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Stability of Superdex 75 prep grade and Superdex 200 prep grade under different chromatographic conditions

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ABSTRACT

The chemical stability of two gel filtration media, Superdex 75 prep grade and Superdex 200 prep grade, was studied in bulk and column experiments. The release of agarose and dextran from these two composite media was measured by three different methods: a specific nephelometric method based on the use of antidextran antiserum, an anthrone method and a gel filtration chromatographic method with a light-scattering detector. Dextran fragments were released from Superdex 75 and 200 prep grade under extreme basic and acidic conditions. However, Superdex withstands many short-term incubations (contact time *ca.* 4 h each time) at pH 14 and 1 without any influence on the chromatographic behaviour. Equilibration of a Superdex column with a neutral buffer after these short-term treatments lowered the concentration of dextran in the eluate to an undetectable level after about three bed volumes. The ability of Superdex columns to withstand practical mistakes such as pumping air into the column was also investigated.

INTRODUCTION

Superdex 75 prep grade and Superdex 200 prep grade are new composite high-performance preparative gel filtration media [1] made by binding dextran covalently to cross-linked agarose beads. The selectivities of the Superdex gels are designed to give separation ranges for proteins similar to those of the corresponding Sephadex media. Thus, Superdex 75 prep grade has the same selectivity as Sephadex G-75 (3–80 kilodalton) and Superdex 200 prep grade has the same selectivity as Sephadex G-200 (5–600 kilodalton) [2]. The mean particle size of the Superdex prep grade matrix is *ca.* 34 μm. The chromatographic properties of these gels have been described in more detail elsewhere [1,3].

It is high important from many points of view that preparative liquid chromatographic (LC) media are chemically stable. The decomposition of such media in different mobile phase buffer solutions is of crucial interest to those involved in purifying pharmacological products. Significant contamination in this area would severely limit the general adoption of the media to process-scale purification. Several studies concerned with the stability of silica-based packings for reversed-phase chromatography have been reported [4–7].

The main aim of this work was to produce quantitative data to demonstrate the

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chemical stability of Superdex 75 prep grade and Superdex 200 prep grade in different solvents. Previously we observed [3] that the K_{av} values of proteins increased slightly on Superdex 75 prep grade treated with solutions of high pH (13–14) and low pH (1–2) for long periods. An increased volume of the agarose pores because of hydrolysis of dextran was assumed to cause this effect. To verify this interpretation, the leakage of polysaccharides from the two media was studied at extreme pH values under static and chromatographic conditions. In addition, the functional resistance to 1.0 *M* NaOH, 0.1 *M* HCl and air pumped into columns packed with Superdex 200 prep grade was studied.

EXPERIMENTAL

Chemicals and apparatus

Superdex 75 prep grade, Superdex 200 prep grade, dextran 70, cross-linked agarose beads (semi-product in the production of Superdex gels) and the dextran used for Superdex were obtained from Pharmacia LKB Biotechnology. Dextran standards with different molecular weights (see Table III) were purchased from Pharmacosmos. The proteins used are listed in Table I and all inorganic compounds were of analytical-reagent grade. The antidextran antiserum was obtained from Pharmacia. The production of antiserum has been described earlier [8]. Tween 20 and polyethylene glycol (PEG) 6000 were provided by KEBO Lab.

Chromatographic measurements were performed on fast protein (FPLC) systems from Pharmacia. Two systems were used, each consisting of an LCC-500 control unit, two P-500 precision pumps, a UV-1 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 a REC-481 recorder. A Shimadzu C-R3A integrator was used to store chromatographic results.

A Shimadzu RF-540 spectrofluorimeter with a DR-3 data recorder and a 10 × 2 mm cell as used for nephelometric measurements.

Determination of polysaccharides with anthrone was carried out with a Technicon AutoAnalyzer II system.

