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ELUTION BEHAVIOUR OF SOME PROTEINS ON FRESH, ACID- OR BASE-TREATED SEPHACRYL S-200 HR

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SUMMARY

The influence of sodium chloride concentration and the pH of the mobile phase on the distribution coefficient of proteins with different p*I* values was studied on Sephacryl® S-200 HR. The non-size-related behaviour of this gel filtration packing is mainly attributed to small amounts of groups that are negatively charged within the pH range investigated (4.2–10.0). These anionic groups on the packing gave rise to ion-exchange or ion-exclusion interactions depending on the charge characteristics of the protein. Hydrophobic interactions at high ionic strength and intramolecular electrostatic repulsive interactions at low ionic strength were also observed for some proteins.

The chemical stability of Sephacryl S-200 HR was studied by comparing the chromatographic results with Sephacryl S-200 HR that had been treated in acidic or basic solutions with those with fresh Sephacryl. After Sephacryl S-200 HR had been stored for 2 weeks in 0.10 *M* sodium hydroxide the chromatographic results at low ionic strengths clearly showed that groups that are positively charged at pH 4.2 had been formed. However, storage for 2 weeks in 0.01 *M* hydrochloric acid did not change the chromatographic behaviour of the proteins from that observed when injected on fresh Sephacryl S-200 HR.

INTRODUCTION

Gel filtration chromatography (GFC) has been used for a long time for the characterization and separation of water-soluble biopolymers[1–3]. In an ideal GFC system molecules are separated on the basis of molecular size[4]. However, other retention mechanisms are also associated with GFC, resulting in non-size-related separation effects[5]. Therefore, to develop reliable GFC methods it is important to understand and document these effects.

Recently, an improved cross-linked copolymer of allyldextran and N,N′-methylenediacrylamide for GFC has become commercially available under the trade-name Sephacryl® S-200 HR. The purpose of this study was to interpret the chromatographic results obtained with this gel under different elution conditions for a number of proteins. Also, the separation characteristics of Sephacryl S-200 HR were studied

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after it has been treated for 2 weeks with 0.10 *M* sodium hydroxide or 0.010 *M* hydrochloric acid.

EXPERIMENTAL

Equipment

Chromatographic measurements were carried out with a Pharmacia FPLC system consisting of an LCC-500 control unit, two P-500 high-precision pumps, a UV-1 UV monitor (280 nm, HR 10 cell), an MV-7 sample injector with a 500-μl loop, an MV-8 sample holder, a P-1 peristaltic pump and an REC-481 recorder. Sephacryl S-200 HR was packed in Pharmacia K 26/40 columns (40 cm × 2.6 cm I.D.) according to the packing instructions for the gel.

Reagents

The mobile phase buffers were prepared from sodium acetate and acetic acid (pH 4.2), sodium dihydrogenphosphate and disodium hydrogenphosphate (pH 7.0) or sodium hydrogencarbonate and disodium carbonate (pH 10.0). The buffers were prepared from 0.020 *M* stock solutions of these acid and base components. Variation of the ionic strength was obtained by addition of sodium chloride to the stock solutions. The aqueous ethylene glycol buffer solutions contained 20% (v/v) ethylene glycol. The pH readings for these buffers were taken in the aqueous ethylene glycol buffer solution with a pH meter standardized against aqueous buffer solutions. The proteins used are listed in Table I. These proteins were dissolved in the mobile phase at a concentration of *ca.* 2 mg/ml.

Treatment of Sephacryl S-200 HR

To study the chemical stability of Sephacryl S-200 HR, the gel (about 300 ml) was stored for 15 days in 0.010 *M* hydrochloric acid or in 0.10 *M* sodium hydroxide. The temperature at these tests was controlled by the ambient temperature (*ca.* 20°C). Before the gel was incubated it was washed with 3 l of the storage solution. The two treated gel samples were packed in K 26/40 columns and the chromatographic behaviour of the acid- and base-treated gel was compared with that of a column packed with fresh Sephacryl S-200 HR.

