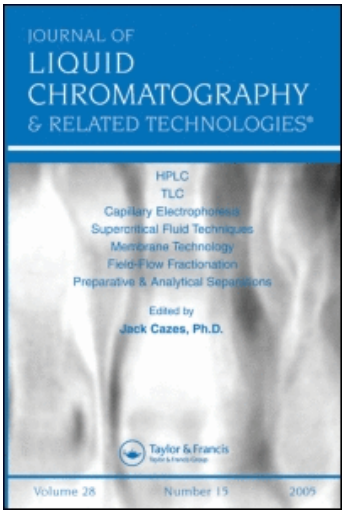

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 30 November 2001

To cite this Article Racaityte, K. , Liesiene, J. and Radzevicius, K.(2001) 'ANION EXCHANGE CHROMATOGRAPHY OF PROTEINS. EFFECT OF CHARGED GROUPS OF THE STATIONARY PHASE ON PROTEIN BINDING', Journal of Liquid Chromatography & Related Technologies, 24: 18, 2749 — 2763
To link to this Article: DOI: 10.1081/JLC-100106946
URL: http://dx.doi.org/10.1081/JLC-100106946

J. LIQ. CHROM. & REL. TECHNOL., 24(18), 2749–2763 (2001)

ANION EXCHANGE CHROMATOGRAPHY OF PROTEINS. EFFECT OF CHARGED GROUPS OF THE STATIONARY PHASE ON PROTEIN BINDING

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ABSTRACT

The purpose of this work was to study how the basicity and density of charged groups of anion exchangers affect protein binding strength and chromatographic performance at different sample loading. For this study, several cellulose-based anion exchangers with different ligand basicity and quantity were synthesized. The binding strength of protein by the ion exchangers, and the chromatographic performance were evaluated.

Investigations showed that a significant part of the protein adsorbed in the high loading mode may be eluted with 0.05-0.1 M NaCl in Tris-HCl buffer pH 7.5. This indicates very weak binding. It may be the result of multi-layer binding of protein molecules to that already adsorbed on the surface of stationary phase. The amount of weakly bound protein decreases with decreasing of sample loading.

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Results of gradient elution of BSA adsorbed at low loading mode showed that the binding strength of protein is dependent on the basicity of ionic groups of the stationary phase.

A tendency towards improved chromatographic performance was observed with increasing density of charged groups on the surface of the stationary phase.

INTRODUCTION

Ion exchange chromatography (IEC) has been routinely used for preparative purification of proteins since Peterson and Sober(1) developed cellulose-based ion exchangers in 1956. Usually, the purification process of any protein involves some chromatographic steps in which the IEC is used twice, or even more, times. At the initial stage of the purification process, the IEC is predominantly used for concentration of proteins, as well as for rough separation of proteins, based on their charge differences. In this case, high dynamic capacity of binding proteins is the major requirement for the ion exchange resins. At later stages, deep resolution of the desired protein from its contaminants is usually sought through the IEC. The demands for high selectivity and resolution are paramount at this stage.

There is a large number of commercially available ion exchangers for protein purification based on various matrices, such as polysaccharides (agarose, dextran, cellulose), synthetic polymers, silica, and composites. The comparison of the functional performance of different ion exchange media showed significant differences between them.(2-5) These differences are manifested even when different companies prepare similar chemistries on related matrices.(3)

The factors that may have impact on protein retention by a charged stationary phase at evaluated internal conditions (pH, buffer composition, ionic strength of buffer, temperature) are as follows:

a) characteristics of the matrix, such as chemistry of the matrix material, pore geometry, pore size and its distribution, accessibility of pores to solute,

b) characteristics of ligands, such as the type of the charged groups, their dissociation constants, surface charge density of the matrix, flexibility of charged groups, i.e., the length of the spacer arm,

c) characteristics of the sorbate, such as charge density on the surface of the protein and the molecular size.

While the influence of the matrix material and its porosity on chromatographic performance has been discussed in several works,(6-8) there has been little attention paid to the effect of the charged groups and their density.(9,10)

The aim of this work was to study how the basicity of positively charged amino groups and their density on the surface of stationary phase affect the pro-

tein retention. For this purpose, we synthesized on the same cellulose matrix Granocel,(11,12) several anion exchangers varying both in the basicity and the quantity of the amino groups.

