were injected using Scientific Glass Engineering glass syringes at a protein concentration of 5 mg/ml, typically as 10–100 μl injections. The protein solutions were prepared in the elution buffer and prefiltered through 0.22-μm ACRO LC13 Gelman filters.

Chromatographic procedures

The composition of the eluent was 20 mM piperazine, pH 9.60 (eluent A), containing up to 300 mM diplacer salt (eluent B). All eluents were filtered through Millipore 0.45-μm cellulose acetate HAWP04700 filters and degassed. Eluents were used for a maximum of two days before replacement. Protein solutions were prepared by dissolving the purified protein in eluent A at a concentration of 5 mg/ml, and filtered through 0.22-μm ACRO LC13 filters (Gelman Sciences, Sydney, Australia). All chromatographic measurements were replicates ($n > 5$). With the gradient elution experiments, the gradient time was varied between 17.1 and 171 min with a constant flow-rate of 1 ml/min. The column dead time was obtained from the retention time of a salt breakthrough peak whilst the gradient elapse time for the different columns was also determined from plots of the conductivity *versus* time. Column effluent conductivity was measured with a Radiometer Cdm3 conductivity meter equipped with a CDC304 glass electrode. All chromatographic measurements were carried out at ambient temperature (*ca.* 20°C). At the start of each day, columns were equilibrated using a step elution protocol with the relevant eluent combination for at least 60 min. The gradient retention parameters b, log $\bar{k}$ and log $(1/\bar{c})$ (where b is the gradient steepness parameter, $\bar{k}$ is the median capacity factor and $\bar{c}$ is the corresponding salt concentration) were calculated using the Chromcalc program, developed in this laboratory and written in BASIC for IBM XT or AT computers as previously described [5]. Retention data (log $\bar{k}$ and log $1/\bar{c}$) were subjected to iterative regression analysis to determine the slope (Z_c) and the log $\bar{k}$ value at $\bar{c}$ (concentration of the displacer ion) $= 10^{-6}$ M (log K_c).

RESULTS AND DISCUSSION

It is well known that a large variety of mobile phase parameters can be used to influence the retention behaviour of proteins in high-performance ion-exchange chromatography (HPIEC). In particular, variation in the pH and the nature and concentration of the displacing salt are widely used to affect improvement in resolution and recovery. Studies over many years have documented the importance of choosing the most appropriate displacing ion and co-ion in HPIEC. Important aspects of this selection relate not only to the relative affinity of the ion/co-ion for binding to the sorbent, but also what kosmotropic or chaotropic characteristics the individual ions manifest with regard to the water structure associated with the protein [5,6]. In the present study, we were thus interested to compare the influence of KBr as a displacing salt with the more conventional, and popular, choice, NaCl, on the retention behaviour of several proteins eluted from the Fractogel-TMAE sorbent and the Li-Chrospher-TMAE sorbent. Further, we wished to compare the behaviour of these so called 'tentacle' sorbents with the widely used microparticulate sorbent, Mono-Q. The physical characteristics of these columns are summarised in Table I. The proteins

TABLE I

ANION-EXCHANGE RESIN COLUMN CONFIGURATIONS

Sorbent	Column length (mm)	Column diameter (mm)	Particle diameter (μm)[a]	Pore size (Å)
Fractogel-TMAE	150	10	25–40	650
LiChrospher-TMAE	50	10	5	1000
Mono-Q	50	5	10	800

[a] Nominal particle diameter as indicated by the manufacturer.

