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Hydrophobic-interaction chromatography of proteins on continuous beds derivatized with isopropyl groups

Cheng-Ming Zeng[1], Jia-Li Liao, Ken'ichi Nakazato[2], Stellan Hjertén*

Department of Biochemistry, Uppsala University, Biomedical Center, P.O. Box 576, S-751 23 Uppsala, Sweden

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Abstract

A new support is described for fast, high-resolution hydrophobic-interaction chromatography of proteins based on a continuous bed containing isopropyl groups. The bed is prepared by a simple procedure: a mixture of appropriate acrylamide monomers, including the monomer with the isopropyl group, is polymerized directly in the chromatographic tube or, alternatively, in a beaker for columns with diameters greater than 1–2 mm. The concentrations of the monomers were adjusted for optimum resolution and low flow resistance. Both standard (6 mm I.D.) and microbore columns (0.015–0.320 mm I.D.) have been employed successfully for the separation of seven model proteins. A linear negative gradient of ammonium sulfate (2.2–0 *M*) in phosphate buffer was used for the elution. These columns share with all continuous bed columns the properties that the resolution is roughly independent of the flow-rate, that the gel is sufficiently rigid to give high flow-rates at moderate pressures and that the efficiency for the separation of proteins is high and independent of the bed height for beds longer than 4–5 cm. The residence time of the proteins on the column has no observable influence on the appearance of the chromatograms.

Keywords: Hydrophobic-interaction chromatography; Stationary phases, LC; Proteins

1. Introduction

Conventional columns for hydrophobic-interaction chromatography are built up of uniform, spherical beads. The preparation of the columns involves many expensive and time-consuming steps. The introduction of continuous beds [1,2] simplified considerably the preparation of high-resolution supports by a one-step polymerization of the monomers directly in the column tube. Elimination of the cumbersome packing procedure makes it easy to prepare these columns with any inside diameter, for instance, 0.005–6 mm [2]. The previously used continuous beds for hydrophobic-interaction chromatography (HIC) were synthesized from butylacrylate and N,N′-methylenebisacrylamide [3]. This paper deals with a new continuous HIC bed made of piperazine diacrylamide, methacrylamide and isopropylacylamide, the latter monomer providing the hydrophobic ligands. The chromatographic properties of this bed in the separation of proteins are described. The bed is prepared in one step.

*Corresponding author.

[1] Permanent address: Department of Bioanalytical Chemistry, Chongqing University of Medical Sciences, 630046 Chongqing, China.

[2] Permanent address: School of Sciences, Kitasato University, Kitasato 1-15-1, Sagamihara-shi, Kanagawa 228, Japan.

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2. Experimental and results

2.1. Materials

The electrophoretic purity reagents piperazine diacrylamide (PDA), N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium persulfate (APS) were from Bio-Rad Laboratories (Richmond, CA, USA); cytochrome *c*, myoglobin, lysozyme, chymotrypsinogen A and trypsinogen from Sigma (St. Louis, MO, USA); and ferritin and ribonuclease A from Pharmacia (Uppsala, Sweden). Methacrylamide (MA) was purchased from Fluka (Buchs, Switzerland); γ-methacryloxypropyltrimethoxysilane (Bind Silane) from Pharmacia; and isopropylacrylamide (IPA) from Tokyo Chemical Industry (Tokyo, Japan). All other chemicals used were of analytical grade.

Fused-silica capillaries with inner diameters of 0.015–0.320 mm were purchased from MicroQuartz (Munich, Germany). The chromatographic tube of I.D. 6 mm, made of Plexiglas and equipped with a movable upper plunger and a 5-μm pore diameter metal frit at the bottom, was made at the Biomedical Center workshop by Mr. Curt Lindh.

The HPLC system consisted of a Model 2150 pump, a Model 2152 LC controller and a Model 2210 recorder from LKB (Bromma, Sweden). The UV monitor (Model 200) was from Linear Instruments (Reno, NV, USA).

