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Chemical properties of and solute–support interactions with the gel filtration medium Superdex 75 prep grade

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ABSTRACT

The influence of the ionic strength and pH of the mobile phase on the distribution coefficient of proteins with different p*I* values was studied on Superdex 75 prep grade. Superdex contains small amounts of negatively charged groups which are responsible for electrostatic interactions between ionic solutes and the gel. Ion-exchange or ion-exclusion interactions were observed at low ionic strengths when the pH of the mobile phase was lower or higher than the p*I* values of the proteins. For some proteins, hydrophobic interactions were also observed at high ionic strengths.

The chemical stability of Superdex 75 prep grade was studied by comparing the chromatographic results from Superdex treated in acidic or basic solutions with those from untreated Superdex. The separation characteristics of Superdex 75 prep grade were unaffected after 25 washes with 1.0 *M* sodium hydroxide solution or 0.1 *M* hydrochloric acid with a contact time of 4 h for each wash. However, storage for 2 weeks in 0.01 *M* hydrochloric acid or 0.1 *M* sodium hydroxide solution partly hydrolysed the covalently bounded dextran in the agarose pores. This hydrolysation resulted in leakage of dextran and an increase in the K_{av} values of the test proteins.

INTRODUCTION

Dextran (*e.g.*, Sephadex) and agarose (*e.g.*, Sepharose) based gel filtration chromatography (GFC) media have played an essential part in the separation of biological samples for many years[1,2]. Dextran gels are known to give high selectivity in GFC as they have an evenly distributed polymer network[3]. However, agarose gels, which have a macroreticular structure[3], tend to have less steep selectivity curves and separate at considerably higher masses than Sephadex gels. On the other hand, agarose gels have a higher matrix rigidity, allowing higher flow-rates to be used.

The GFC medium investigated in this work, Superdex 75 prep grade, is composed of both dextran and agarose and the mean bead size is *ca.* 34 μm. It has been produced to combine high selectivity and high matrix rigidity. The selectivity of

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Superdex 75 prep grade is the same as that of Sephadex G-75, 3000-70 000 daltons. A comprehensive description of the selectivity, efficiency (N), bead structure and flow-rate properties of Superdex is presented elsewhere[4,5].

In addition to the exclusion properties and physical characteristics of a GFC medium, important considerations in GFC are also the non-size-related separation effects and the chemical stability of the medium[6,7]. The purpose of this study was to interpret the chromatographic results obtained with this packing under different elution conditions for a number of proteins. The separation characteristics of Superdex 75 prep grade were also studied after treatment under extreme acidic or basic conditions.

EXPERIMENTAL

Equipment

Chromatographic experiments were carried out with two Pharmacia FPLC systems, each 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. Superdex 75 prep grade was packed in Pharmacia HR 16/50 columns (50 cm × 1.6 cm I.D.) according to the packing instructions for the gel. A Shimadzu C-R3A integrator was used to store data.

Reagents

The mobile phase buffers were made from sodium acetate and acetic acid (pH 4.2), sodium dihydrogenphosphate and disodium hydrogenphosphate (pH 7.0) or sodium hydrogencarbonate and sodium carbonate (pH 10.0). The buffers were prepared from 0.020 *M* stock solutions of these acid and base components. Variations of the ionic strength were obtained by adding different amounts of sodium chloride to the stock solutions. The proteins used are listed in Tables I and II. These proteins were dissolved in the mobile phase at a concentration of *ca.* 2 mg/ml and kept frozen until used. All buffers and protein solutions were filtered through a 0.45-μm filter before use.

TABLE I

PROTEINS USED FOR STUDYING CHROMATOGRAPHIC BEHAVIOUR BEFORE AND AFTER BULK TREATMENT OF SUPERDEX 75 PREP GRADE

Protein	*Source*	*Concentration (mg/ml)*	*Molecular weight*	*Isoelectric point (pI)*
Lysozyme	Egg white	2.0	13 900	11.0
Myoglobin	Horse heart	3.0	17 500	7.0
α-Chymotrypsinogen A	Bovine pancrease	1.0	25 000	8.8
Pepsin	Bovine	8.0	33 000	2.9
Serum albumin	Bovine	2.0	69 000	4.7
Transferrin	Human	2.0	76 500	5.5