EXPERIMENTAL

Materials

1-Chloro-2,3-epoxypropane was obtained from Reachim (Russia) and distilled before using. N-chloroethyl-N,N-diethylamine and N-benzyl-N,N-dimethylamine were purchased from Serva (Germany). N-(1,2-epoxy)propyl-N,N-diethylamine was synthesized in our laboratory. Bovine serum albumin (BSA) MW 68000, was the product of Reanal (Hungary). Tris(hydroxymethyl)-aminomethane (Tris) was obtained from Merck (Germany). Reagent grade chemicals were used without further purification. All buffer chemicals used were of analytical reagent grade.

Preparation of Anion Exchangers

Cellulose matrix Granocel was prepared by saponification of cellulose acetate by the procedure described previously.(11) Crosslinking of the cellulose matrix was performed with 1-chloro-2,3-epoxypropane (CEP) (0,6 mL per 1 g of cellulose). The sample of wet cellulose (cellulose concentration $9 \pm 1\%$) was suspended in the mixture of CEP and 2M solution of sodium hydroxide. The suspension was stirred at 50°C for 2 hours. The resulting product was washed with 0.5 M HCl and then with water.

Amination of the crosslinked material was performed with N-chloroethyl-N,N-diethylamine hydrochloride, N-(1,2-epoxy)propyl-N,N-diethylamine or N-benzyl-N,N-dimethylamine. Amounts of reagents and reaction conditions are presented in Table 1.

The sample of wet cellulose was suspended in a mixture of amination reagent and 1M sodium hydroxide. The suspension was stirred at elevated temperature for an extended time (see Table 1). The product was washed with water, 0.5 M HCl, and then again with water.

Analysis of Anion Exchangers

The total nitrogen content was determined according to the Kjeldahl method. The theoretical ion capacity was calculated from nitrogen analysis.

Table 1. Reaction Conditions for Synthesis of Anion Exchangers

Anion Exchanger	Amination Reagent	Amount Per 1 g Wet Cellulose	Time of Reaction, in Hours	Temperature, °C
DEAE-Granocel	N-chloroethyl-N,N-diethylamine hydrochloride	0.15	1	50
DEAOP-Granocel	N-(1,2-epoxy)propyl-N,N-diethylamine	0.60	2	70
DMBAOP-Granocel	N-benzyl-N,N-dimethylamine 1-chloro-2,3-epoxypropane	2.70 1.85	8	40

The total anion exchange capacity and strong base capacity were analyzed by titration with 0.1 M HCl and 0.1 M NaCl, respectively.

The pK_a value of anion exchanger was determined from potentiometric titration curves. Potentiometric titration was carried out in the presence of 0.1 M NaCl at 25°C, in a nitrogen atmosphere using microprocessor pH-meter pH 537 (WTW, Germany). The capacity of small ions was calculated from equivalence points. Equivalence points for each type of group were determined graphically, from tangential lines drawn through inflection points using standard procedures.(13) The pK_a value of a charged group was calculated by the equation of Hendelsson-Haselbach.

The pore size distribution, pore diameter, pore surface area, and pore volume were determined using inverse size exclusion chromatography, as described.(14) The particle diameter was estimated by optical microscopy.

Feedstock Preparation

Egg white was separated from fresh hen egg and diluted to 14% (v/v) with 0.025 M Tris-HCl buffer pH 7.5. The egg white suspension was clarified using pre-equilibrated cell debris remover (CDR) in a batch mode. CDR was removed by centrifugation and the sample was clarified by filtration. The clear solution containing 10 mg/mL of total protein was used as a model protein mixture for anion exchange chromatography.

Chromatography

The liquid chromatograph consisted of a Ismatec Sams-Reglo pump (Switzerland), a UV detector LCD 2563 operating at 254 nm, a TZ 4620 line recorder (both from Laboratorní prístroje, Czech Republic). A glass column (15

mm x 10 mm I.D.) including adjustable end-pieces was supplied from Whatman International (Maidstone, UK).

Mobile phases for chromatography were prepared from tris(hydroxymethyl)-aminomethane (Tris) and HPLC grade water (purified by a Milli-Q filtration unit from Millipore). A Waters HPLC gradient system was used for egg white protein chromatography.

The inverse size exclusion chromatography system consisted of a pump HPP 5001, a refractometer RIDK 102, and a recorder TZ 4620 (all from Laboratorní prístroje, Czech Republic).

Bovine serum albumin (BSA) or egg white feedstock was applied to an anion exchange column (25 x 10 mm I.D.) pre-equilibrated with 0.025 M Tris-HCl buffer pH 7.5, and unbound material was removed by washing with 5 mL of 0.025 M Tris-HCl buffer pH 7.5. Bond material was eluted with a linear gradient of 0-0.5 M NaCl 0.025 M Tris-HCl buffer pH 7.5. The chromatography was carried out at a flow rate of 0.3 mL/min. All procedures were performed at room temperature (15-20°C).

