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Improvement in flow properties and pH stability of compressed, continuous polymer beds for high-performance liquid chromatography

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ABSTRACT

Continuous polymer beds can be regarded as rods with channels through which the effluent can pass. The original beds had limited pH stability and did not permit especially high flow-rates. These disadvantages were overcome by synthesizing the beds from piperazine diacrylamide (PDA) and methacrylamide (MA) instead of N,N'-methylenebisacrylamide. For high flow-rates, the amounts of PDA and MA and the ratio between them are critical, as is the concentration of ammonium sulphate. The method of synthesis is simple and cost effective. No organic solvents are used. The beds can be prepared either directly in the chromatographic tube or in a beaker in the form of large clusters of small gel particles and then transferred into the tube. The preparation of the beds is described in detail. For a cation exchanger the resolution of model proteins was roughly independent of the bed height (down to 3.5 mm). The resolution was not affected by the flow-rate or even increased when the flow-rate was increased when using a reversed-phase column.

INTRODUCTION

Conventional chromatographic beds are made up of spherical beads of uniform size (see, *e.g.*, refs. 1 and 2). The preparation of the beads involves many critical steps and is both time-consuming and expensive. Beading processes also require the use of organic solvents, the handling, recovery and destruction of which are ever-increasing problems on both the laboratory and industrial scales. There are, accordingly, many reasons for the high cost of chromatographic columns.

Continuous beds, *i.e.*, polymer rods through which buffer can pass, were introduced in order to simplify the preparation of high-resolution chromatographic beds by eliminating the beading step [3–6]; for a short review of compressed continuous beds, see ref. 7. This paper deals with the improvement of the chromatographic properties of continuous beds, particularly with regard to rigidity and pH stability. We hope that further improvements will be introduced by other researchers as they begin to employ continuous beds. An alternative preparation method, in which organic solvents are employed, has already been published [8].

EXPERIMENTAL AND RESULTS

Materials

Piperazine diacrylamide (PDA), N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium peroxodisulphate (electrophoresis purity reagents) and ammonium sulphate (HPLC grade) were obtained from Bio-Rad Labs. (Richmond, CA, USA), methacrylamide (MA) from Fluka (Buchs, Swit-

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zerland) and acrylic acid from Merck (Darmstadt, Germany). Ovalbumin, horse skeletal muscle myoglobin, whale myoglobin, cytochrome *c* from bovine heart and lysozyme from chicken egg white were purchased from Sigma (St. Louis, MO, USA).

Instruments

The chromatographic system, including a Model 2150 HPLC pump, a Model 2152 HPLC pump controller, a Model 2220 recording integrator and a Model 2210 recorder, was obtained from LKB (Bromma, Sweden) and a Model 1306 UV monitor from Bio-Rad Labs. The loop injector was purchased from Rheodyne (Berkeley, CA, USA). A Varian Model 5000 liquid chromatograph was used in a set of experiments performed at flow-rates above 5 ml/min.

Packing of the column

Beds were packed in a Plexiglas tube of 6 mm I.D. with a 5-μm pore diameter metal frit at the bottom and a movable upper plunger [5].

In reversed-phase chromatographic experiments and experiments giving a back-pressure above 50 bar, the chromatographic tube was made of stainless steel. For a meaningful comparison of the flow resistances of beds of different heights we have used in the relevant figures the reduced pressure, defined as $P_r = (P_t - P_0)/L_f$, where P_t is the measured, total pressure, P_0 is the measured pressure with water in the column tube (in the presence of the frit) and L_f is the final bed height.

