Journal of Chromatography, 477 (1989) 337–344
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 572

COLUMN PERFORMANCE OF Q-SEPHAROSE HP IN ANALYTICAL- AND PREPARATIVE-SCALE CHROMATOGRAPHY

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(First received December 19th, 1988; revised manuscript received April 14th, 1989)

SUMMARY

The chromatographic behaviour of two Q-Sepharose® HP HR 16/10 columns was tested under analytical- and preparative-scale conditions. Protein clogging of the top filter and the packing was the main reason for the observed decrease in retention volume, gel bed height and peak height during 300 analytical separations of a protein mixture (5 mg of protein per injection). Different washing procedures proved that adsorbed protein molecules decreased the availability of the sample molecules to the ion-exchange groups. By using a column where the top filter was fixed to the adaptor (XK 16/20 column), the stability of the column bed height was improved.

Purification of ovalbumin from egg white with large sample loads showed that washing with 0.1% pepsin solution maintained the optimum recovery. The column performance was evaluated in 50 purification cycles, corresponding to *ca.* 25 g of purified ovalbumin.

INTRODUCTION

With the development of bioindustries, chromatographic methods have become widely used in the purification of a variety of biological materials. To make the purification processes as inexpensive as possible, chromatographic media that provide a long lifetime are sought. In preparative-scale chromatography, the chemical and physical properties of the media are very important because they determine to a great extent the reusability of the support. It is essential that they can withstand rigorous cleaning and sanitation procedures. Further, it would be an advantage if the support is "user-friendly" so it could be used without a decrease in performance after practical mistakes such as pumping air into the column.

In a series of papers[1–4] it has been shown that media for analytical-scale separations can be used for at least 1000 repetitive injections of a protein or a serum sample. However, it was shown recently[5] that preparative-scale separations sometimes demand a washing procedure between every run in order to sustain optimum sample recovery.

Recently, a new cross-linked agarose-based anion exchanger, Q-Sepharose HP, has become commercially available. This has an average bead size of 34 μm and is

0021-9673/89/$03.50

intended especially for process development and the small-scale production of proteins and peptides. The objective of this work was to determine the ability of Q-Sepharose HP to withstand exposure to operations such as pumping air into the column, compression of the gel bed, different washing procedures, repetitive injection of an analytical protein sample and repetitive protein preparations on a large scale. Further, the importance of having the top filter fixed to the flow adaptor was elucidated.

EXPERIMENTAL

Chemicals and apparatus

Human transferrin was obtained from KabiVitrum (Stockholm, Sweden) and ovalbumin, β-lactoglobulin and pepsin from Sigma (St. Louis, MO, U.S.A.). Piperazine was of purum quality (Fluka, Buchs, Switzerland) and all inorganic compounds were of analytical-reagent grade. Decon 90 was obtained from Decon Labs. (Brighton, U.K.). Q-Sepharose HP, HR 16/10 and XK 16/20 columns and two FPLC® systems were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). An FPLC system consisted of an LCC-500 control unit, two P-500 high-precision pumps, a P-1 peristaltic pump, a UV-1 UV monitor (280 nm, HR 10 cell), MV 7 and MV 8 valves and a FRAC-100 fraction collector. A Shimadzu C-R3A integrator was used to collect and store data. The titrations of chloride were performed on a Mettler Memo Titrator DL 40 RC.

Column packing procedure

A 23-g amount of pre-washed settled gel was gently mixed with an equal amount of distilled water. The gel slurry was poured into an HR 16/10 or an X K16/20 column with packing equipment[6,7], and the flow-rate was set at 2.0 ml/min. When the gel had settled, the flow-rate was set at 8.0 ml/min and after 5 min the packing equipment was removed, the bed height adjusted to 10 cm and the top adaptor mounted. Finally, distilled water was allowed to pass through the gel bed at 8.0 ml/min for 90 min.

Sample preparation

Preparative sample. The egg white was diluted with ten volumes of 0.02 *M* piperazine (pH 6.0) (solvent A) and stirred on a magnetic stirrer for 10 min. It was then left overnight in a refrigerator for insolubles to settle before filtering through a 0.45-μm filter.