Additionally, pooled fractions at various stages of chromatography were assayed for protein content, by measuring the absorbance at 280 nm against standard solutions using a spectrophotometer Spectronic 1201 (Milton Roy, USA).

RESULTS AND DISCUSSION

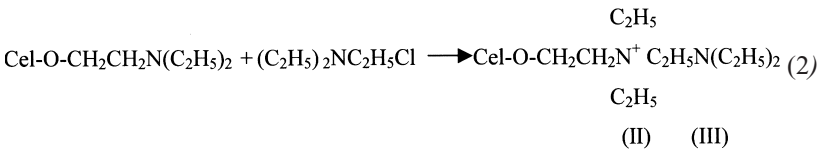
Characterization of Anion Exchangers

Two types of anion exchanger, DEAE- (weak) and Q- (strong), are most commonly used for IEC of proteins. It is to be noted, that both of them are polyfunctional. By evaluation of commercial DEAE-exchangers, it was shown that most of them contain three types of ionic groups-strong, middle, and weak basic groups with dissociation constants pK_a ca. 11, 9 and 6, respectively.(4)

DEAE ligands are usually attached to the matrix by reaction of the matrix polymer with N-chloroethyl-N,N-diethylamine hydrochloride in the presence of sodium hydroxide (1).

$$\text{Cel-OH} + (C_2H_5)_2NC_2H_5Cl \xrightarrow{\text{NaOH}} \underset{\text{(I)}}{\text{Cel-O-CH}_2CH_2N(C_2H_5)_2} \quad (1)$$

This reaction results in a mixed amino derivative because some of the tertiary amino groups are quaternized and converted to quaternary ammonium ones (II) with pK_a ca. 11, and others remain tertiary amino groups (III). Due to blocked dissociation, the latter exhibits weak base properties and its pK_a is ca. 5-6.

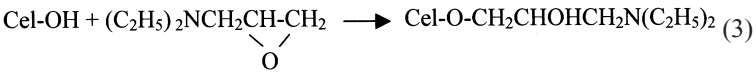


(2)

By controlling the rates of the chemicals, it is possible to regulate the composition of charged groups.(15) Therefore, commercial DEAE-exchangers significantly differ from one producer to another.(2,4)

Q-anion exchangers are usually synthesized by quaternization of tertiary amino groups with alkyl halogenides or propylenoxide in organic solvents. Quaternary ammonium groups are not stable and partly convert to tertiary ones. Therefore, it is difficult to obtain totally monofunctional anion exchangers.

In order to study the impact of the basicity of charged groups on retention of protein by anion exchange, we prepared the anion exchangers with dominant weak (DEAE-Granocel), middle (DEAOP-Granocel), and strong basic groups (DMBAOP-Granocel). DEAOP-Granocel was prepared by the interaction of matrix Granocel with N-(1,2-epoxy)propyl-N,N-diethylamine in the presence of NaOH (3). By this method, the product with dominant tertiary amino groups may be obtained.



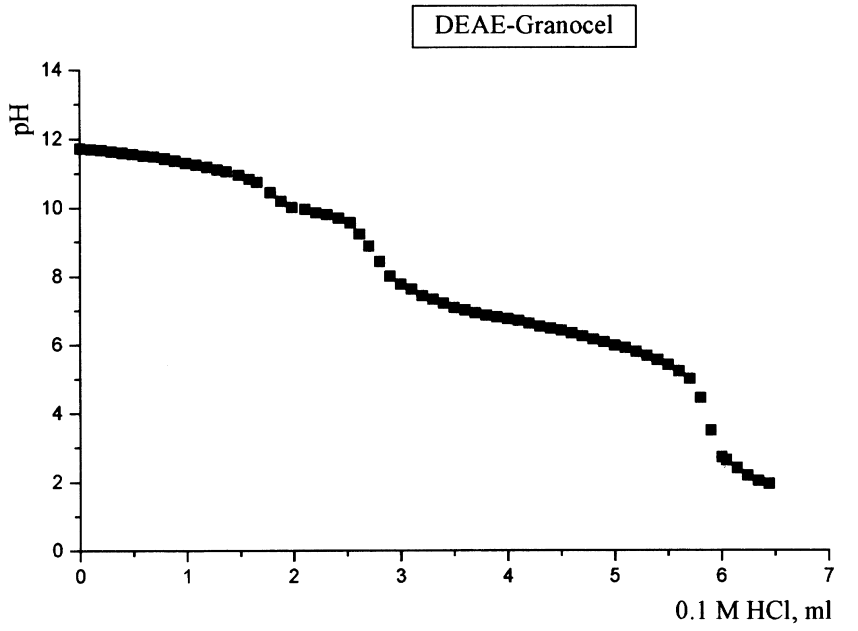
(3)

Some general characteristics of anion exchangers are presented in Table 2.