Flow resistance of the beds as a function of the PDA/MA ratio

Total amounts of 0.25 g of PDA and MA in different proportions were dissolved in 5-ml portions of 0.05 *M* potassium phosphate (pH 6.8) in glass test-tubes. Ammonium sulphate (0.25 g) was dissolved in each of the monomer solutions. Following addition of 50 μl of 10% (w/v) aqueous solutions of ammonium peroxodisulphate and deaeration for 2 min, the solutions were supplemented with 50 μl of an aqueous solution of 5% (v/v) TEMED. The polymerization proceeded at room temperature for 6 h. The bed was broken into large lumps by continuous stirring and then suspended in 5 ml of distilled water and packed at a flow-rate of 2 ml/min into columns of Plexiglas. The packing was finished by compressing the beds manually by means of the upper piston. The back-pressure of the columns was read at flow-rates of 1 and 2 ml/min. The reduced pressure was plotted against the PDA/MA ratio (Fig. 1). A rational way to characterize the monomer solution is to use the parameters *T* (the total concentration of the monomers) and *C* (the cross-linking concentration), routinely employed to define the composition of polyacrylamide gels [9]. With this nomenclature, *T* was constant at 5% (w/v) in this set of experiments, whereas *C* was varied. In Fig. 1 the PDA/MA ratio is also expressed as

$$C = \{(\text{PDA/MA})/[1 + (\text{PDA/MA})]\} \cdot 100\% \text{ (w/w)}$$

From Fig. 1 one can conclude that the minimum flow resistance was obtained for a column prepared with a PDA/MA ratio of 1.27 [$C = 55.9\%$ (w/w)].

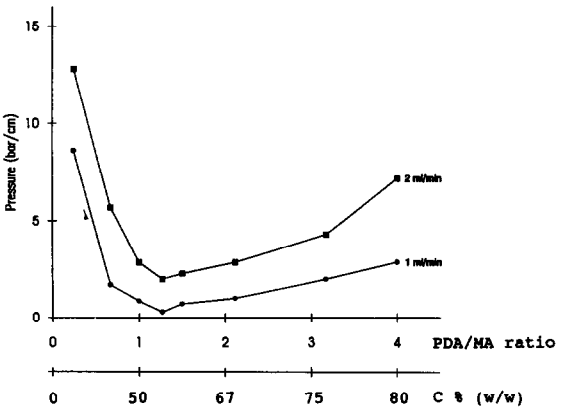

Fig. 1. Relationship between pressure per cm bed height $[(P_t - P_0)/L_f]$ and the PDA/MA ratio [also expressed as cross-linking concentration *C*, % (w/w)] for the compressed continuous beds. Total monomer concentration $T = 5\%$ (w/v); bed dimensions, 3.5 cm × 0.6 cm I.D. (5 ml monomer solution). Ammonium sulphate, 0.25 g per 5 ml of monomer solution.

Flow resistance of the beds as a function of the amount of ammonium sulphate

The beds were prepared as described above with a constant PDA/MA ratio (1.27), but with different concentrations of ammonium sulphate. The columns were packed at a flow-rate of 5 ml/min and then compressed. The flow resistance of the columns was investigated as described in the previous section. The results presented in Fig. 2 show that minimum flow resistance was obtained when the concentration of ammonium sulphate was 0.3 g per 5 ml of monomer solution, *i.e.*, 60 mg/ml.

Stability of the continuous beds at different pH values

It is of interest to know the pH limits at which the amide bonds in the continuous beds begin to hydrolyse, as washing of a chromatographic bed at high and low pH is a routine method for the regeneration of a column to release adsorbed material after a run.

The continuous bed behaves as a cation exchanger on hydrolysis, as free carboxylic groups are formed. The degree of hydrolysis can, therefore, be determined by treating the bed with buffers of different pH for different times and then determining the amount of a basic protein that becomes adsorbed at low ionic strength. The experimental details were as follows: the bed was designed to have a minimum flow resistance, *i.e.*, the PDA/MA ratio was 1.27, C was 55.9% (w/w) (Fig. 1) and the ammonium sulphate concentration was 60 mg/ml (Fig. 2).

The column (3 cm × 0.6 cm I.D.) was equilibrated at a flow-rate of 1 ml/min with 0.01 M potassium phosphate (pH 6.2). A 10-μl volume of human haemoglobin was injected and the column was washed with the equilibration buffer for 2 min. Some haemoglobin became adsorbed and some left the column. The adsorbed fraction was eluted with a 3-ml salt gradient formed from the equilibration buffer and the same buffer containing 1 M sodium chloride. The amounts of non-adsorbed and adsorbed haemoglobin were calculated from the peak areas determined with a recording integrator (detection wavelength = 280 nm). The same procedure was repeated for columns washed continuously at a flow-rate of 0.01 ml/min with buffers of acidic and basic pHs for different times. The results presented in Fig. 3 show that the continuous beds prepared as described here are stable in the pH range 1–11 for at least 3 weeks and at pH 12 for 6 h.

