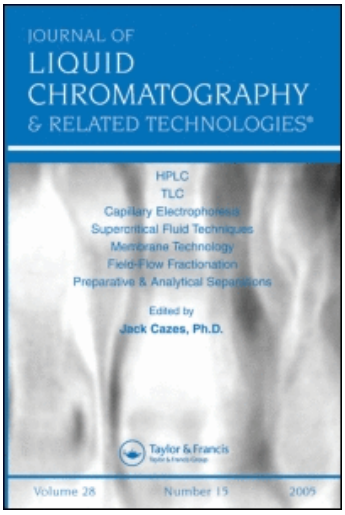

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STUDY OF THE CHROMATOGRAPHIC PROPERTIES OF PROTEINS ON A SILICA-BASED POLYMERIC CATION-EXCHANGE STATIONARY PHASE

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ABSTRACT

A cation exchange stationary phase, a sulfonated copolymer from N,N' dimethylacrylamide and glycidylacrylate deposited on silica, is used for the separation of basic proteins. Their retention properties were studied from the plot of ln k' vs. ln 1/[NaCl] where k' is the retention capacity factor, and [NaCl] the displacing salt concentration in the aqueous phase.

The influence of pH on protein retention was examined. According to the "net charge concept" the basic proteins are retained on the sulfonated copolymer at mobile phase pH below their isoelectric point. However a systematic deviation from this concept was observed at pH > 7 since the retention of these proteins increases with the pH of the eluent.

This behavior, the influence of the amount of copolymer deposited on silica and the comparison with a non sulfonated copolymer stationary phase reveals that the residual hydroxy sites of the silica support play a role in the retention mechanism of basic proteins.

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INTRODUCTION

High-performance cation exchange chromatography is one of the most useful technique for analyzing and purifying basic proteins below their isoelectric point. The separation mechanism is mainly governed by electrostatic interactions and is therefore highly dependent on the pH of the eluent. Kopaciewicz et. al.(1) have shown that the net charge concept may be used to predict partially the mobile phase pH dependent chromatographic behavior on cation-exchange columns : proteins will be retained at eluent pH below their isoelectric point (pI) on cation exchangers (2), their retention being inversely related to pH. However, deviations from this concept (3,4) are often found that are due to the nature of the protein or to the ionisation state of support itself that may influence the shape of the retention map (retention time vs. pH).

The aim of the present work is to describe the elution behavior of basic proteins on a cation-exchange polymeric stationary phase, a sulfonated copolymer of N,N' dimethylacrylamide and glycidylacrylate (DMA-GA) deposited on silica (5). Various types of polymers on silica-based materials were successfully used for the separation of basic proteins in the cation-exchange mode (6,7). Because of its mechanical rigidity, silica supports of low particle size are well suited for HPLC separations. The disadvantages of using silica as a support material are due to its poor chemical stability at pH larger than 8 (8). Also, the silica support material used in the HPLC of proteins may play a role in the retention mechanism of proteins (5,9,10) as shown in size exclusion chromatography (SEC). Although the weak cation-exchange properties of silica at pH>7 are well known (11) and were shown to affect the retention of basic amino compounds on octadecyl stationary phases (12), there are few studies (3) revealing the influence of the residual silanol groups on the retention of basic proteins eluted on a silica-based polymeric cation-exchange material. In this work, we shall examine the influence of the solid support on the cation-exchange retention behavior of basic proteins eluted on the DMA-GA stationary phase deposited on silica. In a previous work the role of the residual hydroxy sites of the silica support was revealed in S.E.C.with silica coated with a neutral DMA copolymer (5). The influence of the silica support in cation exchange chromatography will be studied by comparing the properties of the sulfonated and the non-sulfonated copolymer and by studying the influence of mobile phase pH on the retention behavior of proteins.