Analytical samples. The sample used for the lifetime test with 300 injections was a protein mixture consisting of transferrin (2.3 mg/ml), ovalbumin (3.3 mg/ml) and β-lactoglobulin (3.3 mg/ml). When the XK column was studied, a sample consisting of ovalbumin (10 mg/ml) was utilized. Both samples were solubilized in solvent A and were filtered through a 0.45-μm filter.

All samples were kept frozen until used.

Elution procedures for analytical-scale separations

Gradient elution. A 500-μl volume of the protein mixture was injected on to one of the HR 16/10 columns. The proteins were eluted at a flow-rate of 3.0 ml/min and a

linear gradient from solvent A to solvent B. The gradient time was 30 min. Solvent A consisted of 0.02 *M* piperazine (pH 6.0) and solvent B consisted of solvent A with the addition of 0.3 *M* sodium chloride. This elution procedure was also used when the XK 16/20 column was studied.

Isocratic elution. The two peaks from β-lactoglobulin were isocratically eluted with 55% solvent B at a flow-rate of 3.0 ml/min and acetone (1%, v/v) was eluted with distilled water at a flow-rate of 1.0 ml/min. The injection volume was 500 μl. These tests were performed on the occasions indicated in Table II.

Sample application and elution procedure for preparative-scale chromatography

A 50-ml volume of egg white diluted 10-fold with solvent A was applied to an HR 16/10 column at a flow-rate of 1.0 ml/min. The flow-rate was adjusted to 3.0 ml/min after the sample application. A 90-ml volume of solvent A was allowed to pass through the column before the gradient was started. The gradient and the solvents were the same as for the analytical column. The eluted ovalbumin was collected and a UV spectrum between 190 and 320 nm was recorded after a 10-fold dilution with solvent A. The amount of purified ovalbumin was evaluated from a calibration graph obtained from solutions of pure ovalbumin. The absorption of ovalbumin at 277 nm was used for quantification. Fifty preparations of ovalbumin from egg white were done. The column was rinsed from contaminating materials every six runs by filling it with 0.1% pepsin in 0.01 *M* hydrochloric acid. The enzymatic treatment was performed at room temperature (22°C) and lasted 24 h.

Determination of column ion-exchange capacity

Determinations of the total column ion-exchange capacity were performed by passing 40 ml of 1.0 *M* sodium chloride through the column at a flow-rate of 4.0 ml/min. The excess of ions was washed out with 40 ml of distilled water. The adsorbed ions were desorbed with 1.0 *M* sodium acetate, collected and then titrated potentiometrically with 0.1 *M* silver nitrate by using a silver electrode as the measuring electrode.

RESULTS AND DISCUSSION

In the first part of this investigation, two Q-Sepharose HP HR 16/10 columns were studied with respect to column performance. One column was used under analytical-scale and the other under preparative-scale conditions. In both instances samples containing ovalbumin were utilized. This protein was chosen because it has a strong tendency to denature and adsorb on filters and column packing materials.

Column performance in analytical-scale chromatography

Gradient elution of a protein mixture injected repetitively was used to test the column performance (Fig. 1). To stress the column further it was exposed to different washing procedures according to Table I. The column showed no significant change in performance during the first 50 injections even though air (20 ml) was pumped once into the column. However, after these injections the gel bed started to sink (the top filter was also displaced, see below) continuously during 200 injections (Table II). This behaviour was probably related to clogging of the top filter and the gel bed by