We also studied the pH stability of the previously used methylenebisacrylamide-based continuous beds. Fig. 3 shows that these beds are very susceptible to hydrolysis even at 5.5 and 9.

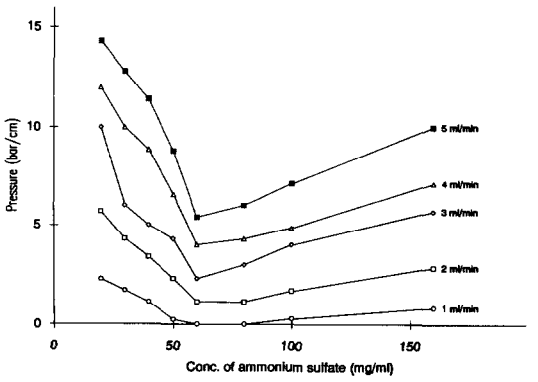

Fig. 2. Relationship between pressure per cm bed height $[(P_t - P_0)/L_f]$ and the concentration of ammonium sulphate for the compressed continuous beds at different flow-rates. PDA/MA ratio = 1.27; T = 5% (w/v); C = 55.9% (w/w). Other parameters as in Fig. 1, except that the concentration of ammonium sulphate was varied between 20 and 160 mg/ml.

Synthesis of cation exchanger

Based on the optimum conditions to reduce the flow resistance of the continuous beds (20 ml of buffer, 1 g of PDA–MA in the ratio 1.27, 1.2 g of ammonium sulphate, 200 μl of a 10% solution of ammonium peroxodisulphate and 200 μl of a 5% solution of TEMED), a cation exchanger was synthesized using 10 μl of acrylic acid as a ligand dissolved in this buffer solution. The gel obtained was mixed with 20 ml of distilled water and packed at a high flow-rate (5 ml/min). In this step the bed was compressed considerably, which increased the resolution. It was then compressed further with the aid of the piston to a final height of 15.5 cm. The flow resistance of the column was investigated by measuring the back-pressure of the column at different flow-rates in the range 1–5 ml/min.

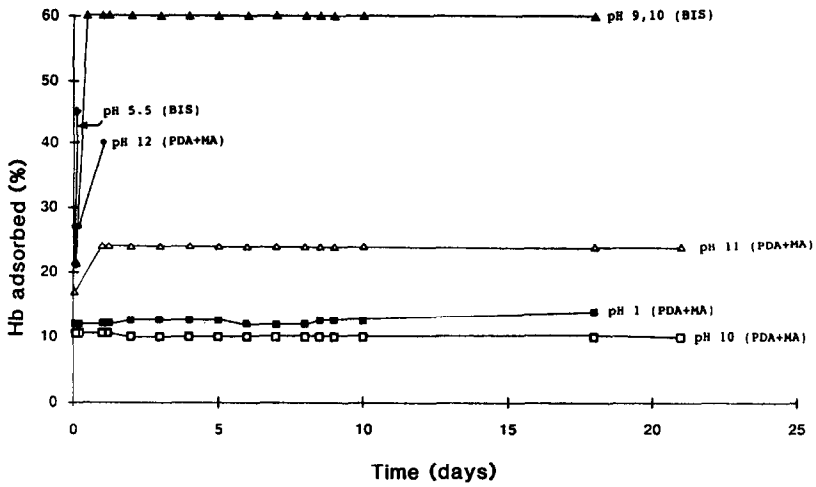

Fig. 3. Effect of pH and time on the stability of continuous beds in terms of adsorption of haemoglobin. The more degraded the bed, the more carboxylic groups are exposed and the more haemoglobin is adsorbed. For details, see the text.

Fig. 4 shows a plot of reduced pressure against flow-rate.