MATERIALS AND METHODS

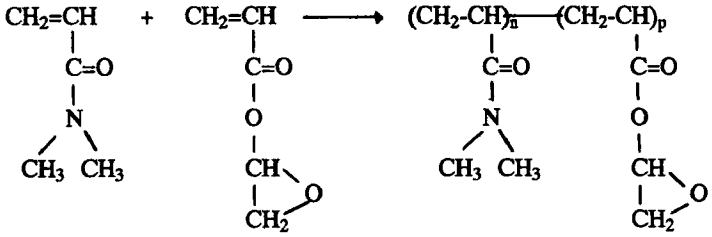
Materials

The porous silica (Nucleosil 300-10, Macherey-Nagel, Duren, Germany) was 10μm particle diameter and 300Å pore size. Glycidylacrylate, N,N' dimethylacrylamide, ethylenediamine and sodium sulfite were from Aldrich-Chemie (Steinheim, F.R.G.) ; α,α'azo-isobutyronitrile and sodium dihydrogen phosphate were from Fluka (Buchs,Switzerland) ; the proteins studied were obtained from Sigma (St. Louis, MO, USA) : cytochrome C (MW=12200 g/mol, pI=9.0), Lysozyme (MW=13930 g/mol, pI=11) and α Chymotrypsinogen A (MW=25000 g/l, pI=9.2), (13,14).

Synthesis of the Copolymer

The hydrophilic polymer, able to form hydrogen bonds and having functionalities for further modifications, a statistic copolymer of N, N' dimethylacrylamide (DMA) and glycidylacrylate (GA) is synthesized in solution by a radical mechanism using α,α'Azo-isobutyronitril (AIBN) as a catalyst.

The monomers (36,3ml of DMA and 13,7ml of GA) were dissolved in 500ml of toluene and polymerized at 70°C under a nitrogen atmosphere for 90 minutes with AIBN (0.31g). The reaction was stopped by diluting the solution at ratio 2.5 with toluene. The polymer was then precipated in diethyl ether, filtered through a sintered-glass funnel and dried under reduced pressure in a dessicator. The molecular weight determinated by osmometric measurement was 36000 ; the GA percentage determinated by quantitative analysis of the elements and by measurement of the quantity of the epoxy functions using sodium thiosulfate (15) was respectively 23% and 21%.



(1)

Polymer Coating Procedure

The strong cation exchange material was synthetized by coating the silica support (Nucleosil 300Å, 10μm) with the cross-linked and sulfonated copolymer. The coating procedure used consists in coating the required amount of copolymer on the support by complete evaporation of the solvent. This was achieved by introducing the porous support in polymer solution of methanol (10ml/g) ; the suspension was sonicated under vacuum for 2 minutes in an ice bath, and the required quantity of ethylenediamine (EDA) for cross-linking was added. About 30% of the epoxy groups of the copolymer are consumed during this reaction. After leaving the mixture at room temperature for 1 hour with constant stirring, it was heated at 60°C for 2 hours to complete the reticulation step and to evaporate the solvent. The coated support was washed with methanol and dried at 60°C.

The sulfonation is then performed as follows : the polymer coated silica was suspended in an aqueous solution of sodium sulfite 1.5 M (16). The mixture was stirred 5 hours at room temperature, the silica was then filtrated on a cellulose acetate membrane under nitrogen pressure and rinsed with water.

Chromatographic Evaluation

A chromatographic system consisting of a pump (HPLC PUMP 420, Kontron Instruments, Zurich, Switzerland), a sample injector (7125, Rheodyne, Berkeley, USA) with a 20μl loop and a UV detector (SPD-6A, Shimadzu, Kyoto, Japan) operating at 280nm was used for the chromatographic evaluation of the support.

The coated copolymer support was slurry packed into a stainless steel column (4.6 mm i.d. x 5 cm) at 200 bars using methylene chloride as the mobile phase. The column was rinsed with methanol and with water before use. The temperature of the column was maintained at 20°C during the experiments, using a temperature controlled water bath. The eluent was phosphate buffer 20mM ; the pH was adjusted with sodium hydroxyde and the ionic strength was fixed with sodium chloride. The injected amount of protein was chosen sufficiently small not to overload the column. The plate heights were determined from measurements of peak width at 0.6 of peak height.

The retention capacity ratio k' was calculated by reference to the retention volume of a non retained protein of about the same molecular weight : α lactalbumine (MW=16000 g/mol, pI=4.8).

RESULTS and DISCUSSION

The retention behavior of proteins was evaluated using the stoechiometric displacement model (1,17) that describes the variation of the retention volume vs. salt concentration. The retention capacity factor is related to the displacing salt concentration C_s according to:

$$\log k' = \log K_z - Z \log C_s \qquad (2)$$

where Z is equal to Z_p/Z_s. Z_p is the number of protein charges and Z_s the valency of the displacing salt. K_z is a constant which is directly proportional to the equilibrium constant describing the association of the protein with the support.