TABLE I

PROTEINS USED FOR STUDYING CHROMATOGRAPHIC PROPERTIES OF SUPERDEX 200 PREP GRADE DURING CLEANING-IN-PLACE EXPERIMENTS

Substance		Source	Concentration (mg/ml)	Molecular weight
A	Cytidine		0.10	323
B	Lysozyme	Egg white	0.62	13 900
C	Cytochrome *c*	Horse heart	1.25	12 400
D	Myoglobin	Horse heart	1.25	17 800
E	α-Chymotrypsinogen	Bovine pancrease	1.25	25 000
F	β-Lactoglobulin	Bovine milk	1.25	35 000
G	Ovalbumin	Egg white	2.50	45 000
H	Serum albumin	Bovine	1.25	67 000
I	Immunoglobulin G	Human	1.25	160 000
J	Ferritin	Horse spleen	0.20	440 000

Analysis of the molecular size distribution of the polysaccharides released from Superdex was performed by using an FPLC solvent delivery system. The separation was performed on three serially coupled columns. The column combination was one TSK G2000PW, one TSK G3000PW and one TSK G5000PW (30 cm × 0.8 cm I.D. (Tokyo Soda, Tokyo, Japan). Column effluents were monitored by using an OPTI-LAB Multiref 901 detector.

Functional stability of Superdex 200 prep grade under different treatments

Treatment with 1.0 M NaOH and 0.1 M HCl. The functional stability of Superdex 200 prep grade was studied by making on-column treatments of the gel with 1.0 *M* NaOH and 0.1 *M* HCl at ambient temperature (22°C). Superdex 200 prep grade was packed in two HR 16/50 columns (50 cm × 1.6 cm I.D.), one for each solution. The solutions were pumped into the columns at a flow-rate of 0.33 ml/min. When the columns were filled with either of the solutions, the flow was stopped. After a specified time (stated below), the gels were equilibrated with three bed volumes of the mobile phase (0.02 *M* phosphate buffer, pH 7.0, containing 0.3 *M* sodium chloride). A series of proteins, listed in Table I, were then injected onto the columns, and the chromatographic behaviour was evaluated by individual injections of the proteins to avoid interactions between the proteins. The total time of exposure to 1.0 *M* NaOH or 0.1 *M* HCl was 2 weeks, divided into *ca.* nineteen treatments of 8 h each and eleven treatments of 16 h. The chromatographic behaviour was tested after each treatment. The flow-rate was 0.5 ml/min and the injection volume was 500 µl. The resulting retention volumes (V_e) of the proteins were used to calculate the distribution coefficient (K_{av}) from the equation

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

where V_o and V_t represent the void volume and the total bed volume of fluid and gel combined, respectively. Blue Dextran 2000 (1.0 mg/ml) was employed as a V_o marker and V_t was calculated from the bed height and the inside diameter of the column. V_t was 103 ml and the void fraction V_o/V_t was 0.36 for both columns. The number of theoretical plates (N) was determined by injection of 500 µl of 1% (v/v) acetone at the start and at the end of the experiments for both columns. The retention volume (V_e) and the peak width at half-height ($W_{0.5}$) were used to calculate N [$N = 5.54(v_e/W_{0.5})^2$].

Resistance to air. A column (HR 16/50) packed with Superdex 200 prep grade was treated with air several times. After each treatment the column was regenerated and the performance evaluated. The amounts of air and buffer for regeneration are given in Table V. The performance of the column was determined in two ways, measuring the plate number and the K_{av} values of β-lactoglobulin and lysozyme.

Determination of leakage from Superdex 75 and 200 prep grade

Leakage in bulk experiments. About 50 ml of homogenized suspension of Superdex 75 or 200 prep grade were transferred to a glass filter-funnel (pore size G3). The gels were washed with about 1 l of distilled water and then sucked dry with a water pump. Portions of 2.00 g gel were transferred to 25-ml conical flasks, 10 ml of the incubation solution were added and the flasks were sealed with a piece of Para-

film. The incubation solutions were hydrochloric acid (pH 1, 2 and 3) and sodium hydroxide (pH 12, 13 and 14) solutions. After an incubation time of 2 weeks at 22°C the supernatant was removed and filtered through a 0.45-μm filter and then analysed for its polysaccharide content (see below). Samples with a high polysaccharide content were diluted with distilled water prior to analysis.