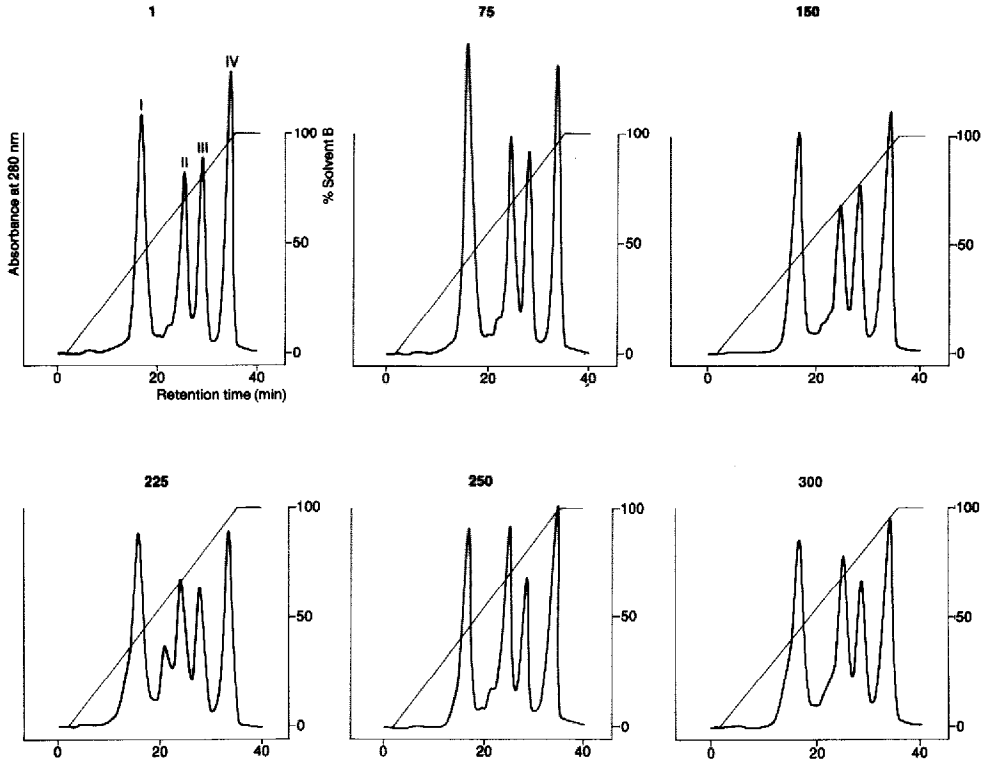

Fig. 1. Chromatograms from a Q-Sepharose HP column (HR 16/10 column) showing the separation of a test mixture containing transferrin (peak I), ovalbumin (peak II) and β-lactoglobulin (peaks III and IV). Events between the chromatograms are given in Table I. See Experimental for chromatographic conditions.

denatured proteins. From Tables I and II it can be seen that cleaning with 0.5 *M* of sodium hydroxide (20 ml) and 13 *M* acetic acid (40 ml) did not affect the continuous compression of the gel bed. However, the cleaning procedures after the 249th injection seemed to stop the reduction of the gel bed height. Moreover, the cleaning procedures with 0.1 *M* sodium hydroxide or 1.0 *M* acetic acid for 7 and 5 days, respectively, were also effective washing steps (Tabel II).

To study more carefully the change in retention characteristics of Q-Sepharose HP during the test, acetone and β-lactoglobulin were isocratically eluted on certain occasions. From Table II it can be seen that the retention volume of acetone decreased as much as the observed reduction in the total bed volume (2.6 ml). However, the bed compression cannot totally explain the decrease in the retention volume (V_R) of the two peaks emanating from β-lactoglobulin with isocratic elution (Table II). Moreover, the cleaning procedures after the 249th injection (Table I) resulted in a temporary increase in V_R for these peaks (Table II). A possible explanation of these results could be that the support matrix was gradually contaminated with proteins and, as a logical consequence, the amount of available ion-exchange groups became reduced. From this explanation the observed increase in V_R after a protein cleaning

TABLE I

WASHING PROCEDURES AND OTHER EVENTS IN ANALYTICAL-SCALE SEPARATIONS ON Q-SEPHAROSE HP

Injection No.[a]	*Washing solution or event*	*Washing conditions*
24	Gel run dry	
74	Adaptor adjusted	
95	Adaptor adjusted	
149	0.5 *M* NaOH	20 ml, 1.3 ml/min
174	Adaptor adjusted	
195	Adaptor adjusted	
199	0.5 *M* NaOH	20 ml, 1.3 ml/min
	13 *M* acetic acid	40 ml, 1.3 ml/min, reversed flow
	Adaptor adjusted	
219	Adaptor adjusted	
244	Adaptor adjusted	
249	2 *M* NaCl	20 ml, 1.3 ml/min
	1 *M* NaOH	80 ml, 1.3 ml/min, reversed flow
	13 *M* acetic acid	40 ml, 1.3 ml/min, reversed flow
	5% Decon	40 ml, 1.3 ml/min, reversed flow and 60 min without flow
	40% ethanol	200 ml, 1.3 ml/min, reversed flow
274	0.1 *M* NaOH	7 days without flow
299	1 *M* acetic acid	5 days without flow

[a] The injection number given is that just before the event indicated.