2.2. Peparation and chromatographic test of the standard column (6 mm I.D.)

Continuous beds containing hydrophobic isopropyl ligands were prepared as follows: PDA (0.250 g), MA (0.150 g), IPA (0.200 g) (see below regarding choice of the optimum concentrations of monomers) and $(NH_4)_2SO_4$ (0.360 g) were dissolved in 6.0 ml of 50 m*M* sodium phosphate (pH 7.0). Following deaeration for 2 min, 60 μl of 10% (w/v) APS and 60μl of 5% (v/v) TEMED aqueous solutions were added. The monomer mixture was allowed to polymerize overnight in a closed test tube. The bed was then transferred to the 6-mm column tube and compressed by pumping with 10 m*M* sodium phosphate (pH 7.0) containing 2.2 *M* ammonium sulfate (buffer B) at a flow-rate of 0.5 ml/min, which was subsequently increased to 1.0 ml/min. The upper plunger was moved down to maintain contact with the bed as soon as a void was created between plunger and bed.

The sample consisted of cytochrome *c*, myoglobin, ribonuclease, lysozyme, trypsinogen, chymotrypsinogen A and ferritin, which were dissolved at a concentration of 0.5–2.0 mg/ml in buffer B. A 15-μl aliquot of the sample was injected after the column was equilibrated with buffer B. The elution was performed at a flow-rate of 0.5 ml/min with a 10-ml linear, negative salt gradient formed from buffer B vs. 10 m*M* sodium phosphate, pH 7.0 (buffer A). The total gradient time was 20 min during which the concentration of ammonium sulfate decreased from 2.2 to 0 *M*. The chromatogram is shown in Fig. 1a.

2.3. Preparation and chromatographic test of microbore columns (0.015–0.32 mm I.D.)

The fused-silica capillaries were pretreated and methacryl-group activated according to Ref. [4]. PDA (0.125 g), MA (0.075 g), IPA (0.100 g) (see the following two sections regarding the choice of the optimum concentrations of monomers) and $(NH_4)_2SO_4$ (0.065 g) were dissolved in 1.0 ml of 50 m*M* sodium phosphate (pH 7.0). Following deaeration, the mixture was supplemented with 18 μl of 10% APS (w/v) and 18 μl of 5% TEMED (v/v), both in water, and was then sucked into the fused-silica capillary. When the diameter of the bed was as small as 0.015 mm, the column was observed under a microscope in order to see the length of the monomer solution. The polymerization proceeded overnight.

The chromatographic experiments were performed as described previously [4]. A 980×0.5 mm I.D. PTFE tube was used for creating a linear, negative salt gradient of 120 μl from buffer B to buffer A. After the column was equilibrated with buffer B for 20 min, the sample (4 μl), at a 5-fold lower concentration than the sample applied on the standard column, was injected and followed by the gradient. The elution was performed at a flow-rate of 6 μl/min. For columns with the inner diameters 0.075, 0.025 and 0.015 mm, the gradient was split before it entered the bed. The actual flow-rates through the beds for these columns were 0.33, 0.036