TABLE I

PHYSICAL PROPERTIES OF THE PROTEINS USED IN THE RETENTION MAPPING STUDY

Protein	*Source*	*Molecular weight*	*Isoelectric point (pI)*
Pepsin	Porcine stomach	33 000	2.9
Bovine serum albumin	Bovine	69 000	4.98, 5.07, 5.18
Transferrin	Human	76 500	5.5
Myoglobin	Horse heart	17 500	6.9, 7.3
α-Chymotrypsinogen A	Bovine pancrease	25 000	8.7, 8.8
Lysozyme	Egg white	13 930	11

Chromatographic procedure

The columns were conditioned by the passage of at least five bed volumes of mobile phase before being used for experimental observations. The chromatographic runs were performed by individual injections of the proteins on the column to avoid interactions between proteins. The flow-rate was 1.5 ml/min and 500 μl of the protein samples were injected. The resulting retention volumes were then used to calculate the distribution coefficient (K_{av}) from the equation

$$K_{av} = (V_e - V_o) / (V_c - V_o)$$

where V_e, V_o and V_c are the solute elution volume, void volume and the total bed volume of fluid and gel combined, respectively. Blue Dextran 2000 was employed as a marker of the void volume. V_o was determined for all ionic strengths investigated at pH 7.0 and 10.0.

RESULTS AND DISCUSSION

It is well known that under certain conditions proteins may deviate from the ideal gel filtration retention mechanism[6–11]. Some of the most important causes of non-size-related separations are (A) hydrophobic, (B) ion-exchange and (C) ion-exclusion interactions between the stationary phase and the sample molecules and (D) intramolecular electrostatic repulsive interactions[12]. Hydrophobic interactions will be favoured if the ionic strength of the mobile phase is increased, whereas at low ionic strength electrostatic interactions (B, C and D) dominate. Ion-exchange or ion-exclusion interactions will be manifested depending on the sign of the net charge on Sephacryl S-200 HR and the proteins. Because proteins are amphoteric, these two interactions can be manipulated by changing the pH of the mobile phase below or above the isoelectric point (p*I*) of the proteins. To test the extent of interactions A, B and C on Sephacryl S-200 HR, the influence of ionic strength on the K_{av} values of various proteins (Table I) was studied at different pH.

Influence of ionic strength and pH on the chromatographic results with Sephacryl S-200 HR

To stress the non-size-related separation mechanisms caused by electrostatic interactions, the protein probes chosen covered a broad p*I* range (Table I).

*Pepsin.*As the p*I* value of pepsin is 2.9 it will have a net negative charge for all mobile phase buffers investigated. Therefore, the increasing K_{av} value of pepsin with increasing ionic strength (Fig. 1) is interpreted as a decreasing ion-exclusion effect. Accordingly, this interpretation also means that Sephacryl S-200 HR consists of groups that are negatively charged in the pH range 4.2–10.0. As the amount of negative charge on pepsin is reduced as the pH decreases it can be expected that the ion-exclusion effect will also be reduced. Consequently, a higher K_{av} value of pepsin should be obtained with decreasing pH. This trend was also observed (Fig. 1). In addition, the K_{av} value of pepsin is not influenced by the concentration of sodium chloride in the range 0.3–0.5 *M* at pH 4.2 This plateau probably is an effect of charge shielding and indicates that a pure size separation dominates under these conditions. Any hydrophobic interactions that may contribute to the separation mechanism at pH 4.2 were also investigated but are discussed later.