Influence of the Amount of Polymer Coating

The influence of the amount of copolymer deposited on silica is shown in Figure 1. With 10% (w/w) of copolymer, the plot of log k' as function of log C_s (solid line) is linear demonstrating that an anion-exchange mecanism controls the retention at pH 6. The corresponding parameters Z and K_z are summarized in table 1.

With a lower amount of polymer deposited on silica (5 %), an important increase of the retention is observed : with [NaCl] = 0.1 M, k' is about 10 times as large with silica coated with 5% than with a 10% DMA-GA copolymer on silica. The values of Z are lower than those observed with 10% sulfonated DMA-GA, but the K_z values are higher. This shows that on the 5 % sulfonated stationary phase, the use of larger salt concentrations does not decrease markedly the retention, as one would expect with a pure ion-exchange chromatography. This reveals a complex retention mechanism with most probably the interplay of hydrophobic interactions (17).

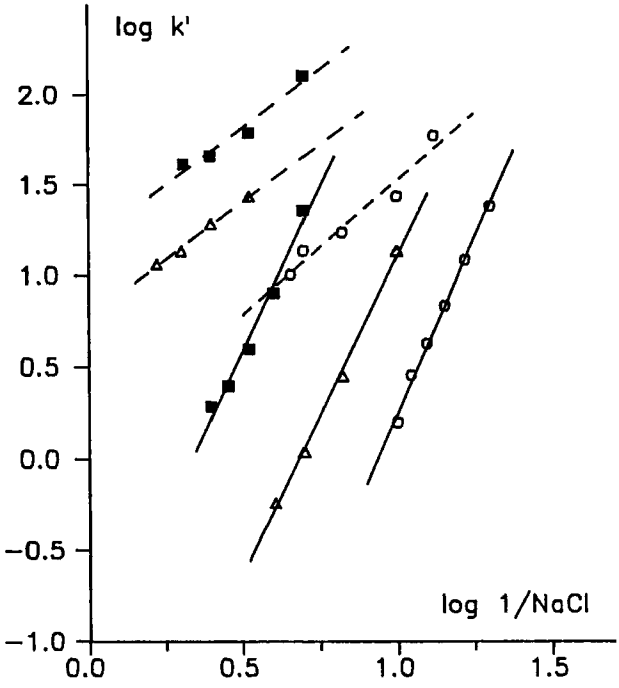


Figure 1 :
Dependence of the logarithmic retention capacity factor on the logarithmic salt concentration. Eluent : phosphate buffer 20mM with NaCl, pH6. Flow rate=1ml/min. Support : 5% copolymer coated silica - - -, 10% copolymer coated silica ——. O α chymotrypsinogen , Δ cytochrome C , ■ lysozyme.

TABLE 1

Coefficients of eqn.2 for 5% and 10% Copolymer Deposited on Silica (pH6).

Copolymer	α chymotrypsinogen		cytochrome C		lysozyme	
%	Z	K_z	Z	K_z	Z	Kz
10	3.6	$0.4\ 10^{-3}$	3.4	$5\ 10^{-3}$	3.4	0.06
5	1.6	0.9	1.2	6.1	1.1	16.5