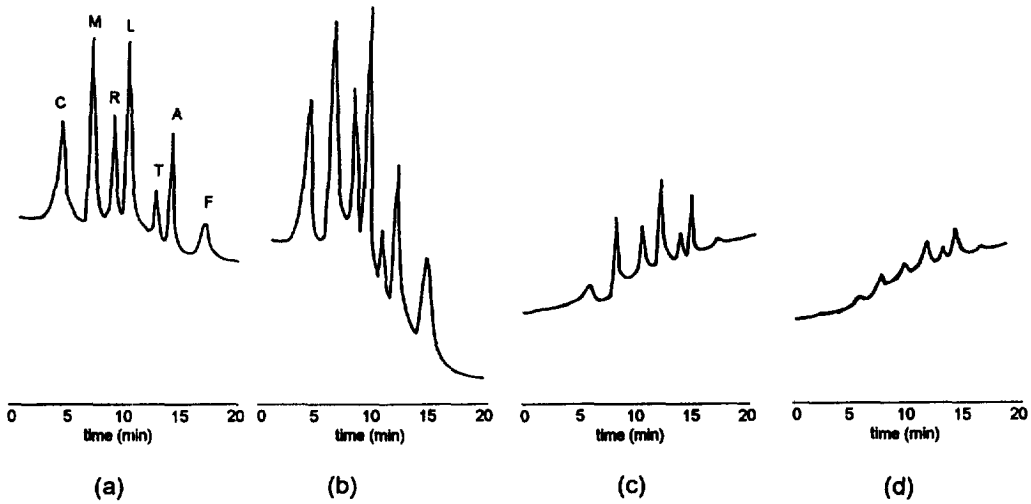

Fig. 1. Chromatograms showing the separation of model proteins on columns filled with C_3-containing continuous HIC bed. The model proteins included cytochrome *c* (C), myoglobin (M), ribonuclease (R), lysozyme (L), trypsinogen (T), chymotrypsinogen A (A) and ferritin (F). Bed dimensions: (a) 10.5 cm×6 mm I.D., (b) 12 cm×0.32 mm I.D., (c) 10 cm×0.025 mm I.D. and (d) 8 cm×0.015 mm I.D.. The absorbance measurements were made at 220 nm in a flow cuvette (a) or on-tube (b–d). Chart speed: 2 mm/min. For other chromatographic conditions, see the text.

and 0.013 μl/min (with the split ratio of 1:18, 1:165 and 1:450, respectively). The separation of model proteins by the microbore columns is shown in the chromatograms in Fig. 1b–d. The detection was made on-tube following removal of the non-UV transparent polyimide coating; for details, see Ref. [4].

2.4. Flow resistance of the gel as a function of the content of methacrylamide in the monomer solution

Microcolumns, 12 cm×0.32 mm I.D., were prepared from the monomer solutions consisting of 0.125 g PDA, 0.100 g IPA and different amounts of methacrylamide (0–0.130 g) in 1.0 ml of 50 m*M* sodium phosphate buffer (pH 7.0). The back pressures of these columns were tested at flow-rates of 6 and 12 μl/min. As shown in Fig. 2, the column with the minimum pressure has a concentration of methacrylamide of 0.075 g/ml or a PDA/MA ratio of 1.74 (by weight), which is higher than the value of 1.27 obtained previously when only PDA and MA were used as monomers [5].

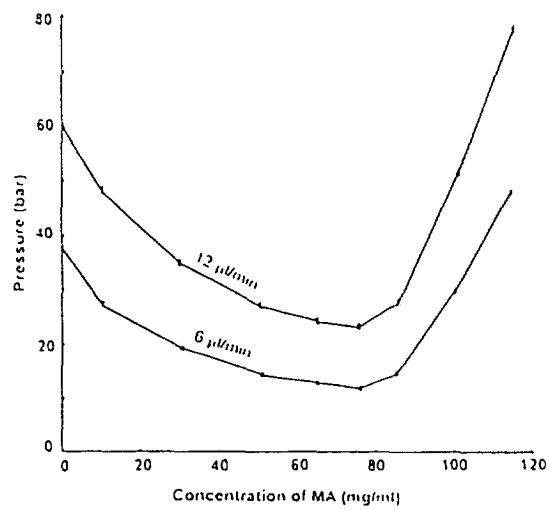

Fig. 2. Relationship between flow resistance (back pressure) and the content of methacrylamide (MA) in the monomer solution (w/v) used for the preparation of the HIC gel. The concentrations of PDA and IPA were kept constant at 125 and 100 mg/ml, respectively. Bed dimensions: 12 cm×0.32 mm I.D.. Flow-rate: 6 μl/min. Mobile phase: 10 m*M* phosphate buffer (pH 7.0).

2.5. Resolution as a function of the content of isopropylacrylamide in the monomer solution

Microcolumns with the dimensions 12 cm×0.32 mm I.D. were prepared from the above optimum monomer solution (0.125 g PDA and 0.075 g MA) with the difference that the contents of isopropylacrylamide were varied from 0–0.160 g/ml. After the column was equilibrated with the starting buffer for 20 min, the sample (4 μl) containing 0.4 μg of lysozyme and 0.4 μg of chymotrysinogen A was injected and then eluted for 20 min at a flow-rate of 6 μl/min with a linear, negative salt gradient of 120 μl from buffer B to buffer A. The resolution of these two proteins was calculated from the following equation:

$$R_s = (t_2 - t_1)/0.5(t_{w2} + t_{w1})$$

where t_2 and t_1 are the retention times and t_{w2} and t_{w1} are the widths of the peaks at the base line of chymotrypsinogen A and lysozyme, respectively. Fig. 3a shows that the resolution increases with an increase in the content of isopropylacrylamide in the monomer solution and that the degree of enhancement becomes smaller when the concentration of isopropylacrylamide exceeds 0.120 g/ml.