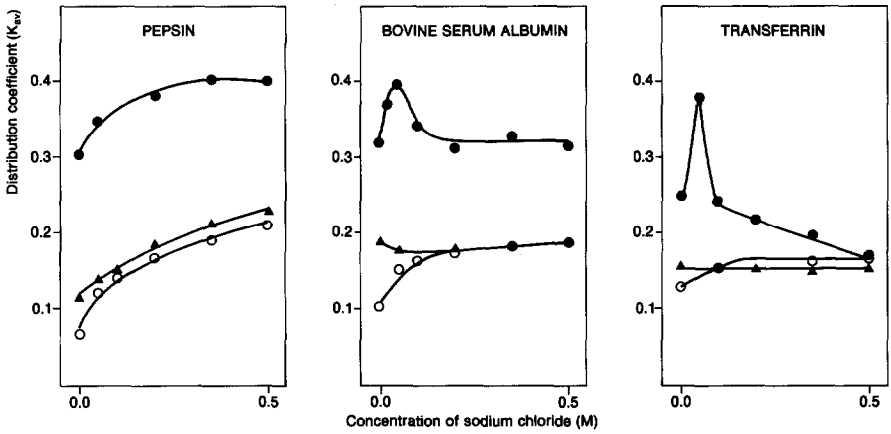

Fig. 1. Influence of sodium chloride concentration on K_{av} of pepsin, bovine serum albumin and transferrin on fresh Sephacryl S-200 HR with various mobile phase buffers. Mobile phase: (●) 0.020 *M* acetate, pH 4.2; (▲) 0.020 *M* phosphate, pH 7.0; (○) 0.020 *M* carbonate, pH 10.0

Bovine serum albumin (BSA). Owing to the higher p*I* value of BSA (Table I), the ion-exclusion interaction should not influence the retention of BSA to such a great extent as for pepsin. Fig. 1 shows that this interaction is only observed at low ionic strength at pH 10.0. At high ionic strength ([NaCl] > 0.2 *M*) at pH 10.0 the K_{av} values of BSA merge with the results at pH 7.0 (Fig. 1). Therefore, it can be concluded that this K_{av} plateau (*ca.* 0.18) represents an ideal size separation behaviour. On the other hand, at pH 4.2, where BSA has a positive net charge, the results in Fig. 1 suggest that several separation mechanisms are involved. Ion-exchange between positively charged BSA molecules and anionic groups on Sephacryl S-200 HR explains why K_{av} increases when the sodium chloride concentration decreases from 0.2 to 0.05 *M*. In addition, an increasing hydrophobic interaction superimposed on a decreasing ion-exchange interaction probably explains the constant K_{av} value of BSA at high ionic strength ([NaCl] > 0.2 *M*) at pH 4.2 (Fig. 1). Further, it is known that BSA molecules expand in acidic solutions because of intramolecular repulsive interactions[13,14]. As these interactions are most pronounced at low ionic strength, this expansion of BSA can explain the decrease in K_{av} at low concentrations of sodium chloride (0.05–0 *M*).

Transferrin. Ion exclusion influences the chromatographic behaviour of this protein at pH 10.0 only when the sodium chloride concentration is low ([NaCl] < 0.1 *M*). At higher ionic strengths the K_{av} value for transferrin coincides with the result at pH 7.0 (Fig. 1). This indicates that the ideal K_{av} value of this protein is about 0.16 on Sephacryl S-200 HR. At pH 4.2 transferrin is positively charged and the gel is negatively charged. Therefore, ion-exchange interactions probably cause the variation in K_{av} with ionic strength (between 0.05 and 0.5 *M* NaCl) at this pH. The sharp decrease in K_{av} of transferrin at 0 *M* sodium chloride (Fig. 1) is explained by intramolecular repulsive interactions, in accordance with the interpretation of the results for BSA at low ionic strengths. However, in contrast to BSA, no hydrophobic interactions were observed for transferrin at high ionic strengths.