Resolution as a function of flow-rate for the cation exchanger

The column described in the previous section was equilibrated with 0.01 *M* potassium phosphate (pH 6.2). The sample (20 μl) containing 5–10 μg of each of the proteins ovalbumin, horse skeletal muscle myoglobin, whale myoglobin, cytochrome *c* and lysozyme was eluted at a flow-rate of 5 ml/min with a constant gradient volume (6 ml) formed from the equilibration buffer and the same buffer containing 0.5 *M* sodium chloride. The experiment was repeated at flow-rates of 2.5, 0.5 and 0.25 ml/min. The results presented in Fig. 5 show that the resolution of the proteins was unaffected by the flow-rate.

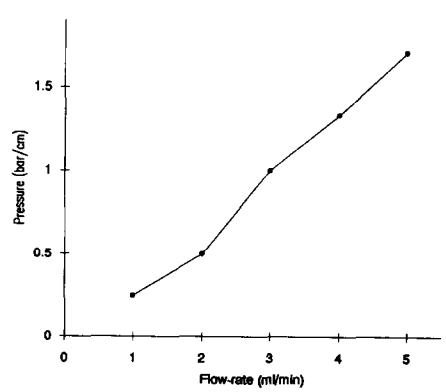

Fig. 4. Pressure $[(P_t - P_0)/L_f]$–flow-rate dependence of the cation exchanger. Bed dimensions, 15.5 cm × 0.6 cm I.D.

Influence of the bed height on the separation pattern for the cation exchanger

The cation exchanger was packed into columns of different bed heights (15.5–0.15 cm). The columns were used for the separation of proteins at a flow-rate of 5 ml/min (the sample and other experimental conditions were the same as those

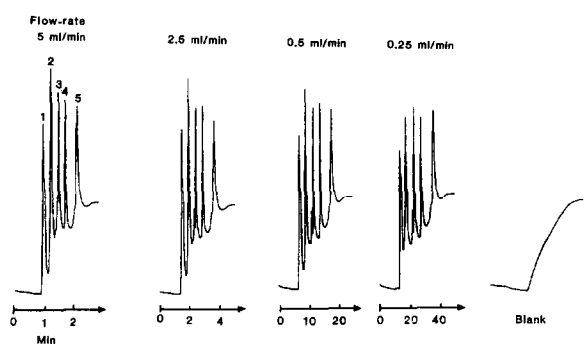

Fig. 5. Cation-exchange chromatography at various flow-rates at constant gradient volume. Bed dimensions, 15.5 cm × 0.6 cm I.D. Sample: 1 = ovalbumin; 2 = horse skeletal muscle myoglobin; 3 = whale myoglobin; 4 = cytochrome *c*; 5 = lysozyme. For experimental conditions, see text. Comparison of the chromatograms shows that the resolution is independent of the flow-rate.

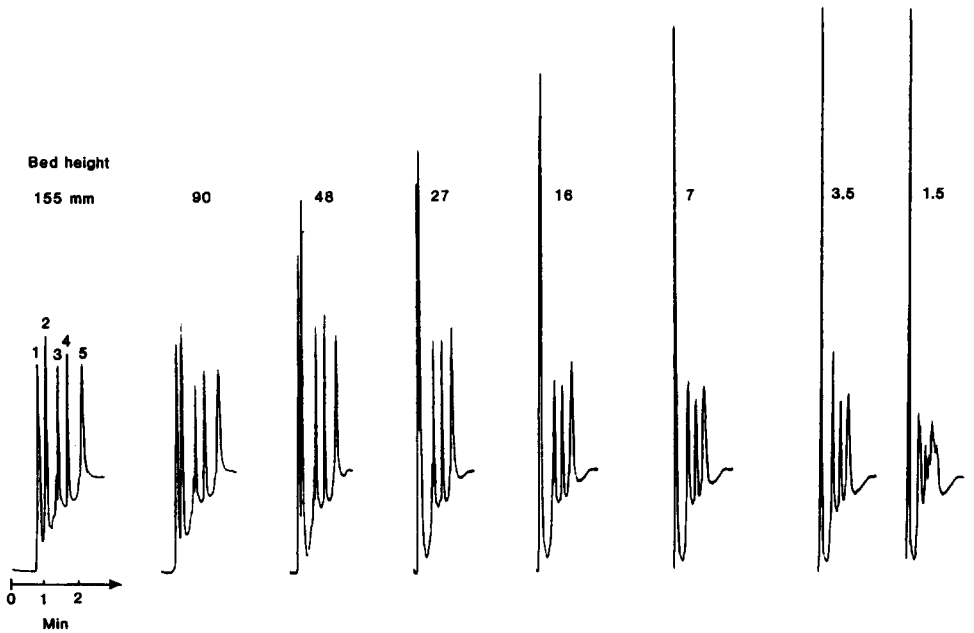

Fig. 6. Influence of the bed height on the separation of proteins on the continuous-bed cation exchanger. Flow-rate, 5 ml/min. Column and experimental conditions as in Fig. 5.