Leakage in on-column experiments. The gels were packed in Pharmacia K 16/10 columns (10 cm × 1.6 cm I.D.) at a flow-rate of 2 ml/min to settle the gel. After the end adaptor had been inserted the gel bed was stabilized by pumping water through the column at a flow-rate of 7 ml/min for 1 h. Sodium hydroxide solution (1 *M*) was then pumped into the columns at a flow-rate of 1 ml/min. The flow was stopped after passage of 40 ml of 1.0 *M* NaOH. After a contact time of 4 h the columns were equilibrated with ten bed volumes of 0.02 *M* sodium phosphate buffer (pH 7.0) at a flow-rate of 1 ml/min. Fractions of the eluate were continuously sampled during the equilibration of the columns and analysed with the nephelometric method (see below) to determine any released amount of dextran. The treatment of the columns with sodium hydroxide solution was then repeated twice. All experiments were performed at room temperature (22°C).

Nephelometric determination of dextran. The antidextran antiserum was diluted 50-fold with 0.05 *M* phosphate buffer containing 0.1 *M* NaCl, 0.15 ml/l of Tween 20 and 100 g/l PEG 6000 [8]. After 20 min at room temperature, the solution was filtered through a 0.22-μm Millipore filter. A 360-μl volume of this antiserum–polymer solution was mixed with 30 μl of the sample solution in a 600-μl quartz cuvette. After a reaction time of 4 min the light scattering was measured at 312 nm by scanning the excitation and emission monochromators in a synchronous manner ($\Delta\lambda$ = 0 nm) between 250 and 350 nm. Dextran used in the production of Superdex was used as a standard and gave linear calibration graphs in all incubation solutions in the range 0–10 μg/ml.

Determination of dextran and agarose with anthrone. The selection of sulphuric acid concentration, reaction time, reaction temperature and anthrone concentration was made in accordance with the conditions recommended by Scott and Melvin [9]. The wavelength for the absorption measurements was adjusted to 620 nm. Dextran 70 was used as the calibration substance and linear calibration graphs were obtained for all incubation solutions in the working range 0–30 μg/ml.

RESULTS AND DISCUSSION

The exact lifetime of Superdex columns will depend on the nature of the sample and the stability of the matrix with respect to the elution and cleaning conditions. The stability of gel filtration packings in typical FPLC separations is often not considered to be an important problem [10,11]. Relatively mild conditions are often met in many analytical applications. However, separation media for preparative applications must be periodically renewed after exposures to large amounts of protein. A common approach for cleaning or regeneration of contaminated preparative media is to wash them with acidic or alkaline solutions [12]. It is therefore important that gel filtration media for preparative separation can withstand extreme pH conditions to ensure a long column lifetime.

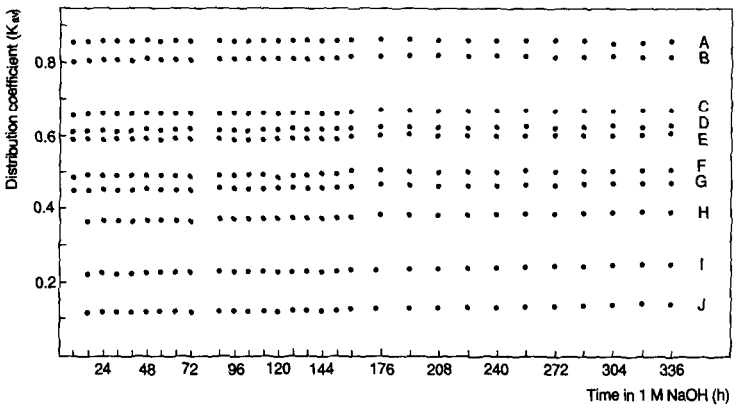

Fig. 1. Influence of repeated CIP treatments with 1.0 *M* NaOH on the distribution coefficients of a series of proteins (Table I) on Superdex 200 prep grade in an HR 16/50 column.