listed in Table II were eluted from the two 'tentacle-type' anion exchangers by gradient elution from 0–300 mM of displacing salt, using gradient times ranging from 17.1 to 171 min at a constant flow-rate of 1 ml/min. Similar conditions were used for the Mono-Q experiments. The retention data were evaluated according to the dependency of log $\bar{k}$ on log $1/\bar{c}$ according to the relationships derived [5,6] from the Linear Solvent Strength Model, namely,

$$\log \bar{k} = \log K + Z_c \log 1/\bar{c}$$

where $\bar{k}$ is the median capacity factor of the biosolute, *i.e.* the capacity factor of the biosolute at the mid-point of the column under gradient elution and $\bar{c}$ is the median concentration of the displacing salt. The slope parameter (Z_c) can be taken as a measure of the average number of charges located in the contact area (ionotope) established between the protein and the sorbent surface. Tables III and IV summarise the results of this evaluation using NaCl and KBr, respectively. These Tables also include comparative data from the published literature for several of the proteins eluted with the same salts and gradient system from the Mono-Q sorbent. Values of

TABLE II

PHYSICAL PROPERTIES OF PROTEINS

Protein (source)	Abbreviation	pI	Molecular weight
Ovalbumin (hen egg white)	OV	4.70	43 000
Insulin (bovine pancreas)	INS	5.32	5 700
Carbonic anhydrase (bovine erythrocytes)	CA	5.89	30 000
Growth hormone (porcine recombinant)	GH	6.70	22 100
Myoglobin (sperm whale muscle)	MYO	7.68	17 500
Ribonuclease A (bovine pancreas)	RIB	9.60	12 640
Lysozyme (hen egg white)	LYS	11.00	14 300

TABLE III

RETENTION PARAMETERS FOR PROTEINS ELUTED WITH NaCl

Protein	Mono-Q		Fractogel-TMAE		LiChrospher-TMAE	
	Z_c	log K_c	Z_c	log K_c	Z_c	log K_c
OV	9.4	51.3	7.0	37.4	1.4	6.7
INS	5.4	28.8	3.2	17.1	1.3	5.7
CA	3.1	17.3	2.4	11.8		Not retained
GH	3.1	16.0	4.6	23.5	0.8	3.5
MYO	4.6	17.0	3.3	16.1		Not retained
RIB	1.6	7.7	2.7	12.8	0.7	2.1
LYS	1.5	6.0		Not retained	4.9	24.3

log K_c, which were determined by extrapolation of the log $\bar{k}$ *versus* log $(1/\bar{c})$ plots to the limit case of $\bar{c} \rightarrow 10^{-6}$ M, are also included in Tables III and IV. Log K_c values also provide some indication of the relative influence of experimental conditions on the affinities of protein solutes for the particular sorbent. Figs. 1–4 show the results of the elution behaviour of the test proteins over a range of ionic strengths for the Fractogel-TMAE and LiChrospher-TMAE sorbents.

Several observations can be made on the basis of the results shown in these figures and the derived values of Z_c and log K_c shown in Tables III and IV, about the retention behaviour of the selected proteins with these sorbents. The net-charge concept [7] has been widely used as a basis to predict the retention properties of proteins in both anion- and cation-exchange chromatography. According to this model, for strong anion-exchange systems, a protein will be retained when the solvent pH is greater than the pI of the protein. Inspection of the data, listed in Table III with NaCl as the displacer salt, reveals that this concept is not universally followed, with anomalous results observed for this group of proteins with the Mono-Q resin. For example, both the Z_c and log K_c values of the proteins decreased as the pI of the protein solute approached the pH of the solvent (pH 9.60). However, RIB and LYS, which have pI values equal to 9.60 and 11.00 respectively, exhibited significant retention on the Mono-Q sorbent. These results are in accord with the non-uniform distribution of

TABLE IV

RETENTION PARAMETERS FOR PROTEINS ELUTED WITH KBr

Protein	Mono-Q		Fractogel-TMAE		LiChrospher-TMAE	
	Z_c	log K_c	Z_c	log K_c	Z_c	log K_c
OV	9.5	48.9	7.2	37.7	1.6	7.2
INS	3.9	20.9	2.8	14.8	1.6	6.5
CA	5.6	28.4	2.4	7.0		Not retained
GH	3.9	20.2	4.0	20.6	0.7	2.8
MYO	4.8	24.2	1.9	8.5		Not retained
RIB	0.8	3.5	1.6	7.1	0.4	1.7
LYS	3.3, 1.0	10.2, 5.3		Not retained	1.9	10.1