The potentiometric acid-base titration was used for the characterization of the charged groups of the anion exchangers (Fig. 1). The results obtained are summarized in Table 3.

Table 2. Characteristics of Anion Exchangers

Characteristics	DEAE-Granocel	DEAOP-Granocel	DMBAOP-Granocel
Small-ion capacity, meq/g	1,08	1,21	0,93
Protein (BSA) adsorption capacity, mg/mL	95,0	100,0	75,7
Pore volume, mL/g	18,65	13,40	14,90
Pore surface area, m^2/mL	139,0	198,9	119,8
Particle size d_p, μm	65–100		



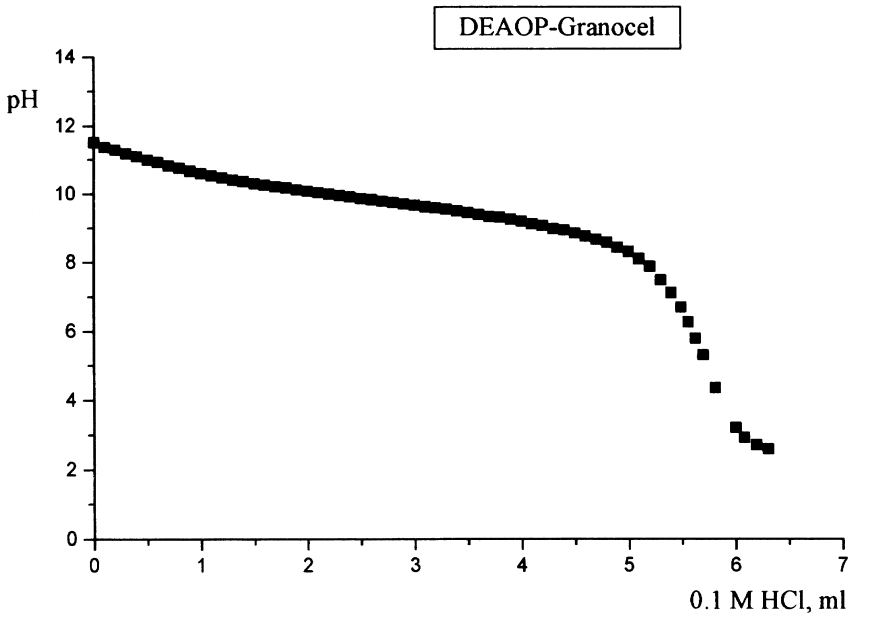


Figure 1. Potentiometric titration curves of anion exchangers.

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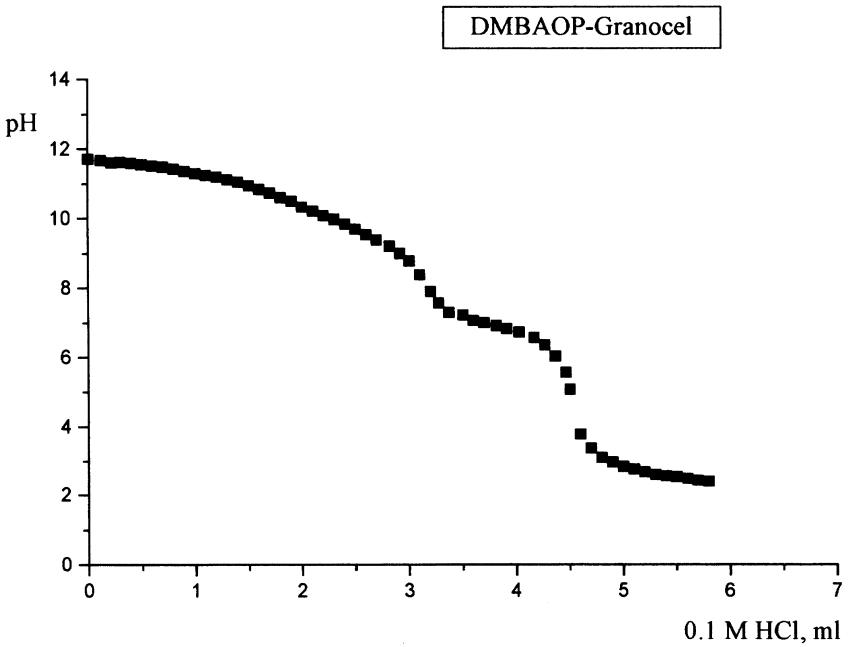

Figure 1. Continued.