Chemical stability of Superdex 200 prep grade on repeated column washes at pH 1 and 14

Recently, we showed that Superdex 75 prep grade withstands at least 25 short-term treatments (exposure time 4 h each time) with 1.0 *M* NaOH or 0.1 *M* HCl without any changes in chromatographic behaviour [3]. Figs. 1 and 2 show that Superdex 200 prep grade was also functionally stable during all cleaning-in-place cycles with 1.0 *M* NaOH or 0.1 *M* HCl with a contact time of 8 h per cycle. These results demonstrate that Superdex 75 and 200 prep grade may be cleaned with solutions that effectively regenerate and purify the matrix. No effect on the efficiency of the columns was observed during the tests. However, a small increase in retention volumes was observed for the proteins, but not for cytidine, when the cleaning time was prolonged to 16 h (Figs. 1 and 2). This behaviour was also observed with Superdex 75 prep grade [3]. We previously suggested [3] that hydrolysis of dextran in the

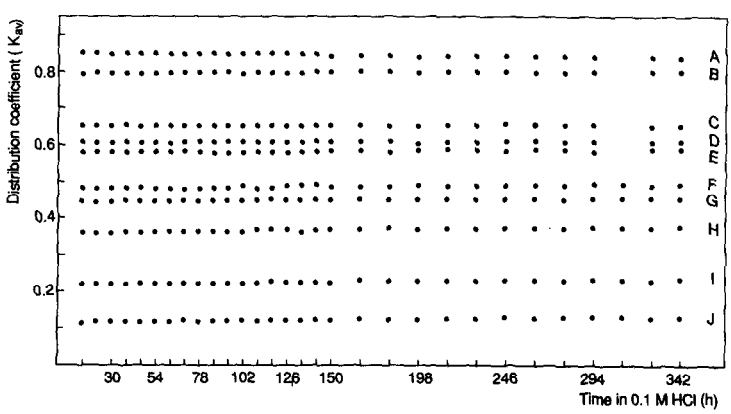

Fig. 2. Influence of repeated CIP treatments with 0.1 *M* HCl on the distribution coefficients of a series of proteins (Table I) on Superdex 200 prep grade in an HR 16/50 column.

agarose pores can give rise to increased K_{av} values. To verify this hypothesis and to investigate the risk of contamination of fractionated proteins with polysaccharide fragments, a study of possible leakage products from Superdex media was undertaken.

Leakage of polysaccharides from Superdex 75 and 200 prep grade

To determine the amount of dextran and agarose released from Superdex 75 and 200 prep grade, a specific nephelometric method for dextran [8], based on the use of antidextran antiserum, and the well documented anthrone method [9] were used.

Static experiments. From the agarose base matrix, to which dextran is coupled in the production of Superdex 75 and 200 prep grade, only small amounts of agarose were released during static experiments at pH 1 (Table II). However, polysaccharides were released from the Superdex gels at all pHs investigated (Table II). These results show that the polysaccharide leakage from Superdex mainly originated from the dextran structures of the gel. The dextran leakage was highest at extremely high pH (14) and low pH (1) (and decreased when the incubation pH was changed towards neutral values). Also, the leakage of dextran was higher from Superdex 75 than Superdex 200 prep grade. The results from the antrone and the nephelometric measurements were in good accord (Table II) and also support the suggestion that dextran is the main leakage product.

The leakage of dextran is probably caused by base- and acid-promoted hydrolysis of glycosidic linkages between glucose units. This means that the molecular weight–size distribution curve of the dextran fragments released ought to be displaced

TABLE II

POLYSACCHARIDE LEAKAGE FROM SUPERDEX 75 AND 200 PREP GRADE AND SEMI-PRODUCT TO SUPERDEX IN STATIC EXPERIMENTS DURING 2 WEEKS IN DIFFERENT SOLUTIONS

Incubation medium	Method[a]	Superdex 75 prep grade (μg/ml)	Superdex 200 prep grade (μg/ml)	Sepharose[b] (μg/ml)
1 *M* NaOH	ANT	1500	150	<2
0.1 *M* NaOH	ANT	740	86	<2
0.01 *M* NaOH	ANT	80	31	<2
0.001 *M* HCl	ANT	41	4	<2
0.01 *M* HCl	ANT	43	16	<2
0.1 *M* HCl	ANT	295	70	5
1 *M* NaOH	NEP	960	540	n.a.[c]
0.1 *M* NaOH	NEP	850	208	n.a.
0.01 *M* NaOH	NEP	2	5	n.a.
0.001 *M* HCl	NEP	3	5	n.a.
0.01 *M* HCl	NEP	55	48	n.a.
0.1 *M* HCl	NEP	220	152	n.a.