TABLE II

PROTEINS USED FOR STUDYING CHROMATOGRAPHIC BEHAVIOR ON SUPERDEX 75 PREP GRADE DURING CLEANING-IN-PLACE EXPERIMENTS

	Protein[a]	*Source*	*Concentration (mg/ml)*	*Molecular weight*	*Isoelectric point (pI)*
A	Cytidine		0.1	323	
B	Lysozyme	Egg white	1.2	13900	11.0
C	Cytochrome C	Horse heart	1.2	12400	10.2
D	Myoglobin	Horse heart	1.2	17500	7.0
E	α-Chymotrypsinogen A	Bovine pancrease	1.2	25000	8.8
F	β-Lactoglobulin	Bovine milk	1.2	35000	5.2
G	Ovalbumin	Egg white	2.5	45000	4.7
H	Serum albumin	Bovine	2.5	69000	4.7
I	IgG	Human	1.2	160000	7.7

[a] The letters A–I refer to the designation of the proteins in Figs. 5 and 6.

Treatment of Superdex 75 prep grade before packing

The chemical stability of Superdex 75 prep grade under static conditions was studied by incubating the gel (about 150 ml) for 14 days in 0.01 *M* hydrochloric acid or 0.1 *M* sodium hydroxide solution. Before the gel was incubated it was washed with 300 ml of the incubation solution. The incubation was done at ambient temperature (*ca.* 20°C). The two treated gel samples were packed in HR 16/50 columns and their chromatographic behaviour was compared with that of untreated Superdex 75 prep grade.

Chromatographic procedure for treated and untreated Superdex 75 prep grade

The columns were equilibrated by passage of at least three bed volumes of mobile phase before being used for experimental observations. The chromatographic runs were performed by individual injections of the proteins in Table I to avoid possible interactions between proteins. The flow-rate was 1.0 ml/min and the injection volume was 500 μl. The resulting retention volumes were used to calculate the distribution coefficient (K_{av}) from the equation

$$K_{av} = (V_e - V_0)/(V_t - V_0)$$

where V_e, V_0 and V_t represent solute elution volume, void volume and the total bed volume of fluid and gel combined, respectively. Blue Dextran 2000 (8.0 mg/ml) was employed as a marker of the void volume. V_0 was determined for all three buffers used: the relative standard deviation of V_0 was 0.75% ($n = 18$) when calculated from V_0 data acquired from all mobile phases investigated. V_t was calculated from the bed height and the inner diameter of the column. V_t was about 102 ml and the void fraction (V_0/V_t) was 0.34 for all packed columns.

Treatment of Superdex 75 prep grade after packing

The chemical stability of Superdex 75 prep grade was also studied by treating the gel after it had been packed in the column. One packed HR 16/50 column was

repeatedly treated with 0.1 M hydrochloric acid and another with 1.0 M sodium hydroxide solution. The solutions were pumped into the columns at a flow-rate of 1.0 ml/min for the acid and 0.67 ml/min for the sodium hydroxide solution. The flow was stopped when 100 ml had passed and after a specified time stated below and in Figs. 5 and 6 the gels were equilibrated with 0.02 M sodium phosphate buffer (pH 7.0) containing 0.3 M sodium chloride. A series of proteins, listed in Table II, were then injected onto the columns and the chromatographic behaviour was evaluated. The total time of exposure was 2 weeks, divided into 25 treatments of 4 h each, five treatments of 10 h, three of 24 h and finally two lasting 48 h. The chromatographic behaviour was tested after each treatment and the chromatographic procedure was the same as above except that 1.0 mg/ml of Blue Dextran 2000 was used as a V_0 marker. The number of plates (N) was determined by injection of 500 μl of 1% (v/v) acetone after each treatment. The retention volume (V_e) and the peak width at half-height ($w_{0.5}$) were used to calculate N [$N = 5.54 \cdot (V_e/w_{0.5})^2$].