TABLE II

VARIATION OF BED HEIGHT, CHLORIDE ION CAPACITY AND RETENTION VOLUME IN ISOCRATIC ELUTIONS IN ANALYTICAL-SCALE SEPARATIONS ON Q-SEPHAROSE HP

Injection No.	*Bed height (cm)*	*Chloride ion capacity*[a] *(mmol)*	*Retention volume (ml)*		
			Acetone	*β-Lactoglobulin*[b]	
				Peak I	*Peak II*
1	[c]	[c]	19.0	36.0	92.5
50	9.9	3.56 ± 0.07	18.0	35.4	93.0
100	9.8	n.d.[d]	17.4	33.6	84.9
150	9.5	3.53 ± 0.08	17.2	32.7	79.5
200	9.1	n.d.	17.0	32.4	84.0
250	8.9	n.d.	16.4	33.0	88.8
275	8.9	n.d.	16.4	n.d.	n.d.
300	8.9	3.54 ± 0.07	16.2	32.4	87.0

[a] The capacity is given for the total gel volume. The values are given with a 95% confidence interval. The pooled standard deviation is 0.05 mmol (degrees of freedom = 6).

[b] The β-lactoglobulin peaks are eluted at 55% solvent B.

[c] Prior to the start the bed height was 10.2 cm and the chloride ion capacity was 3.53 ± 0.08 mmol, corresponding to 0.173 mmol/ml gel.

[d] n.d., not determined.

TABLE III

VARIATION OF THE RETENTION VOLUME OF THE FOUR MAIN PEAKS DURING 300 INJECTIONS OF A PROTEIN MIXTURE ON A Q-SEPHAROSE HP COLUMN

Injection No.	*Peak I (ml)*	*Peak II (ml)*	*Peak III (ml)*	*Peak IV (ml)*
1	48.87	74.04	84.69	100.74
25	48.24	73.77	84.75	100.53
50	47.97	73.65	84.58	100.26
75	47.85	73.41	83.85	100.05
100	46.77	72.15	82.74	98.73
125	46.53	72.12	82.08	98.64
150	47.19	72.81	82.89	99.48
175	46.47	71.85	82.41	99.21
200	46.59	71.46	82.17	98.52
225	46.83	71.28	82.05	98.13
250	46.98	71.70	82.62	99.27
275	48.00	73.11	84.00	99.57
300	47.91	73.14	84.00	99.12

step can also be rationalized. The fact that the chloride capacity remained constant during the lifetime test (Table II) indicates that the ion-exchange groups are chemically stable.

From Tables I and III it can be seen that the retention volumes under gradient elution also decreased with the number of sample injections until the column was cleaned. However, no effect on V_R from the cleaning process after the 199th injection was observed because the adaptor was adjusted at the same time (Table I). The column cleaning procedure with 0.1 *M* sodium hydroxide for 7 days gave rise to the largest changes in V_R of the four peaks (Table III), indicating that this was the most effective cleaning procedure investigated.

The peak heights had also decreased after 300 injections (Fig. 1). This reduction may reflect a decrease in the column plate count due to bed compression. In addition, peak II was split at the 225th injection. From separate experiments it was concluded that this extra peak resulted from denaturation of ovalbumin. The chromatographic pattern was restored after the injection of a fresh sample.

The results presented indicate that if the performance of a Q-Sepharose HP column is to remain unchanged over a long period of use, it must be cleaned regularly.

Column lifetime in preparative-scale chromatography

Repetitive injections of 50 ml of egg white, diluted 10-fold with solvent A, were used to investigate the fouling of Q-Sepharose HP under preparative conditions. A preparative and an analytical separation of egg white are depicted in Figs. 2 and 3, respectively. During each preparative run about 0.5 g of ovalbumin was purified but the recovery of ovalbumin had decreased by about 25% after six repetitive preparations (Fig. 4). Moreover, the gel bed height was reduced by 3 cm. After the first six preparations the column was treated with 1.0 *M* sodium hydroxide for 2 h. As this treatment did not restore either the recovery or the bed height, a 0.1% pepsin solution was used to clean the column. It was found that enzymatic cleaning of the column after every sixth preparation restored the recovery to 100% (Fig. 4) and the gel bed