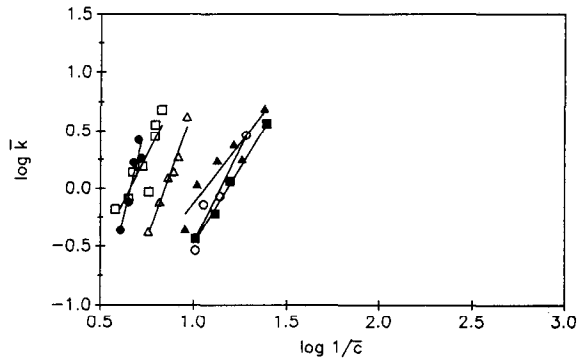

Fig. 1. Plots of log $\bar{k}$ *versus* log $1/\bar{c}$ for proteins eluted on the Fractogel-TMAE column with NaCl as the displacer salt. The plots were derived from gradient elution data at pH 9.60, a flow-rate of 1 ml/min, with gradient times between 17.1 and 171.1 min. See Table II for code to protein solutes, and Table III for the Z_c and log K_c values obtained from these plots. ○ = MYO; ● = OV; △ = GH; ▲ = CA; □ = INS; ■ = RIB.

negatively charged amino acids on the protein surface which generate patches of negative charge through which binding of the protein to the stationary phase surface may occur.

Comparison of retention properties of the test proteins on the Fractogel-TMAE and LiChrospher-TMAE sorbents reveals several interesting behavioural features. Firstly, OV shows similar retention behaviour on the Fractogel-TMAE and Mono-Q sorbents, but the Z_c and log K_c values for OV diminished significantly with the LiChrospher-TMAE sorbent. This finding is surprising considering the p*I* of OV (4.70) is almost 5 p*I* units lower than the solvent pH. Similar behaviour was also observed with GH, and also for CA and MYO which were both unretained on the LiChrospher-TMAE sorbent. In contrast, the more basic proteins RIB and LYS were still retained on the LiChrospher-TMAE sorbent. Clearly, the density and/or the accessibility of the positively charged ligands on the silica based LiChrospher-TMAE tentacle support differ from that on the Fractogel-TMAE sorbent. These differences

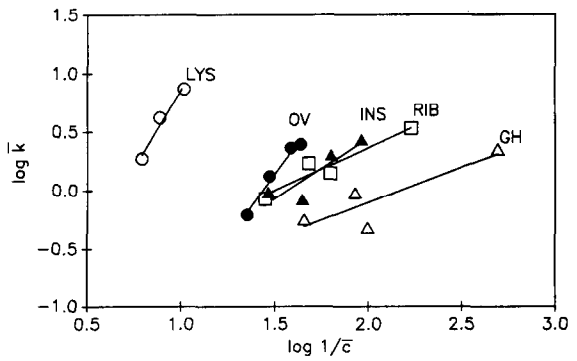

Fig. 2. Plots of log $\bar{k}$ *versus* log $1/\bar{c}$ for proteins eluted on the LiChrospher-TMAE column with NaCl as the displacer salt. See legend to Fig. 1 for other details.

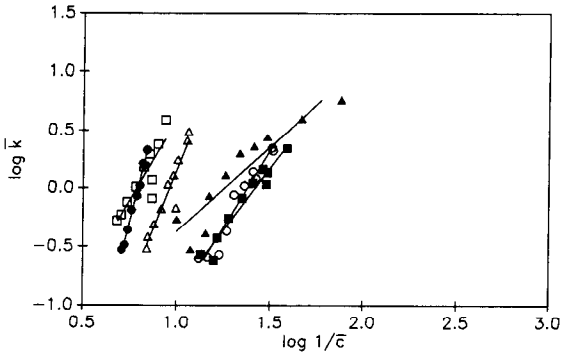

Fig. 3. Plots of log $\bar{k}$ *versus* log $1/\bar{c}$ for proteins eluted on the Fractogel-TMAE column with KBr as the displacer salt. See legend to Fig. 1 for other details and Table IV for the Z_c and log K_c values obtained from these plots.