described in the previous section). The chromatograms are shown in Fig. 6. As demonstrated, proteins can be separated very rapidly even on a bed as short as 2.7 cm without a decrease in resolution, except for the first two peaks (see Discussion). The resolution obtained with a bed height of 0.35 cm is, however, acceptable in many instances.

Further improvement of flow properties

A minimum of flow resistance was obtained when the mass ratio between PDA and MA was about 1.27 (corresponding to a cross-linking concentration $C = 55.9\%$) (Fig. 1) and the concentration of ammonium sulphate was 60 mg/ml (Fig. 2). In these experiments, the total concentration, T, of the monomers was kept constant at 5% (50 mg/ml). We also studied how the flow resistance was affected by the total concentration of the monomers at the above optimum values of the cross-linking concentration C (55.9%) and of the concentration of ammonium sulphate (60 mg/ml). The experimental conditions were similar to those given under *Flow resistance of the beds as a function of the PDA/MA ratio*, with the exception that the polymerization was not performed in a test-tube but directly in the chromatographic tube. The volume of the monomer solution was 4 ml. Following polymerization for 14–16 h, water was pumped into the column, first at a flow-rate of 1 ml/min, then at 5 ml/min and finally at 10 ml/min. In this step the bed height decreased from about 13.2 to 1.6–8.0 cm, depending on the total concentration of the monomer (Table I). In each experiment, the upper plunger was moved down to make contact

TABLE I

BED HEIGHT (L, cm) FOR COLUMNS PREPARED FROM VARYING TOTAL CONCENTRATIONS T (%, w/v) OF THE MONOMERS

L_f = Final bed height; L_i = initial bed height.

T (%)	L_i	L_f	L_f/L_i
5	13.2	1.6	0.12
10	12.7	3.8	0.30
15	12.7	6.2	0.49
17.5	13.0	6.5	0.50
20	12.7	8.0	0.63

with the bed when the bed height became constant. The pressure (P_t) at different flow-rates and at different total concentrations of the monomers was then determined, in addition to blank values (P_0) of the pressure.

The pressures in the reduced form (P_r) are plotted against the total concentration T of the monomers in Fig. 7. Fig. 7 shows that a total monomer concentration of 150 mg/ml ($T = 15\%$) is much preferable to that of 50 mg/ml ($T = 5\%$), the concentration used in the experiment shown in Fig. 4.

The reversed-phase chromatographic experiment presented in Fig. 8 illustrates that a gel bed with this high total concentration permits both high flow-rates and high resolution (for the preparation of the column, see ref. 10, which deals with reversed-phase chromatography on continuous beds). Lysozyme often appears as two peaks in reversed-phase chromatography [11,12], which we also found with the continuous bed columns [10]. Interestingly, at a flow-rate of 1 ml/min (Fig. 8a) only one peak was observed, whereas 10 ml/min gave two peaks (Fig. 8b) on this bed with the composition $T = 15\%$, $C = 55.9\%$ (see Discussion).

The following experiment was done to determine whether two consecutive polymerizations could increase further the rigidity of the bed. A gel bed was prepared directly in the chromatographic tube as described above. The total concentration of the monomer was 150 mg/ml ($T = 15\%$). Following washing with water with the aid of a syringe, monomer solution of concentration 50 mg/ml [$T = 5\%$ (w/v)], *i.e.*, that used in the experiment shown in Fig. 4, was pressed into the bed with the same syringe (when a syringe is used the bed is not compressed) and allowed to polymerize for 16 h. The gel was then compressed at a flow-rate of 10 ml/min. As shown in Fig. 9, the flow resistance was reduced considerably on a second polymerization.