The form of titration curves reflects three different basic groups, which means that all of synthesized anion exchangers are polyfunctional. However, DEAE-Granocel contains more than 50% of weak basicity groups (pK_a~6.2) and we can describe it as a weak anion exchanger. Sorbent DEAOP-Granocel is the most "monofunctional" one–81.7% of the groups are of intermediate basicity

Table 3. Characteristics of Anion Exchangers Obtained from Potentiometric Titration Curves

	Amount of Ionizable Groups, %			Ionization Constant pK_a		
Exchanger	Strong Basicity	Intermediate Basicity	Weak Basicity	Strong Basicity	Intermediate Basicity	Weak Basicity
DEAE-Granocel	32.8	15.7	51.4	11.0	9.2	6.2
DEAOP-Granocel	18.3	81.7	0.0	10.9	9.4	—-
DMBAOP-Granocel	74.5	0.0	25.5	10.7	—-	6.4

(pK_a~9.4). Strong basicity groups (pK_a~11) are dominant (74.5%) in DMBAOP-Granocel.

The Influence of Column Loading on Protein Retention

In these investigations, the influence of column loading on protein binding strength was examined. The two modes of loading have been used for this examination. Firstly an anion exchange column was loaded with protein (BSA) till saturation. Then the gradient elution of BSA was performed.

Results showed that a significant part of the protein adsorbed at high loading mode may be eluted with 0.05-0.1 M NaCl in 0.025 M Tris-HCl buffer pH 7.5 (Fig. 2a, c, e). This indicates very weak protein binding. With decreasing of sample loading (down to 10 - 13.5 mg/mL), the amount of weakly bound protein significantly decreases (Fig. 2b, d, f). That phenomena may be caused by occurrence of several types of interactions: (i) binding between the protein and binding sites on the adsorbent (ii) protein-protein interaction involving the changes in protein conformation. Approaching the maximal adsorption capacity of the anion exchanger, protein molecules are able to form a so called "multi-layer," i.e., binding of protein to that already adsorbed on the surface of the stationary phase.

This nonspecific binding has already been observed by several authors.(16-18) The experimental results show that the interaction between protein molecules is much weaker than binding between the protein and binding sites on the adsorbent. Thus, even minor changes in the strength of the eluent (amount of salt) may disarrange that "protein-protein" interaction. There was also no weak binding observed when the salt (0.05-0.1 M NaCl) was added to the loading buffer (results are not shown).

All kinds of interaction are profitable for concentration of target proteins from crude extracts. However, the investigations of the protein interactions with ionic groups may be performed only at minimal loading.

The Effect of Basicity of Charged Groups on Protein Binding Strength

The experimental data show that the binding strength between protein molecules and the stationary phase depends on the basicity of charged groups (Fig. 3). This relationship has been observed at very low loading of BSA (~10 mg/mL). The elution of BSA from three different anion exchangers requires different ionic strength of the eluent. For instance, DEAE-Granocel is a weak anion exchanger with dominant weak basicity ionic groups, therefore, most of BSA (85%) was eluted with 0.1-0.15 M NaCl solution in buffer. DEAOP-Granocel