RESULTS AND DISCUSSION

In gel filtration chromatography, the influence of mechanisms other than the size-separation mechanism is well documented. The most important non-size-related mechanisms are ion-exchange, ion-exclusion and hydrophobic interactions between the stationary phase and the sample molecules[7,8]. In addition, intramolecular electrostatic repulsive interactions[8] can also influence the separation behaviour of the sample molecule. Manipulation of these interactions by changing the mobile phase can increase selectivity in GFC. To test the extent of these separation mechanisms of Superdex 75 prep grade the influence of ionic strength on the K_{av} values of various proteins (Table I) was studied at different pH.

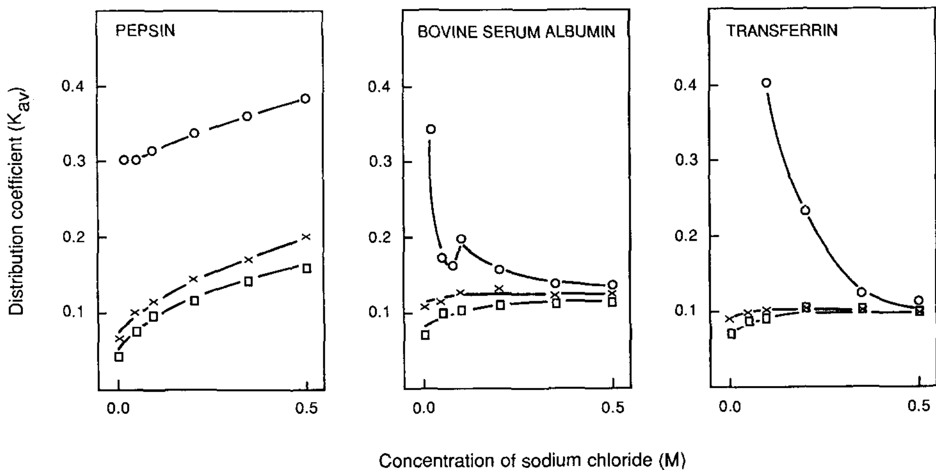

Fig. 1. Influence of sodium chloride on K_{av} of pepsin, bovine serum albumin and transferrin on untreated Superdex 75 prep grade with various mobile phase buffers. Mobile phases: ○ = 0.020 M acetate, pH 4.2; × = 0.020 M phosphate, pH 7.0; □ = 0.020 M carbonate, pH 10.0.

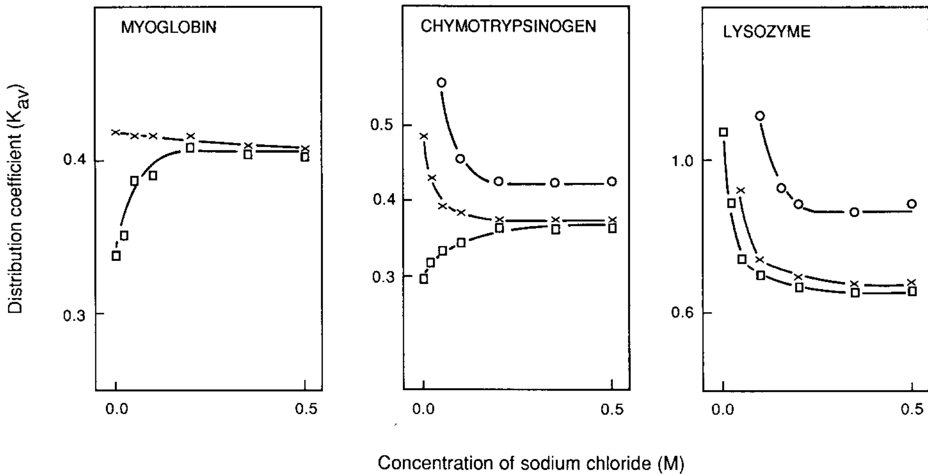

Fig. 2. Influence of sodium chloride on K_{av} of myoglobin, α-chymotrypsinogen and lysozyme on untreated Superdex 75 prep grade with various mobile phase buffers. Mobile phases: ○ = 0.020 *M* acetate, pH 4.2; × = 0.020 *M* phosphate, pH 7.0; □ = 0.020 *M* carbonate, pH 10.0.

Influence of ionic strength and pH on the performance of Superdex 75 prep grade

Figs. 1 and 2 show that the K_{av} values of the six investigated proteins were strongly influenced by the pH and the ionic strength of the mobile phase buffers used.