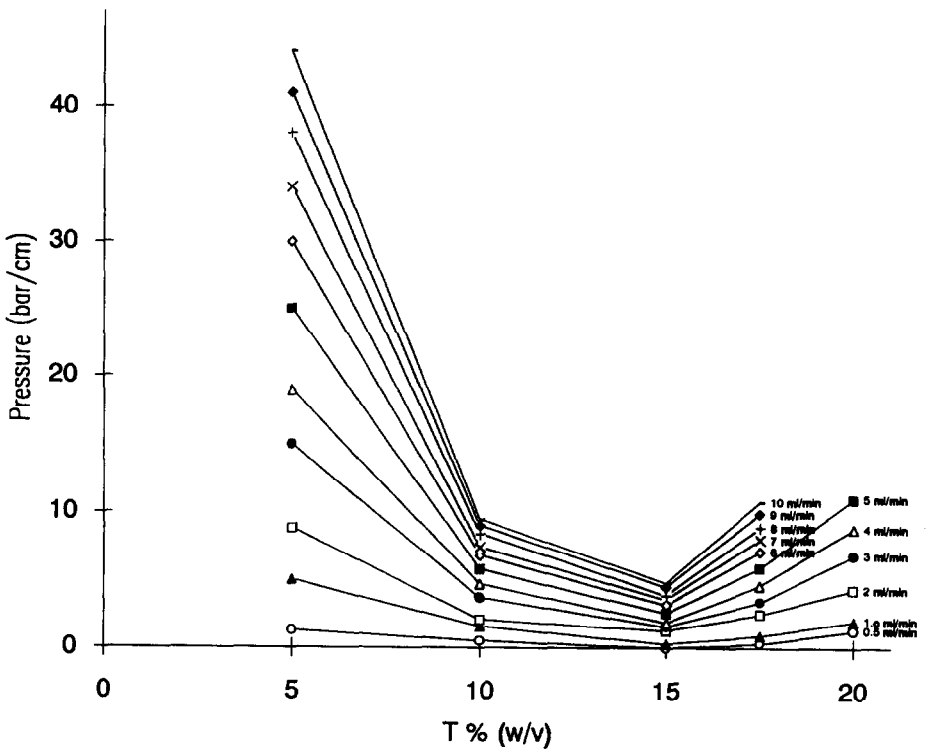

Fig. 7. Plot of pressure per cm bed height $[(P_t - P_0)/L_f]$ against the total monomer concentration T (%, w/v) for different flow-rates. $C = 55.9\%$ (w/w). For a minimum in flow resistance the gel bed should, accordingly, have a total monomer concentration $T = 15\%$ (= 150 mg/ml).

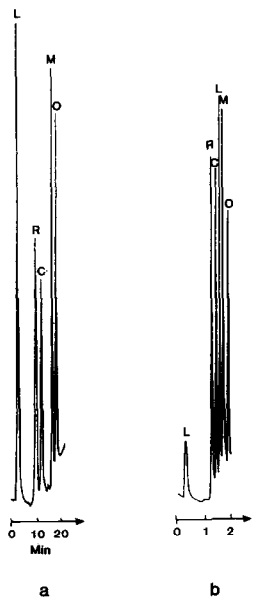

Fig. 8. High-performance reversed-phase chromatography of model proteins at flow-rates of (a) 1 and (b) 10 ml/min on a compressed continuous dextran-coated bed derivatized with octadecyl groups. Total concentration of the monomers T = 15% (see Fig. 7); ammonium sulphate, 60 mg/ml (see Fig. 2); bed dimensions, 14.9 cm × 0.6 cm I.D. Sample: R = Ribonuclease; C = cytochrome *c*; L = lysozyme; M = myoglobin; O = ovalbumin. Sample concentration, 5 mg/ml of each protein; gradient, from 10 to 70% acetonitrile in 0.1% (v/v) TFA in water; gradient volume, 20 ml. The results illustrate that continuous beds prepared as described here permit high flow-rates (10 ml/min = 60 cm/min) and afford high resolution, which may increase with increase in flow-rate. Lysozyme gives only one peak at the lower flow-rate (a).