in retention behaviour observed for these tentacle sorbents reflect the composite effect of the ligand environment. In the case of the LiChrospher-TMAE sorbent, the N-trimethylaminomethylacrylamide chains are introduced via a pre-existing glyceridylpropylsilane coverage of the silica surface, which will clearly represent a different chemical environment of hydroxyl groups to that occurring with the polydisperse hydroxy environment of the Fractogel HW65. In addition, the polymer characteristics of the silica or Fractogel matrix itself will engender secondary effects. A possible consequence of these effects is that other forces such as hydrophobic interactions may contribute to the interactive process. Depending on the quality of the glyceridylpropylsilane coverage of the silica surface, residual silanol groups could also influence the retention behaviour, particularly with basic proteins such as LYS. In an associated study [3] we have shown that high coverage glyceridylpropyl silicas similar to those used in the present investigation do not however exhibit significant adsorption of proteins attributable to free silanols under elution conditions commonly used for ion exchange.

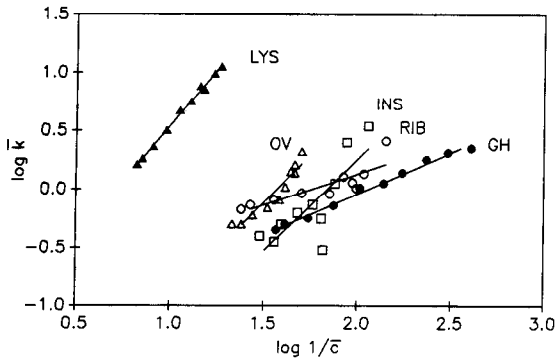

Fig. 4. Plots of log $\bar{k}$ *versus* log $1/\bar{c}$ for proteins eluted on the LiChrospher-TMAE column with KBr as the displacer salt. See legend to Fig. 3 for other details.

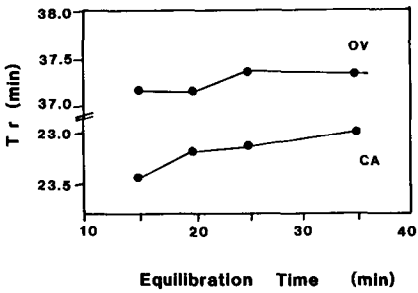

Fig. 5. Plots of retention time (T_r) *versus* equilibration time for OV and CA eluted on the Fractogel-TMAE column. The data were derived with a gradient time of 34.5 min and flow-rate of 1.0 ml/min and NaCl as the displacer salt.

The influence of the chaotropic salt KBr on the Z_c and log K_c values of the test proteins is listed in Table IV. According to the stoichiometric displacement model, the displacement of a protein solute from the charged surface is accompanied by the adsorption of a stoichiometric amount of displacer counter-ion. As a consequence, Z_c values should be independent of the chemical nature of the displacer ion and differences in log K_c should reflect the relative affinity of the counter-ion for the anion-exchange sorbent. Comparison of the Z_c and log K_c values for Fractogel-TMAE with both displacer salts reveals that similar behaviour was observed for all proteins on this resin. Similar Z_c and log K_c values were also observed for all proteins except LYS when eluted from the LiChrospher-TMAE sorbent. Once again, this result would not be anticipated for these proteins on the basis of their respective p*I* values *i.e.* their retention behaviour is not in accord with the classical Boardman-Partridge model. The Z_c and log K_c values for LYS decreased by a factor of 2 when KBr was employed as displacing salt. Similar results were also observed previously for LYS with the Mono-Q sorbent, and have been suggested [8] to be related to the conformational changes associated with the interaction of KBr with the lysozyme molecule.