Pepsin. The increase in the K_{av} value of pepsin with increasing ionic strength at pH 10.0 and 7.0 (Fig. 1) is probably related to a decreasing ion-exclusion effect. This interpretation is supported by the fact that dextran and agarose gels contain small amounts of carboxyl groups[2,9] which are negatively charged at pH 7.0 and 10.0 [like pepsin (p*I*=2.9)]. However, at pH 4.2 pepsin and Superdex 75 prep grade are charged to a lesser extent because the acid groups are ionized to a lower degree. The much higher K_{av} values observed at pH 4.2 than at pH 7.0 and 10.0 indicate that ion-exclusion effects did not influence the result to a great extent. Therefore, the increase in K_{av} with increasing ionic strength at pH 4.2 is probably related to hydrophobic interations. Such interactions between dextran-based gels and pepsin have been observed previously at acidic pH values[6].

Bovine serum albumin (BSA). Ion exclusion seems to influence significantly the K_{av} value of BSA only at low ionic strenght at pH 10.0 (Fig. 1). This may be attributed to the higher p*I* value of BSA compared with pepsin (Table I). Minimal interactions between Superdex 75 prep grade and BSA occurred at pH 7.0 and 10.0 at [NaCl] > 0.2 *M*, according to the K_{av} plateau (0.11). On the other hand, at pH 4.2, where BSA has a positive net charge, the results in Fig. 1 suggest that several non-size-related mechanisms are involved. Ion exchange between the positively charged BSA and anionic groups on Superdex 75 prep grade explains why K_{av} increases with decreasing ionic strength. In addition, it is known that BSA molecules expand in acidic solutions because of intramolecular repulsive interactions[10,11]. These interactions are most pronounced at low ionic strength and will be superimposed on the ion-exchange mechanism. The molecular expansion counteracts the ion-exchange effect and can therefore explain the decrease in K_{av} of BSA at about 0.05 *M* NaCl (Fig. 1).

Transferrin. In contrast to BSA, no effects from intramolecular interactions were observed for transferrin. However, like BSA, both ion exclusion and ion exchange influenced the retention time of transferrin (Fig. 1). Ion-exchange interactions affect the retention of transferrin more than for BSA because of the higher p*I* value of transferrin (Table I).

Myoglobin. The effect of ionic strength on the K_{av} of myoglobin at pH 10.0 is caused by ion-exclusion interaction, as discussed previously for pepsin, BSA and transferrin (Figs. 1 and 2). No significant trend of the retention of myoglobin was observed at pH 7.0 (Fig. 2), indicating that only an ideal size separation mechanism is involved. In the acidic mobile phase buffer myoglobin was denatured and precipitated in the column top filter.

Chymotrypsinogen. The high p*I* value of chymotrypsinogen means that it is positively charged at pH < 8.8. The increasing K_{av} value of chymotrypsinogen with decreasing ion strength at pH 4.2 and 7.0 (Fig. 2) is therefore attributed to an ion-exchange mechanism. The K_{av} plateau at pH 4.2 is higher compared with the K_{av} plateau at pH 7.0 (Fig. 2). This indicates an increasing hydrophobic interaction superimposed on a decreasing ion-exchange interaction when the sodium chloride concentration increases from 0.2 to 0.5 *M* at pH 4.2. The ideal K_{av} value of chymotrypsinogen is probably about 0.37, as the K_{av} plateaux at pH 7.0 and 10.0 merge at this value when [NaCl] > 0.2 *M*. However, at low ionic strength at pH 10 chymotrypsinogen is excluded from the pores because of electrostatic repulsion between the protein and the negatively charged Superdex 75 prep grade.

Lysozyme. Fig. 2 shows that the K_{av} of lysozyme increases with decreasing ionic strength for all the mobile phase buffers used. As lysozyme is positively charged at pH < 11 (Table I) and Superdex 75 prep grade is negatively charged at pH $>$ *ca.* 4, ion exchange can explain this behaviour. The levels of the K_{av} plateaux at [NaCl] > 0.4 *M* are much higher for all investigated buffers than could be expected from the molecular weight of lysozyme alone. It has been shown[12] that lysozyme exhibits a strong hydrophobic nature. Therefore we postulate that hydrophobic interactions also contribute to the retardation of lysozyme at high ionic strengths. Fig. 2 indicates that these interactions are highest at pH 4.2.