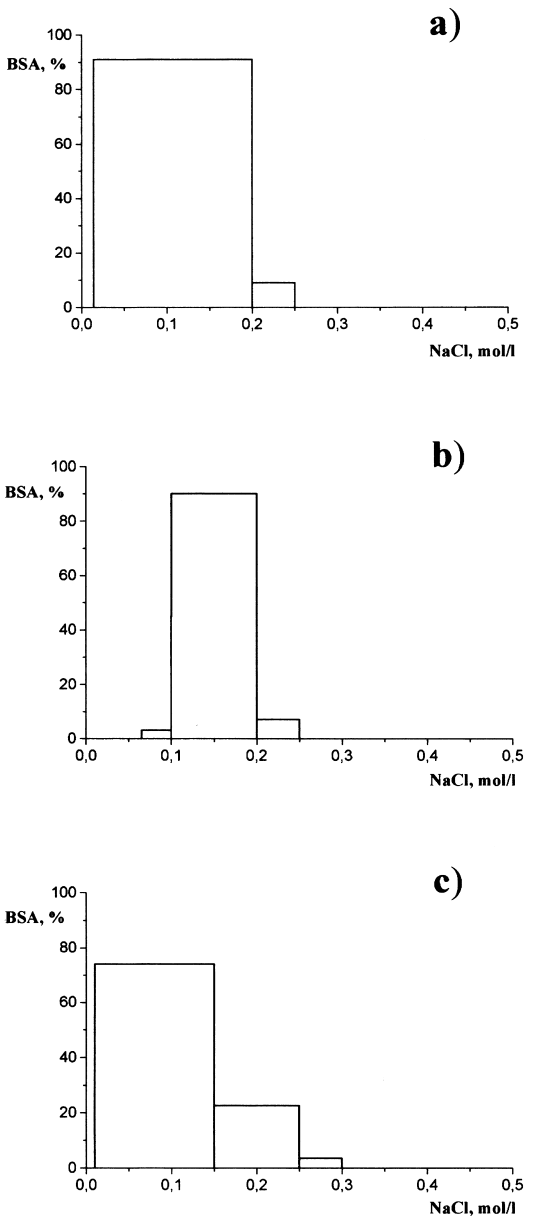

Figure 2. Effect of the NaCl concentration on the elution of BSA at different loading mode: DEAE-Granocel-a) high loading mode (95 mg/mL BSA), b) 10 mg/mL of BSA loaded; DEAOP-Granocel-c) high loading mode (100 mg/mL), d) 12.5 mg/mL loaded; DMBAOP-Granocel-e) high loading mode (75.75 mg/mL), f) 13.5 mg/mL loaded.

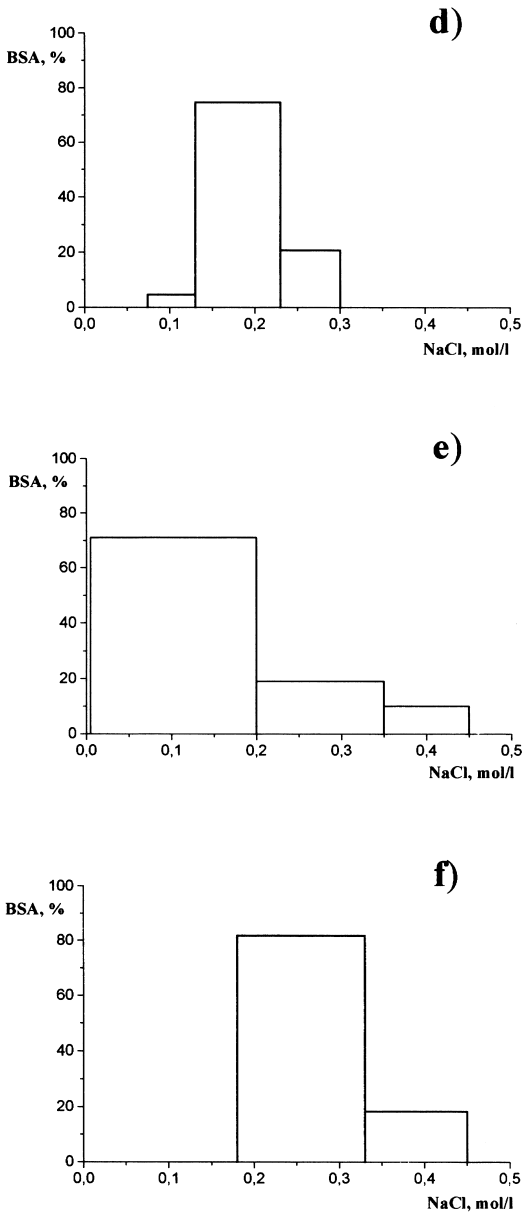

Figure 2. Continued.