Myoglobin. The effect of ionic strength on K_{av} of myoglobin at pH 10.0 is

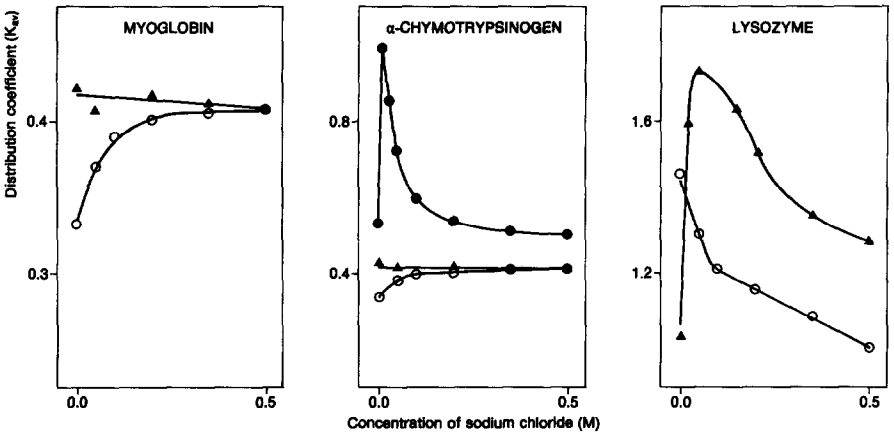

Fig. 2. Influence of sodium chloride concentration on K_{av} of myoglobin, α-chymotrypsinogen and lysozyme on fresh Sephacryl S-200 HR with various mobile phase buffers. Mobile phase: (●) 0.020 *M* acetate, pH 4.2; (▲) 0.020 *M* phosphate, pH 7.0; (○) 0.020 *M* carbonate, pH 10.0

caused by ion-exclusion interactions as for the previously discussed proteins (Figs. 1 and 2). At pH 7.0 no significant trend of the K_{av} of myoglobin was observed (Fig. 2), indicating that only an ideal size separation mechanism determines the retention time. In the acidic mobile phase buffer myoglobin was denatured and precipitated in the column top filter.

Chymotrypsinogen. Chymotrypsinogen shows a general K_{av} trend with all the mobile phase buffers investigated, which is in agreement with the results for serum albumin (Fig. 2). Consequently, as for BSA, four non-size-related separation mechanisms are involved, namely, ion-exclusion, ion-exchange, hydrophobic and intramolecular repulsive electrostatic interactions. The higher amount of positive charge on chymotrypsinogen at pH 4.2 compared with BSA is probably the cause of the greater influence of ion-exchange and intramolecular interactions on K_{av} of chymotrypsinogen at this pH (Fig. 2).

Lysozyme. The high p*I* value of lysozyme means that this protein has a net positive charge in all the mobile phase buffers used. As Sephacryl S-200 HR contains anionic groups, it can be expected that ion-exchange interactions will influence K_{av} of lysozyme to a great extent. As shown in Fig. 2, the expected increase in K_{av} of lysozyme with decreasing ionic strength was also observed. However, K_{av} was greater than 1 even at 0.5 *M* sodium chloride at pH 7.0 and 10.0, which suggests that at least one more non-size-related mechanism influenced the retardation of lysozyme. It has been shown[9] that lysozyme has a strong hydrophobic nature. Therefore, we postulate that hydrophobic interactions also contribute to the retardation. This interpretation will be discussed in more detail later. The dominating non-size-related interaction at acidic pH (4.2) is probably an ion-exchange mechanism, which is illustrated by the very high K_{av} values of 2.5, 4.1 and 6.6 at 0.5, 0.2 and 0.1 *M* sodium chloride, respectively. At low ionic strengths at pH 7.0 K_{av} decreases with decreasing ionic strength. This is probably an effect of intramolecular electrostatic repulsion[6]. Fur-

TABLE II

INFLUENCE OF ETHYLENE GLYCOL ON THE RETARDATION OF PROTEINS ON SEPHACRYL S-200 HR

Protein	*Distribution coefficient* K_{av}*			
	A_1	A_2	B_1	B_2
Pepsin	0.399	0.361	0.299	n.d.**
Bovine serum albumin	0.326	0.263	0.187	0.203
Transferrin	0.193	n.d.	0.155	0.176
Myoglobin	n.d.	n.d.	0.406	0.483
α-Chymotrypsinogen A	0.509	0.477	0.409	0.452
Lysozyme	3	n.d.	1.29	0.929

* A_1 = 0.020 *M* acetate (pH 4.2) and 0.35 *M* sodium chloride; A_2 = A_1 in 20% (v/v) ethylene glycol; B_1 = 0.020 *M* phosphate (pH 7.0) and 0.35 *M* sodium chloride; B_2 = B_1 in 20% (v/v) ethylene glycol.

** n.d. = not determined.

ther, at pH 4.2 K_{av} of lysozyme decreased from 6.6 to 1.7 when the concentration of sodium chloride was changed from 0.1 to 0 *M*. This phenomenon cannot be explained only by the expansion of the lysozyme molecules. However, if the ion-exchange interaction also decreases because of restricted accessibility to the anionic groups on Sephacryl S-200 HR when the lysozyme molecules are expanded, the result may be rationalized.