Chemical stability of Superdex 75 prep grade during long-term incubation at pH 2 and 13

The chromatographic behaviour of six proteins (Table I) on Superdex 75 prep grade treated with 0.01 *M* hydrochloric acid or 0.1 *M* sodium hydroxide solution for 14 days is shown in Figs. 3 and 4. Fig. 3 and 4 show that the K_{av} values of the proteins are influenced by the ionic strength in the same way on untreated and on acid- and base-treated Superdex 75 prep grade. This similiar behaviour indicates that no charged groups were formed on Superdex 75 prep grade during long-term incubation at pH 2 or 13. On the other hand the K_{av} values obtained after acid or base treatment of the gel are about 0.05 units higher than for the untreated Superdex 75 prep grade (Figs. 3 and 4). These results can be explained if the available pore volume has increased after the long-term incubation (see also the next section). Superdex 75 prep grade consists of spherical agarose beads to which dextran has been covalently bonded. Therefore, an increase in the available pore volume of Superdex 75 prep grade can be caused by a certain breakdown of dextran. The observed release of dextran at pH 2 and 13, verified with an immunochemical method[13], proves that dextran decomposes. This

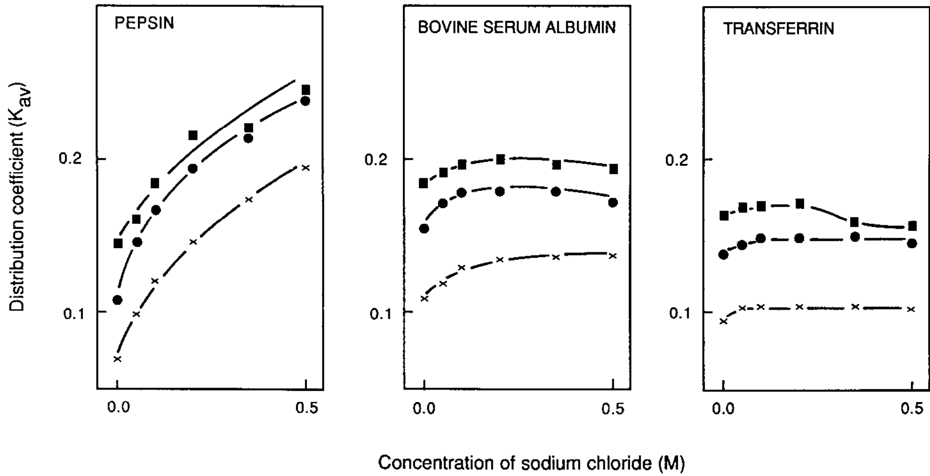

Fig. 3. Influence of sodium chloride on K_{av} of pepsin, bovine serum albumin and transferrin on (×) untreated, (●) acid-treated and (■) base-treated Superdex 75 prep grade. The mobile phase was 0.020 *M* phosphate (pH 7.0).

leakage will be investigated more carefully in a forthcoming study. Figs, 3 and 4 also show that the changes in available pore volume were higher after incubation at pH 13 than at pH 2.

Chemical stability of Superdex 75 prep grade with repeated column washes at pH 1 and 14

Cleaning procedures for prepacked gel filtration columns normally consist of rinsing with sodium hydroxide solution or hydrochloric acid. For example, cleaning-