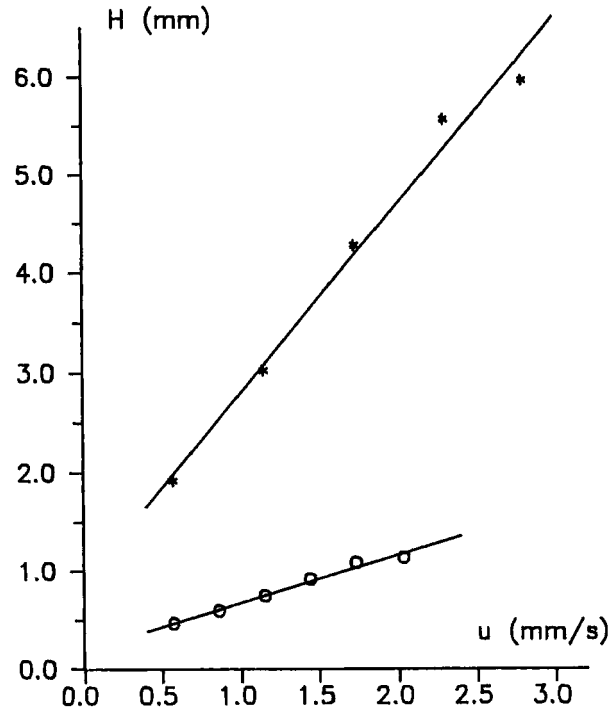


Figure 2 :
Plate height versus mobile phase velocity for isocratic elution of α chymotrypsinogen : influence of the amount of polymer deposited on silica. Eluent : phosphate buffer 20mM with NaCl, pH6. Support : 5% copolymer coated silica * , 10% copolymer coated silica O.

The corresponding efficiencies for α chymotrypsinogen are given in Figure 2, in terms of plate height variations vs. the interstitial mobile phase velocity (u). For these experiments the salt concentration in the eluent was chosen so as to obtain close retention capacity factor. An important loss of the efficiency is observed with the 5% DMA-GA and reveals the heterogeneity of the polymer coating.

TABLE 2

Coefficients of eqn. 2 for Sulfonated and Non Sulfonated Copolymer Coated Silica at pH 7 and 8.

Copolymer	pH	α chymotrypsinogen		cytochrome C		lysozyme	
		Z	$K_z(10^3)$	Z	$K_z(10^3)$	Z	$K_z(10^3)$
Sulfonated	7	2.4	8.5	2.8	32.3	3.1	107.1
	8	2.7	12.9	3.9	66.1	3.3	104.7
Non Sulfonated	7	2.2	1.7	3.1	2.6	2.0	26.9
	8	2.4	4.4	2.9	28.1	2.2	32.3

Influence of the pH of the Mobile Phase

The influence of the eluent pH was studied for 10% DMA-GA deposited on silica. In Figure 3 are given the dependence of k' with the displacing salt concentrations for α chymotrypsinogen and cytochrom C at three different mobile phase pH. The corresponding values of Z and K_z are listed in Table 1 and 2. At the lower pH a larger value is observed for Z in agreement with the increase of the ionisation state of the protein related to a larger difference between the pH of the eluent and its pI.

For a given salt concentration, the retention volumes are larger at pH 8 than at pH 7 (Figure 3) except for α chymotrypsinogen at low salt concentration where the retention is larger at pH 6 than at pH 7. This is due to the larger Z value at pH 6 and the straight lines log k' vs. log 1/[NaCl] for pH 6 and pH 7 intercept (Figure 3). Indeed one would expect for cation-exchange chromatography a decrease of the retention at pH closer to the isoelectric point of the proteins. Therefore the increase of retention with pH reveals a complex adsorption mechanism. This is also observed for lysozyme but the retention volume is 100 times as large that of the other proteins. This may be explained by a higher hydrophobic contribution. To elute this protein of high pI, it was necessary to use high salt concentrations that increase the hydrophobic effects (17).

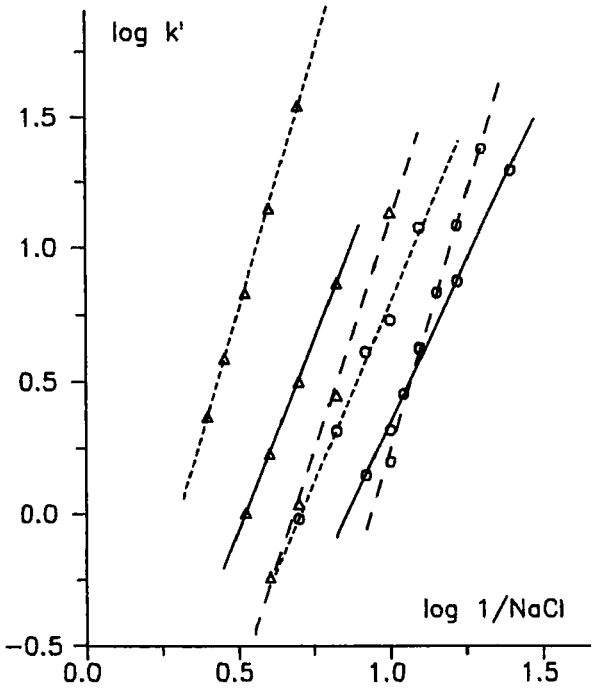


Figure 3 :
Dependence of the logarithmic retention capacity factor on the logarithmic salt concentration. Eluent : phosphate buffer 20mM with NaCl. Flow rate=1ml/min. Support : 10% sulfonated copolymer coated silica. O α chymotrypsinogen ; Δ cytochrome C. pH 6- - - ; pH 7 —— ; pH 8 -----.