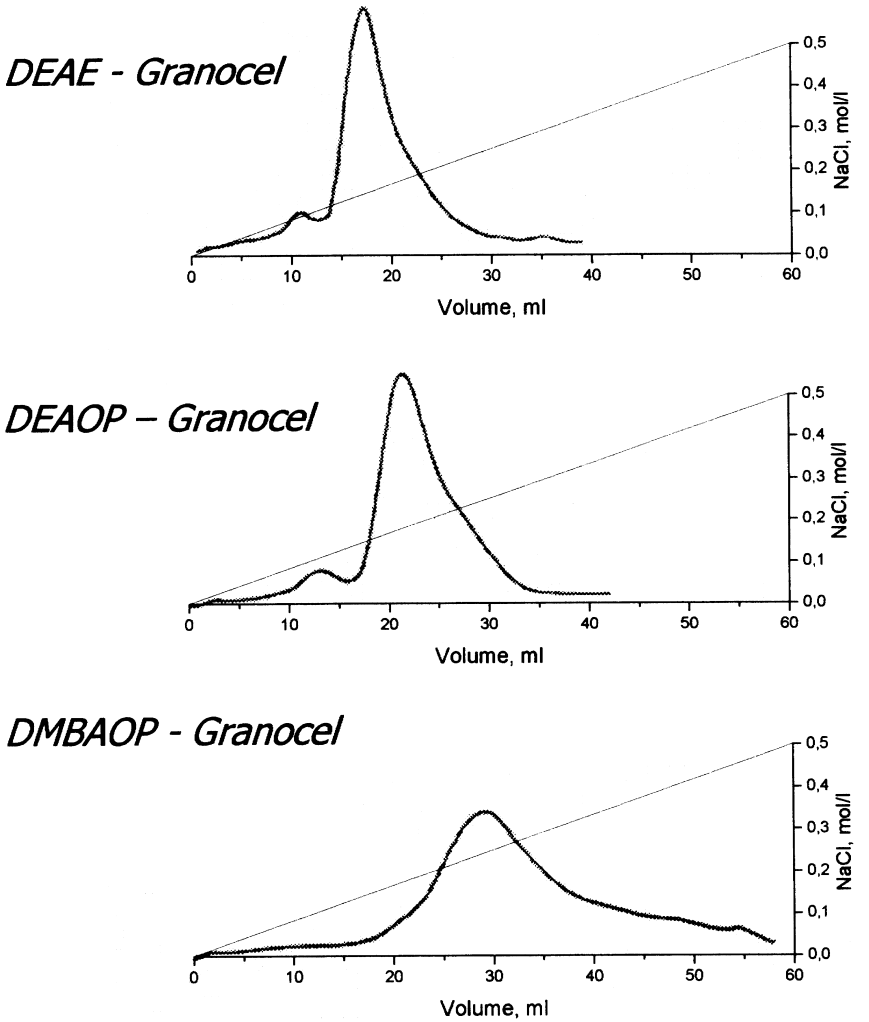

Figure 3. Effect of basicity of functional groups on binding strength.

has been characterized as an intermediate basicity anion exchanger, thus, about 80% of BSA was eluted with 0.14-0.2 M NaCl. The highest ionic strength (0.2-0.3 M NaCl) has been applied to perform elution (82% of BSA) from the strong anion exchanger (DMBAOP-Granocel).

The above data suggest that polyfunctional anion exchanger may exhibit lower chromatographic performance than a monofunctional one.