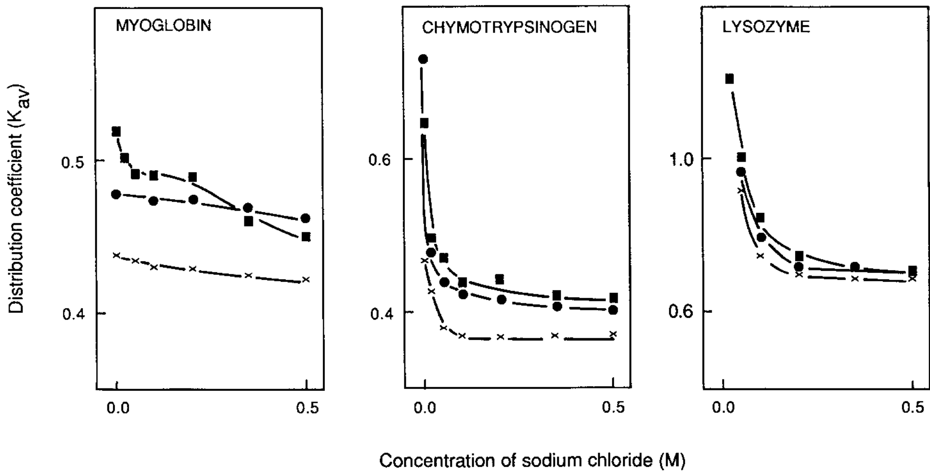

Fig. 4. Influence of sodium chloride on K_{av} of myoglobin, α-chymotrypsinogen and lysozyme on (×) untreated, (●) acid-treated and (■) base-treated Superdex 75 prep grade. The mobile phase was 0.020 *M* phosphate (pH 7.0).

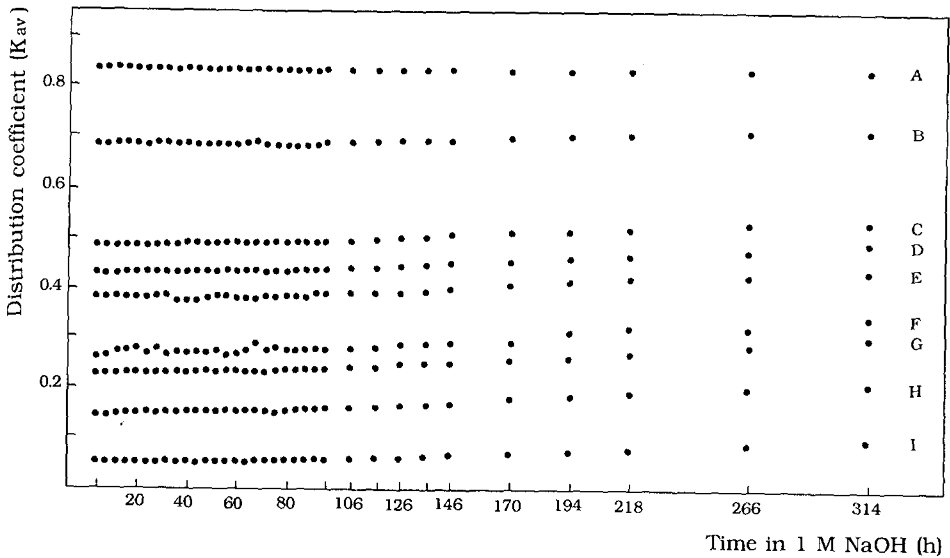

Fig. 5. Influence of repeated CIP treatments with 1.0 *M* NaOH on the distribution coefficients of a series of proteins (Table II) on Superdex 75 prep grade in an HR 16/50 column.

in-place (CIP) with 1.0 *M* sodium hydroxide for *ca.* 2 h is a very effective means of removing bacterial contamination from the purification system[14]. Therefore it is very important that a liquid chromatographic medium intended for purification of biomolecules can withstand many short-term treatments at extreme pH values.