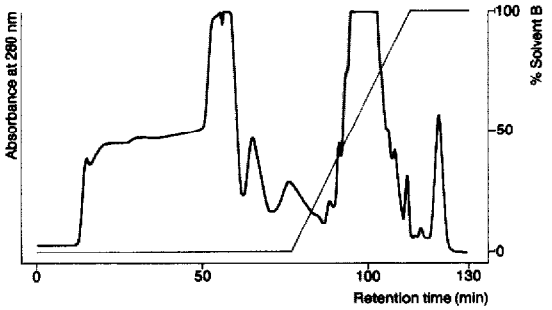

Fig. 2. Preparative-scale chromatogram of egg white on Q-Sepharose HP packed in an HR 16/10 column. A 50-ml sample of 10-fold diluted egg white was injected at a flow-rate of 1.0 ml/min. See Experimental for details of chromatographic conditions.

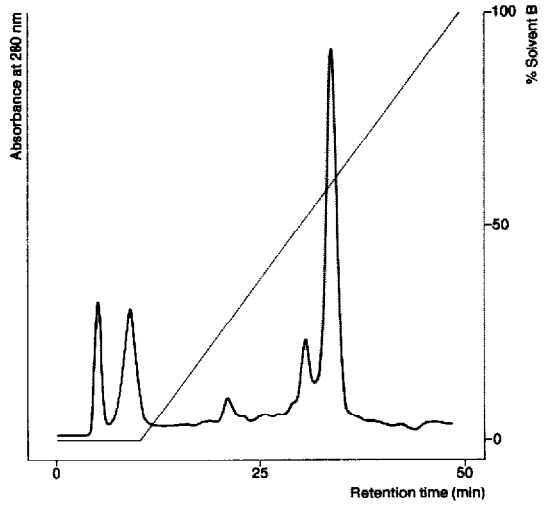

Fig. 3. Analytical-scale chromatogram of egg white on Q-Sepharose HP packed in an HR 16/10 column. A 500-μl sample of 10-fold diluted egg white was injected at a flow-rate of 3.0 ml/min. See Experimental for details of chromatographic conditions.

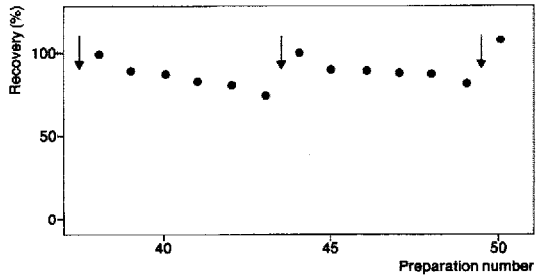

Fig. 4. Recovery of ovalbumin from egg white using a Q-Sepharose HP column (HR 16/10). The recovery of the original preparation was set at 100%. Arrows indicate that the gel has been rinsed with 0.1% pepsin in 0.01*M* hydrochloric acid between the runs.

height to a great extent. The only effect observed after 50 preparations was a 10% decrease in bed height.

Bed compression

The results presented above indicate that the movable top filter may be an important cause of bed compression. To investigate this, an XK 16/20 column, which has a top filter attached to the flow adaptor, was packed with Q-Sepharose HP. It was found that after 100 repetitive injections of 10 mg/ml ovalbumin (injection volume 500 μl), the bed height was the same as at the beginning of the test (10.0 cm). This result indicates that a column with the top filter attached to the adaptor is to be preferred for semi-rigid gels when clogging proteins are to be separated.

CONCLUSION

It can be conluded that the durability of column performance in the preparative and analytical chromatography of proteins such as ovalbumin depends to a great extent on the choice of cleaning solution and how often the column is cleaned. Therefore, it is important that the packing material can withstand different washing solutions. In this investigation it has been shown that Q-Sepharose HP withstands treatment for long periods of time at both high and low pH values. However, the most effective cleaning solution in this instance was a pepsin solution. This cleaning procedure is probably also applicable to common analytical LC columns.

Further, to reduce the bed compression caused by filter clogging, it is important to use a top filter adaptor to which the filter is attached or to change the top filter regularly.

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