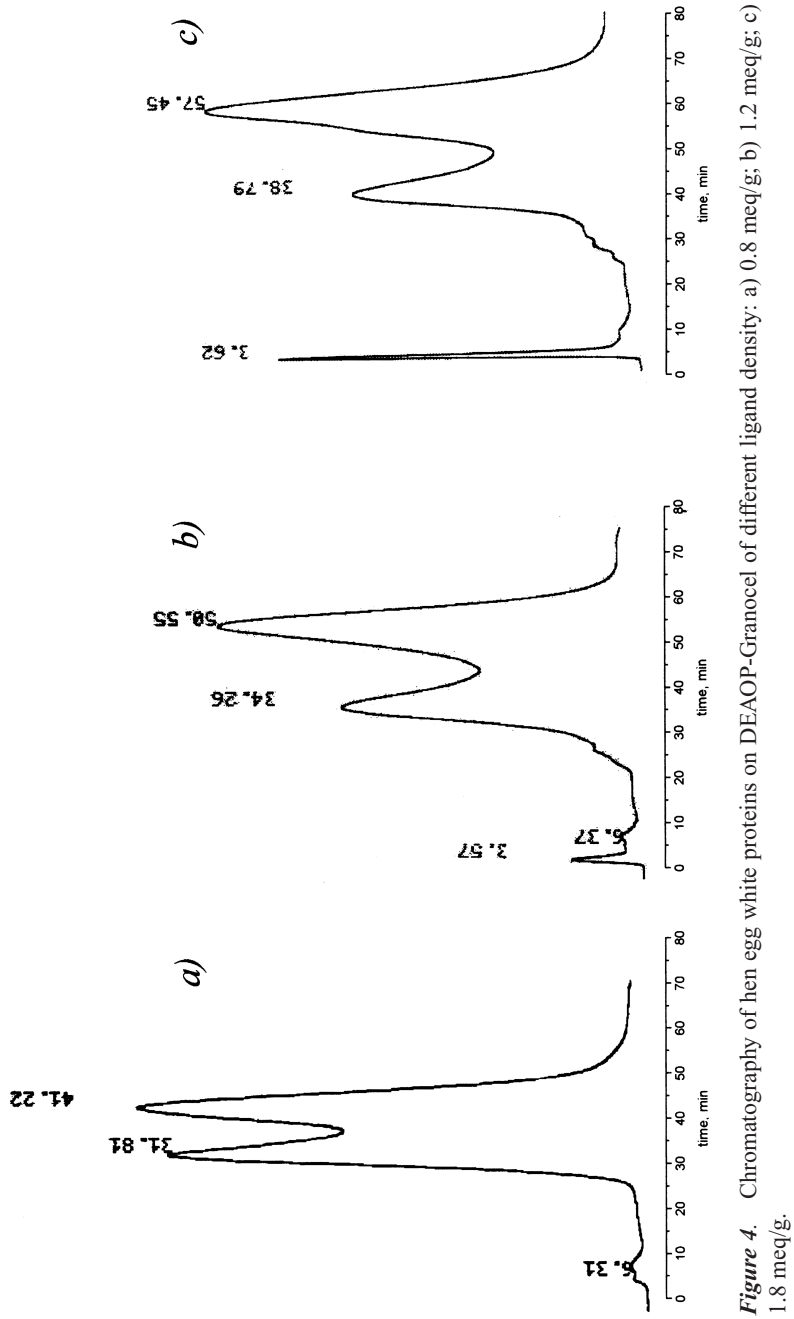

Figure 4. Chromatography of hen egg white proteins on DEAOP-Granocel of different ligand density: a) 0.8 meq/g; b) 1.2 meq/g; c) 1.8 meq/g.

The Effect of Ligand Density on Resolution of Proteins

Fig. 4 shows the obtained chromatographic profile of hen egg white proteins on anion exchanger DEAOP with different charge densities. The elution was achieved by using a gradient of 0-0.5 M NaCl in 0.025 M Tris-HCl buffer pH 7.5.

In order to compare the chromatographic performance of different anion exchangers, a degree of resolution, R, of ovalbumin and conalbumin may be calculated.(3):

$$R = 2 \times (V_2 - V_1) / (W_2 + W_1)$$

Where V_2-elution volume of ovalbumin, V_1-elution volume of conalbumin, W_2-peak width of ovalbumin at 0.5 peak height and W_1-peak width of conalbumin at 0.5 peak height.

The data obtained for the cellulose-based anion exchanger DEAOP-Granocel (Fig. 5) shows that the higher ligand density matrices exhibited better chromatographic resolution.

CONCLUSION

The above data exemplifies the importance of understanding the ligand characteristics of anion exchangers. Retention of proteins by anion exchangers depends on the type of the charged groups and the density of charge on the surface of the matrix. Thus, it is possible to optimize the chromatographic performance by varying both the basicity and the quantity of the amino groups on the surface of an ion exchanger.

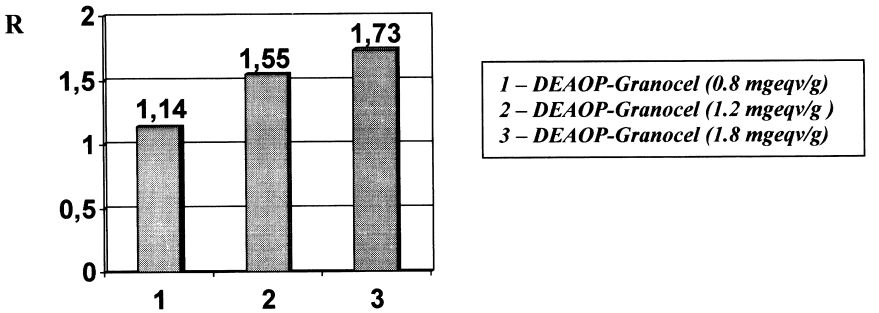

Figure 5. Effect of ligand density on chromatographic resolution.

The loading mode of protein also plays a significant role in protein-adsorbent interaction. The "non-specific" protein adsorption increases by increasing the amount of loaded protein.

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Received October 10, 2000 Manuscript 5411
Accepted January 26, 2001