Influence of ethylene glycol on K_{av} for some proteins

At high ionic strengths relatively high K_{av} values were observed for BSA and chymotrypsinogen at pH 4.2 and for lysozyme at pH 10.0 and 7.0 (Figs. 1 and 2). In order to verify that these high K_{av} values were caused by hydrophobic interactions, 20% (v/v) ethylene glycol was added to the mobile phase. Table II shows that K_{av} of BSA and chymotrypsinogen at pH 4.2 decreased when ethylene glycol was added. This behaviour was also observed for lysozyme at pH 7.0 (Table II). These results support the suggestion that hydrophobic interactions contribute to the retardation of these proteins. In addition, it was also noted that K_{av} of pepsin at pH 4.2 decreased after the addition of ethylene glycol. Consequently, the influence of ionic strength on the retardation of pepsin at pH 4.2 (Fig. 1) can be partly explained by hydrophobic interactions and not only by ion exclusion (see above).

Under certain mobile phase conditions it was also observed that K_{av} of some proteins increased after addition of ethylene glycol. For example, at high ionic strengths at pH 7.0 BSA, transferrin, myoglobin and chymotrypsinogen behave in this way (Table II). Therefore, the effect of ethylene glycol on hydrophobic interactions can be confusing. However, more experiments need to be performed in order to elucidate the mechanism that increases the retardation of some proteins after addition of an organic modifier.

Chemical stability of Sephacryl S-200 HR at pH 2 and 13

Chemical structure of Sephacryl. Sephacryl is produced via a polymerization

process between N,N'-methylenediacrylamide and allyldextran[15]. The production procedure means that N,N'-methylenediacrylamide polymerizes to seven-membered ring units or reacts with allyldextran[15–17]. The chromatographic behaviour of the six proteins investigated (Figs. 1 and 2) clearly proves that Sephacryl S-200 HR contains anionic groups. As these groups are charged even at pH 4.2 they must have a pK_a value that is lower than *ca.* 4. It is known that dextran gels (Sephadex) contain small amounts of carboxyl groups[15]. Therefore, it is reasonable to assume that the observed ion-exchange and ion-exclusion interactions are caused by carboxyl groups in the dextran structures of Sephacryl S-200 HR. However, it is also possible that both carboxyl groups and amino groups can be formed because of the cleavage of amide bonds in polymerized N,N'-methylenediacrylamide units in Sephacryl S-200 HR after acid or base treatment.

*Chromatographic behaviour of Sephacryl S-200 HR after treatment at high or low pH.*The chemical stability of Sephacryl S-200 HR was studied by suspending the gel in 0.010 *M* hydrochloric acid or 0.10 *M* sodium hydroxide for 15 days. The stability of the gel was then evaluated by comparing the chromatographic results obtained after the treatment with the chromatographic behaviour of fresh Sephacryl S-200 HR (Figs. 1 and 2). Fig. 3 shows that the chromatographic results (at pH 4.2) from three representative proteins were different, depending on whether the gel had been treated at pH 2 or 13. It can also be noted that the chromatographic results with fresh gel (Figs. 1 and 2) are in agreement with those obtained from the gel that had been treated at pH 2. This indicates that Sephacryl S-200 HR is chemically stable at pH 2.0. However, the increasing K_{av} value of pepsin with decreasing ionic strength (Fig. 3 B) indicates that groups that are positively charged at pH 4.2 are formed on the base-promoted hydrolysis of Sephacryl S-200 HR. These cationic groups that are formed retard pepsin via an ion-exchange mechanism. Conversely, it can be expected that transferrin and chymotrypsinogen, which are positively charged at pH 4.2, should be eluted earlier on base-treated Sephacryl S-200 HR because of ion-exclusion interactions. Accordingly, Fig. 3 shows that the ion-exchange effect observed for