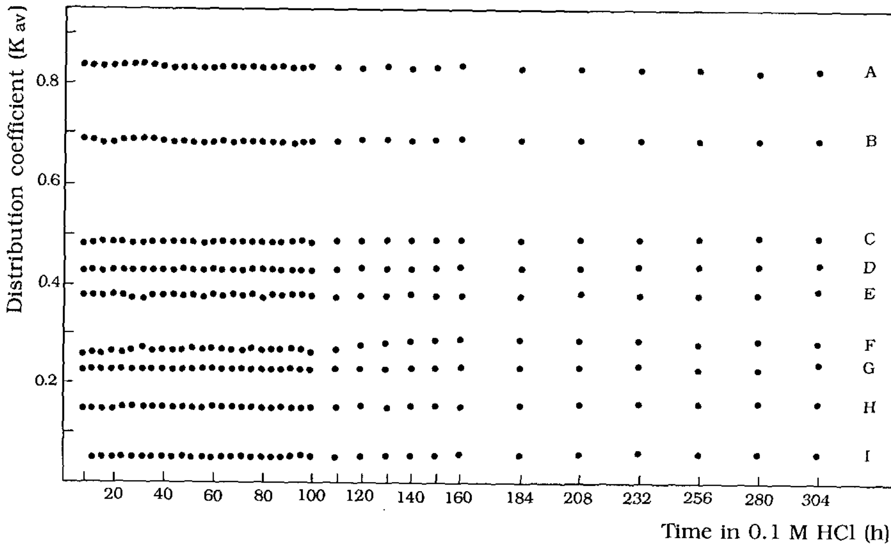

Fig. 6. Influence of repeated CIP treatments with 0.1 *M* HCl on the distribution coefficients of a series of proteins (Table II) on Superdex 75 prep grade in an HR 16/50 column.

The change in the K_{av} values of eight different proteins (Table II) after repeated short-term treatments of two Superdex 75 prep grade columns with 1.0 *M* sodium hydroxide solution and 0.1 *M* hydrochloric acid is shown in Figs. 5 and 6, respectively. A slight and gradual increase in retention volume was observed for all proteins in CIP with 1.0 *M* sodium hydroxide solution (Fig. 5). This trend increased when the washing time was prolonged. It can also be noted that the K_{av} of cytidine and V_0 (determined using Blue Dextran) were not influenced by the washing procedure. These results can be rationalized if the pore volume is constant and the available pore volume for protein molecules increases after the gel has been treated with 1.0 *M* sodium hydroxide solution. Partial breakdown of dextran (as suggested in the previous section) in the agarose pores at extreme pH values can cause such an effect on the separation behaviour. The total increase in the K_{av} values of the proteins after repeated washings with 1.0 *M* sodium hydroxide solution resulting in a total contact time of 14 days was in the range 0.04–0.09 (Fig. 5). This increase can be compared with the long-term treatment of the medium at pH 13 (experiments before packing reported in Figs. 3 and 4). Even though the pH value in the long-term treatment experiments was one unit lower, th K_{av} values of the proteins increased as much as for the total increase in the repeated washes experiments. The cause of these results has not been elucidated but may be attributed to the catalytic action of a hydrolysis product of dextran or the decomposition of dextran being independent of the sodium hydroxide concentration in the range investigated.

The results obtained after repeated column washes with 0.1 *M* hydrochloric acid (Fig. 6) show that the increase in the K_{av} values of the proteins was less than with 1.0 *M* sodium hydroxide solution (Fig. 5). However, long-term treatment with 0.01 *M* hydrochloric acid (pH 2) resulted in about the same effect on the K_{av} values (Figs. 3 and 4) as the total effect observed in the repeated CIP treatments with 0.1 *M* hydrochloric acid (Fig. 6). This effect can probably be explained similarly to for the results at high pH (see above).

The number of plates per metre for both columns were randomly varied between 13 500 and 16 500 during the tests. Further, the bed heights and the column back-pressure were constant throughout the experiments. The results indicate that the rigidity of Superdex 75 prep grade was unchanged after repeated CIP cycles with 0.1 *M* hydrochloric acid and 1.0 *M* sodium hydroxide solution.

CONCLUSION

The objectives of this work were to investigate the interactions of various proteins with the gel filtration medium Superdex 75 prep grade and to investigate the chemical stability of the gel.

It was shown that the presence of negatively charged groups on Superdex 75 prep grade can explain the electrostatic characteristics of the gel at low ionic strengths of the mobile phase. Ideal gel filtration chromatography was observed for proteins with intermediate p*I* values (5–9) using mobile phases of pH 7.0 and 10.0 at sodium chloride concentrations higher than about 0.2 *M*. In addition, the results obtained with lysozyme and chymotrypsinogen at acidic pH also show that Superdex 75 prep grade has hydrophobic properties at high ionic strengths.

The study of the chemical resistance of Superdex 75 prep grade shows that

dextran in the agarose pores was partly hydrolysed under extreme acidic and basic conditions. Nevertheless, a column packed with Superdex 75 prep grade can withstand at least 25 CIP cycles with 1.0 *M* sodium hydroxide solution or 0.1 *M* hydrochloric acid (contact time 4 h for each) without any significant change in separation performance.

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