After 250 h of continuous chromatographic elution under isocratic low or high salt conditions at 1 ml/min at pH 9.6, the LiChrospher-TMAE sorbent did not show

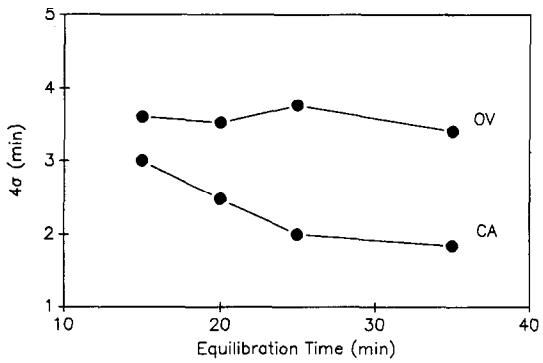

Fig. 6. Plots of solute bandwith (4σ) *versus* equilibration time for OV and CA eluted on the Fractogel-TMAE column. See legend to Fig. 5 for other details.

any significant change in performance in terms of retention or peak shape variation. Similar stability of retention and peak shape was noted with continuous elution at pH 9.6 with the Fractogel-TMAE sorbent. However, changes in peak shape, which were protein specific, were noted as a consequence of the choice of re-equilibration time following a gradient experiment. Figs. 5 and 6 show examples of the effect of re-equilibration time with OV and CA as test proteins with the Fractogel-TMAE sorbent. In these experiments, a constant linear gradient time of 34.5 min at a flow-rate of 1 ml/min was used with a mixture of OV and CA injected at the same concentration. Following each gradient run, the time taken to re-equilibrate the column from the 100% buffer B conditions was varied from 15 to 35 min. As is evident from Figs. 5 and 6, no significant change in the retention time for either protein occurred following re-equilibration irrespective of the re-equilibration time. However, a change in peak width (as $4\sigma_v$) was evident for CA whilst the $4\sigma_v$ for OV showed more limited variation although the trend towards smaller $4\sigma_v$ values with longer re-equilibration times also occurred. It can be concluded from these results that re-equilibration volumes of approximately two-times the column volume will permit adequate re-conditioning. Such behaviour is consistent with the flexible nature of the tentacle surface but clearly the origin of this phenomenon requires further characterisation. However, it can be noted that similar kinetic variations have been noted with other fuzzy surface ion-exchange adsorption systems following regeneration, *e.g.* polymeric membranes following removal of fouling components [9].

CONCLUSIONS

The experimental data obtained in this study provides the first documentation of the retention dependencies, as revealed from the log $\bar{k}$ *versus* log $1/\bar{c}$ plots, of several proteins with the so called 'tentacle-type' sorbents Fractogel-TMAE and LiChrospher-TMAE. As also noted in our earlier studies on the batch adsorption and kinetics of protein adsorption with similar tentacle-type sorbents [3], the surface properties and mode of chemical modification of the support material can strongly influence the chromatographic performance. The LiChrospher-TMAE is derived from the corresponding glycidylpropoxy-bonded silica with the oxirane ring opened by acidic hydrolysis. The matrix used for the Fractogel-TMAE sorbent is, in contrast, derived from polymerisation of various methacrylate esters with vinyl alcohol emulsifiers. These two hydrophilic support materials would thus be expected to generate two discrete classes of 'tentacle-type' sorbents following introduction of the individual, linear polyelectrolyte chains of N-trimethylaminomethyl acrylamide by radical grafting polymerisation initiated by Ce^{4+} ions. Differences in the chemical characteristics of the surface of the support material, including the hydroxyl group distribution, the effect of acrylamide monomer to hydroxyl group ratio, and the permeation of the monomer and Ce^{4+} initiator to the inner interstices of the porous particle will all affect the 'tentacle' polymer chain length, and ultimately the sorbent characteristics. Besides confirming that these new classes of anion-exchange sorbents exhibit log $\bar{k}$ *versus* log $1/\bar{c}$ dependencies compatible with other microparticulate sorbents in current use for the analysis and purification of proteins and polypeptides, these studies also highlight the need for additional, detailed investigations on protein adsoption behaviour with chromatographic sorbents in order to allow further improvements in sorbent design to be affected.

ACKNOWLEDGEMENTS

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