[a] The leakage was analysed using two different methods: ANT = anthrone method; NEP = nephelometry.

[b] Sepharose is the agarose bead on which Superdex 75 and 200 prep grade are based.

[c] n.a. = Not analysed.

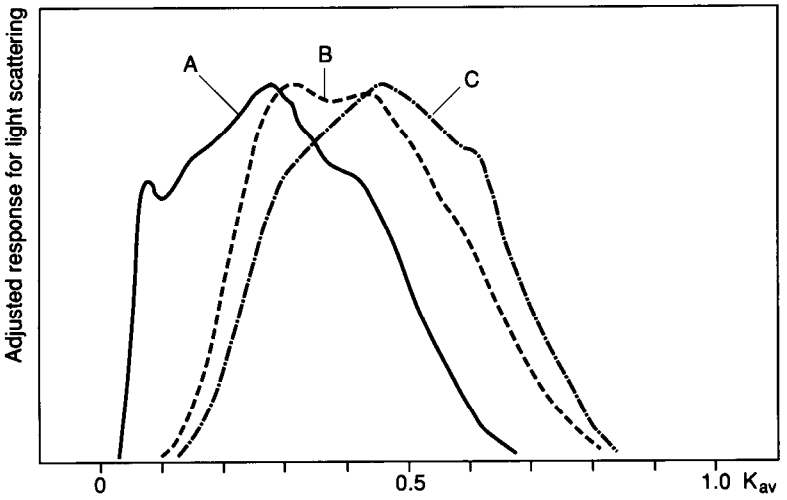

Fig. 3. Gel filtration elution pattern of (A) dextran used in the production of Superdex, and polysaccharides released from Superdex 75 prep grade incubated for 2 weeks in (B) 0.1 *M* HCl and (C) 1.0 *M* NaOH.

towards lower molecular weights compared with the dextran used in the production of Superdex. This was also verified when the dextran leakage was analysed by gel filtration chromatography after incubation of the gels in 1.0 *M* NaOH or 0.1 *M* HCl (Fig. 3). Fig. 3 also shows that the size distribution of released dextran fragments covered the range from $2.4 \cdot 10^6$ (K_{av} = 0.1) to $2.0 \cdot 10^3$ (K_{av} = 0.80).

To study the effect of the molecular size of dextran on the results from the nephelometric and the spectrophotometric (anthrone) methods, different dextran standards were analysed (Table III). The results of the anthrone method were not

TABLE III

COMPARISON BETWEEN NEPHELOMETRIC (NEP) AND ANTHRONE (ANT) ANALYSIS OF DEXTRANS WITH DIFFERENT MOLECULAR WEIGHTS IN 0.1 *M* NaOH

Molecular weight of dextran standard	True dextran concentration (μg/ml)	Found dextran concentration (μg/ml)	
		NEP[b]	ANT[b]
1080	5.1	0.2 (0.04)	4.9 (0.96)
4440	5.3	3.1 (0.58)	4.9 (0.92)
9890	5.0	4.9 (0.98)	n.a.
21 400	5.0	6.7 (1.34)	4.5 (0.90)
43 500	5.1	6.4 (1.25)	4.9 (0.96)
66 700	5.0	6.3 (1.26)	n.a.
123 600	5.0	5.7 (1.14)	n.a.
196 300	5.0	5.5 (1.10)	n.a.
276 500	5.1	5.2 (1.02)	4.6 (0.90)
401 300	5.0	5.0 (1.00)	n.a.
Dextran[a]	5.0	5.0 (1.00	4.7 (0.94)

[a] Dextran used in the production of Superdex 75 and 200 prep grade.

[b] Values in parentheses show the ratio between found and true dextran concentration; n.a. = not analysed.

influenced by the size of the dextran fragments. On the other hand, the nephelometric method gave too low results at molecular weights below $1.0 \cdot 10^3$ and slightly too high values between $2 \cdot 10^4$ and $2 \cdot 10^5$ (Table III). Although the results from the anthrone and the nephelometric measurements were in good accord (Table II), these findings indicate that the most reliable results for determination of the dextran leakage were achieved with the anthrone method.