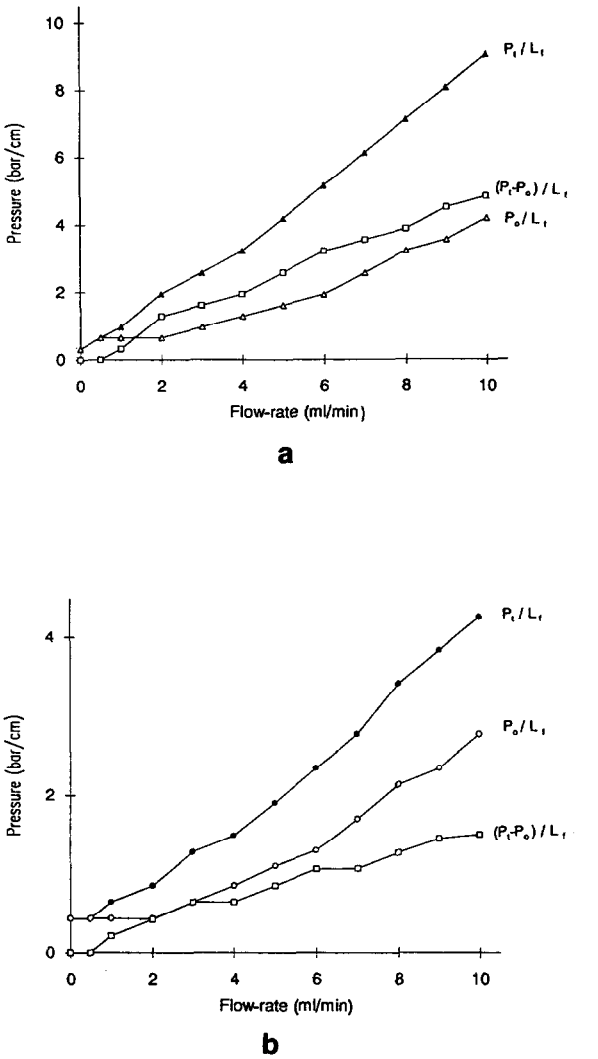

Fig. 9. Plot of pressure per cm bed height against flow-rate for a gel bed designed to give a low flow resistance. The bed was prepared in the chromatographic tube. Curve (▲) in (a) corresponds to a bed prepared from an aqueous solution of total monomer concentration T = 15% (see Fig. 7), cross-linking concentration C = 55.9% (see Fig. 1) and ammonium sulphate concentration 60 mg/ml (see Fig. 2). The bed height was 13 cm. This polymer bed was saturated with a solution of total monomer concentration T = 5% and ammonium sulphate concentration 60 mg/ml. Following polymerization, curve (●) in (b) was obtained. The bed height after compression was 9.4 cm. The blank curves (△) in (a) and (○) in (b) correspond to pressure per cm bed height (P_0/L_f) when the gel was replaced with water (the frit was not removed). A comparison of (a) and (b) shows that the flow resistance decreased on a second polymerization, although the bed in (b) was more compressed. The pressure over the bed $[(P_t - P_0)/L_f]$ in (b) (□) is lower than the blank pressure (P_0/L_f) (○).

DISCUSSION

The continuous beds prepared as described here share with the continuous beds synthesized according to the previous method [3–5] the property that the resolution is independent of the flow-rate (Fig. 5). The same highly desirable feature characterizes compressed beds of non-porous agarose and silica beads [13–17].

Fig. 6 shows that the ovalbumin zone (peak 1) approaches and finally co-elutes with the muscle myoglobin zone (peak 2) as the bed height decreases, probably because the concentration of the buffer is lower at the outlet of the longer bed owing to a continuous uptake of potassium and sodium ions during passage of the buffer through

the column. However, for the more strongly adsorbed proteins (peaks 2–5) the pattern is almost unchanged down to a bed height of only 3.5 mm, *i.e.*, that of a thick membrane.