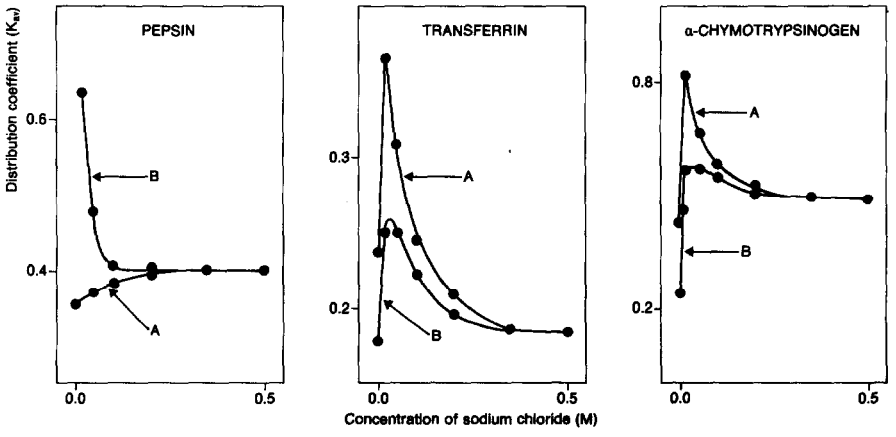

Fig. 3. Influence of sodium chloride concentration on K_{av} of pepsin, transferrin and α-chymotrypsinogen on (A) acid- and (B) base-treated Sephacryl S-200 HR. The mobile phase buffer was 0.020 *M* acetate (pH 4.2).

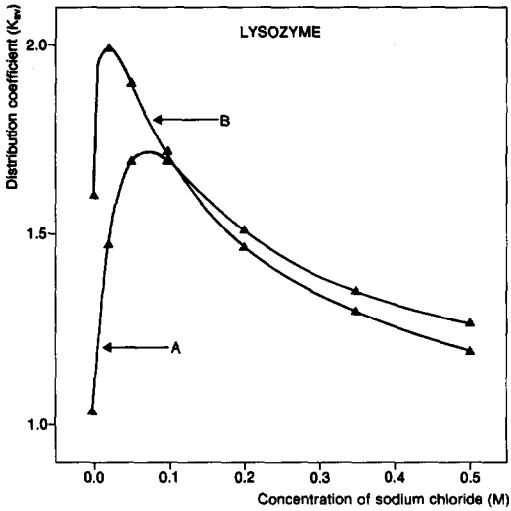

Fig. 4. Influence of sodium chloride concentration on K_{av} of lysozyme on (A) acid- and (B) base-treated Sephacryl S-200 HR. The mobile phase buffer was 0.020 phosphate (pH 7.0).

these proteins on fresh gel at pH 4.2 has decreased considerably on a sodium hydroxide-treated gel because of the counteracting ion-exclusion interactions.

The cationic groups formed on base treatment of Sephacryl S-200 HR only influenced the chromatographic results when an acidic mobile phase was used. This behaviour can probably be related to decreasing ionization of these cationic groups with increasing pH. However, with basic mobile phases one of the proteins investigated, lysozyme, was retarded to a greater extent at low ionic strength when a base-treated gel was used (Fig. 4). This could indicate that also a small amount of anionic groups had been formed during the treatment with 0.10 *M* sodium hydroxide. As fresh Sephacryl S-200 HR contains anionic groups, a small increase in this amount, as a result of base treatment, may significantly influence the K_{av} of lysozyme, probably because it has the highest net positive charge of the proteins investigated.

CONCLUSIONS

The objective of this work was to examine the interactions of various proteins with the gel filtration packing Sephacryl S-200 HR. From the results obtained it may be concluded that both pH and ionic strength must be carefully optimized in order to eliminate or reduce the non-size-related exclusion separation behaviour. Proteins with intermediate p*I* values (5–9) seem not to give rise to any interactions with Sephacryl S-200 HR when the pH of the mobile phase is 7.0 or 10 and the sodium chloride concentration is higher than 0.2 *M*. However, lysozyme (p*I* = 11) and pepsin (p*I* = 2.9) interact with the support under all the conditions investigated. Further, as a general rule, protein–support interactions are most dominant at acidic pH (4.2).

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