It can also be noted that the released amounts of dextran reported in Table II are low compared with the total amount of dextran in Superdex media. This conclusion is verified by the small K_{av} effects observed for Superdex 200 prep grade after treatment with 1.0 *M* NaOH or 0.1 *M* HCl for 2 weeks (Figs. 1 and 2).

On-column experiments. Chromatographic separations of proteins are normally not performed at pH 14 and 1. However, solutions with high or low pH values are usually used to clean packed chromatographic columns contaminated with proteins, endotoxins or microorganisms [13]. In cleaning-in-place (CIP) experiments with 1.0 *M* NaOH, the leakage level of dextran at the column outlet was lower for Superdex 200 prep grade than for Superdex 75 prep grade (Table IV). This was also observed in the static bulk experiments (see above) and is probably related to the fact that the amount of dextran in the two gels is highest for Superdex 75 prep grade. Also, for both gels the dextran leakage decreased with the number of CIP cycles (Table IV). The dextran released in a CIP procedure must be thoroughly washed out if eluates are not to be contaminated with dextran. From Table IV it can be seen that after passage of three bed volumes of mobile phase buffer through the column no dextran fragments can be observed in the eluate.

Further, as the sodium hydroxide solution was stagnant in the column for 4 h, the concentration of released dextran obtained was higher than that in a typical CIP

TABLE IV

LEAKAGE OF DEXTRAN FROM SUPERDEX 75 AND 200 PREP GRADE COLUMNS (K 16/10) AFTER CLEANING-IN-PLACE (CIP) WITH 1.0 *M* NaOH

CIP cycle	Equilibration fraction[a]	Dextran leakage (μg/ml)	
		Superdex 75 prep grade[b]	Superdex 200 prep grade[c]
First	1st	193	51
	3rd	7.8	n.a.
	4th	3.9	n.a.
	5th	n.d.	n.a.
Second	1st	132	23
	3rd	5.2	n.a.
	4th	3.0	n.a.
	5th	n.d.	n.a.
Third	1st	110	17
	3rd	4.3	n.a.
	4th	n.d.	n.a.

[a] After treatment for 4 h with 1 *M* NaOH the columns were equilibrated with phosphate buffer (pH 7.0) where the first fraction corresponded to one bed volume (20 ml) and the other fractions to 10 ml.
[b] n.d. = Not detected.
[c] n.a. = Not analysed.

TABLE V

VARIATION OF EFFICIENCY WITH VOLUME OF AIR PUMPED THROUGH AN HR 16/50 COLUMN PACKED WITH 100 ml OF SUPERDEX 200 PREP GRADE

Volume of air pumped through the column (ml)	Equilibration volume of buffer[a] (ml)	Plate number (m^{-1})
New packing	100	11 500
1	100	11 600
1	100	12 100
30	100	10 450
30	100	9800
90	100	9000
90	750	8800
	1250	10 500
720	500	6600
	1500	8900

[a] After each air treatment the column was treated with different amounts of buffer before the efficiency test.

procedure with continuous flow through the column. It has been shown for other media that the concentration of leakage products in the eluate is inversely proportional to the flow-rate [14].

Functional stability of a Superdex 200 prep grade column after pumping air through the column

A common mistake in chromatographic experiments is to pump air into the column because the mobile phase in the eluent reservoir has been used up. To study how this influences the column efficiency and the retention properties of Superdex 200 prep grade, a column was treated with different amounts of air. Table V shows that when small amounts of air (1 ml) were pumped into the column no significant effect on the column efficiency was observed. However, when 30 ml or more of air were applied the N value decreased (Table V). The magnitude of this decrease was related to the volume of equilibration buffer pumped through the column after air exposure. The bed height after equilibration and the K_{av} values of the proteins (Table V) remained unchanged during the test.

In conclusion it has been shown that Superdex 75 prep grade and Superdex 200 prep grade are functionally stable to short-term treatments with 1.0 M NaOH and 0.1 M HCl. Nevertheless, Superdex media release small amounts of dextran under these conditions. However, released dextran can be easily washed away by equilibration with the buffer normally used in the separation procedure.

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