Comparison with a Non-Sulfonated Stationary Phase

In order to reveal the non specific interactions, the retentions of various proteins on the 10% sulfonated DMA-GA silica and on the 10% non-sulfonated one were compared. The non-sulfonated copolymer deposited on silica differs from the sulfonated one not only by the presence of charges due to the sulfonated functions, but also by the presence of α diols. On the non-sulfonated column, at pH 6, the retention capacity ratio for all the proteins is small (<0.1) even at very low salt concentrations. Therefore the residual adsorption on the silica support is negligible at pH6 with 10% coating. As with the sulfonated column, at higher

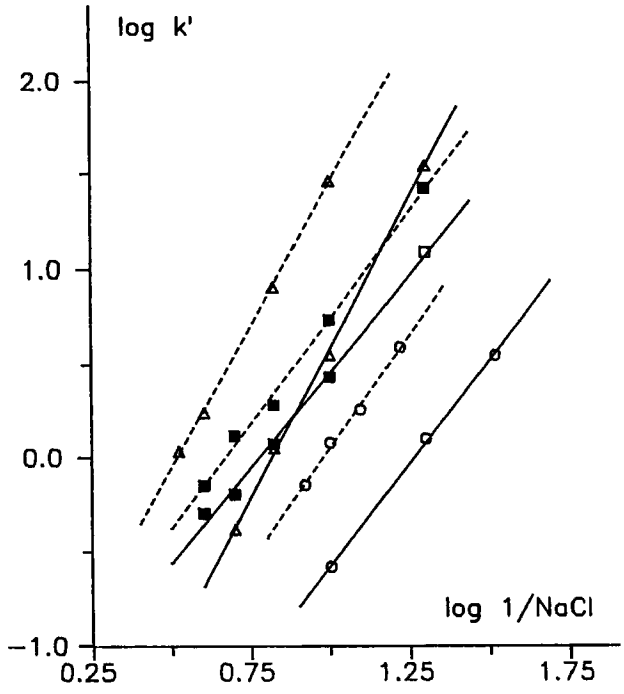


Figure 4 :
Dependence of the logarithmic retention capacity factor on the logarithmic salt concentration. Eluent : phosphate buffer 20mM with NaCl. Flow rate=1ml/min. Support : 10% non sulfonated copolymer deposited on silica. O α chymotrypsinogen ; Δ cytochrome C ; ■ lysozyme. pH 7 —— ; pH 8 -----.

eluent pH the retention increases and a linear dependence of log k' with log C_s is observed (figure 4). The corresponding Z and K_z values are listed in Table 2. The number of protein charges is of comparable magnitude for the elution on the sulfonated and the non-sulfonated support.

Therefore a cation exchange mechanism governs basic proteins retention at pH 7 and 8 on the non-sulfonated column. The residual OH groups present on the silica surface are ionized at pH>7 and probably participate to the protein adsorption via a cation exchange mechanism.

CONCLUSION

This work shows that on silica-based cation-exchangers the residual silanols can participate to the retention mechanism of the basic proteins. The increase of the retention time with the pH is an easy procedure to reveal this residual adsorption due to an incomplete passivation of the support. On the opposite of the material studied in this work, Kopaciewicz and Regnier (2) have synthesised several silica based cation-exchange materials where the retention of lysozyme decreases with increasing eluent pH. This shows that there was no residual adsorption of the protein on their support.

Although the material described in this work is not as suitable as one would expect for the separation of basic proteins, we give a methodology for testing silica-based cation exchange materials. Moreover this work shows that the deviations of retention maps are not only due to the nature of the protein but may also have their origin in a variation of the ionisation state of the support.

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