Fig. 8 illustrates that the continuous beds prepared as described here permit high flow-rates (10 ml/min = 60 cm/min) and afford high resolution, which may increase with increase in flow-rate. Interestingly, the two lysozyme components are resolved at the higher but not at the lower flow-rate. We have not investigated whether this increase in resolution (caused by an increase in the relative retention time of the slower component) with an increase in flow-rate reflects that the flow pattern at the higher flow-rate is non-laminar in some parts of the column [7,13]. Only the first non-retarded peak (L) in Fig. 8a was affected by an increase in flow-rate (Fig. 8b), which is not unexpected on a transition from laminar to non-laminar flow. Similarly, in an experiment with a chiral continuous bed we found that only the enantiomer that eluted at the void volume showed an increase in plate number when the flow-rate was increased [18].

The flow resistance of the continuous beds is low also at high flow-rates, which is evident from Fig. 9b [$(P_t - P_0)/L_f < P_0/L_f$, *i.e.*, the pressure over the bed is smaller than the pressure over the rest of the system]. The pump did not show zero pressure when the flow-rate was zero, which reflects an offset error in the recording of the pressure.

CONCLUSIONS

A few examples have been presented of separations of proteins on compressed continuous beds. Many more have been reported previously [3–6] and a short review has recently been published [7]. The latter should be consulted by those who want a brief introduction to continuous beds with regard to their preparation, properties and application. We have shown here that the nature of the monomers, their concentrations (Figs. 1 and 7) and the concentration of ammonium sulphate (Fig. 2) strongly affect the chromatographic properties of continuous beds. The suggested composition of the monomer solution ($T = 5\%$, $C = 55.9\%$ or, more favourably, $T = 15\%$, $C = 55.9\%$) gives increased pH stability (Fig. 3) and lower flow resistance than with beds based on N,N′-methylenebisacrylamide [3,4]. However, there are certainly other experimental conditions that will give beds with still better chromatographic properties. Studies toward that goal are in progress. We are also investigating the usefulness of continuous beds for electrochromatography and for microchromatography.

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REFERENCES

1 S. Hjertén, *Methods Immunol. Immunochem.*, 2 (1968) 142.
2 S. Hjertén, *Methods Immunol. Immunochem.*, 2 (1968) 149.
3 S. Hjertén, J.-L. Liao and R. Zhang, *J. Chromatogr.*, 473 (1989) 273.
4 J.-L. Liao, R. Zhang and S. Hjertén, *J. Chromatogr.*, 586 (1991) 21.
5 S. Hjertén, J. Mohammad and J.-L. Liao, *Biotechnol. Appl. Biochem.*, 15 (1991) 247.
6 S. Hjertén, Y.-M. Li, J.-L. Liao, J. Mohammad, K. Nakazato and G. Pettersson, in H.J. Schneider (Editor), *Proceedings of Würzburg Colloquium, Würzburg, Germany, 24–26 February 1992*, GIT Darmstadt, 1992, pp. 93–96.
7 S. Hjertén, Y.-M. Li, J.-L. Liao, J. Mohammad, K. Nakazato and G. Pettersson, *Nature*, 356 (1992) 810.
8 F. Svec and J.M.J. Fréchet, *Anal. Chem.*, 64 (1992) 820.
9 S. Hjertén, *Arch. Biophys., Suppl.*, 1 (1962) 147.
10 S. Hjertén, K. Nakazato, J. Mohammad and D. Eaker, *Chromatographia*, submitted for publication.
11 N. Nimura, H. Itoh, T. Kinoshita, N. Nagae and M. Nomura, *J. Chromatogr.*, 585 (1991) 207.
12 K. Benedek, S. Dong and B.L. Karger, *J. Chromatogr.*, 317 (1984) 227.
13 S. Hjertén, Y. Kunquan and J.-L. Liao, *Makromol. Chem., Macromol. Symp.*, 17 (1988) 349.
14 S. Hjertén and J.-L. Liao, *J. Chromatogr.*, 457 (1988) 165.
15 J.-L. Liao and S. Hjertén, *J. Chromatogr.*, 457 (1988) 175.
16 S. Hjertén, J.-P. Li and J.-L. Liao, *J. Chromatogr.*, 475 (1989) 177.
17 S. Hjertén, J. Mohammad, J.-L. Liao and K.-O. Eriksson, *Chromatographia*, 15 (1990) 85.
18 J. Mohammad, Y.-M. Li, M. El-Ahmad, K. Nakazato, G. Petersson and S. Hjertén, *Chirality*, (1993) in press.