The same columns were used to test the relationship between the flow resistance of the gel and the content of IPA at flow-rates of 6 and 12 μl/min. The results presented in Fig. 3b show that the flow resistance of the gel increases with an increase in the content of isopropylacrylamide.

2.6. Recovery

The standard column with the dimension 10.5 cm×6 mm I.D. was equilibrated with buffer B and 40 μl of chymotrypsinogen A in the equilibration buffer (2.5 mg/ml) was applied. Desorption was effected with buffer A. The eluate was monitored at 280 nm and the fractions were collected for spectrophotometric determinations. The recovery was estimated from the volume and the absorbance of the sample and of the eluate. The results indicated a recovery of 99%.

In similar experiments, the recoveries of ribonu-

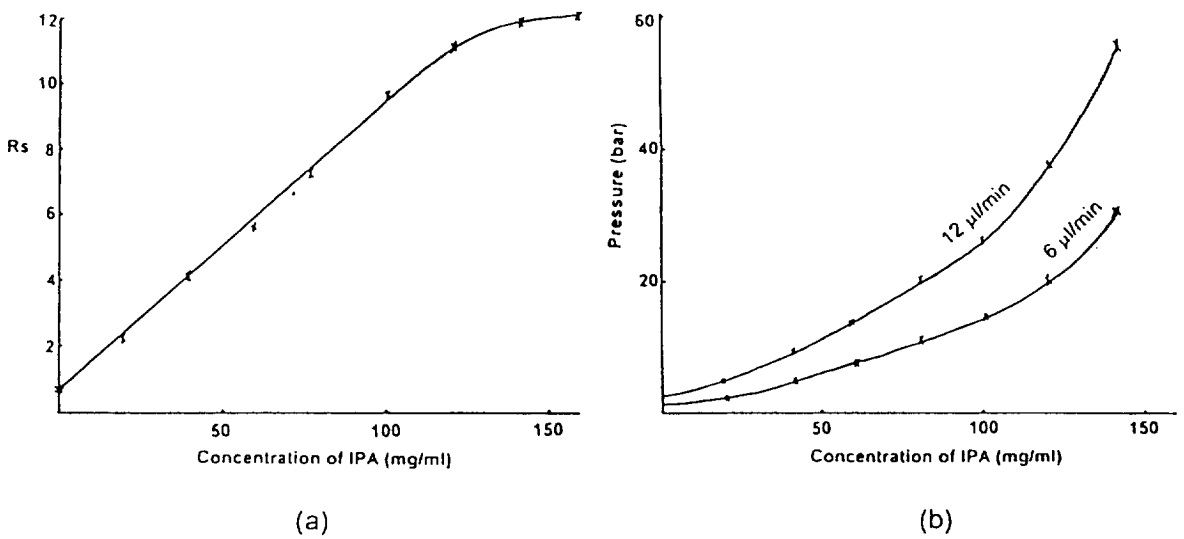

Fig. 3. Resolution (a) and flow resistance (b) as a function of the content of isopropylacrylamide in the monomer solution (w/v) employed for the synthesis of the HIC gel. Bed dimensions: 12 cm×0.32 mm I.D.. The concentrations of PDA and MA were kept constant at 125 and 75 mg/ml, respectively. Flow-rate: 6 μl/min. For details, see the text.

clease, myoglobin, trypsinogen and lysozyme were determined to be 99, 97, 96 and 91%, respectively.

2.7. Influence of flow-rate at constant gradient volume on the appearance of the chromatograms

A microcolumn with the dimensions 12 cm×0.32 mm I.D. was used for a series of experiments which were performed at flow-rates from 3 to 30 μl/min with a 120-μl gradient volume. The experimental conditions were the same as described in Section 2.3. In each experiment, the chart speed was kept proportional to the flow-rate to maintain the volume scale constant, which makes it easier to compare visually the patterns and the resolution of the protein peaks at different flow-rates. Fig. 4 shows that the appearances of the chromatograms are the same at low and high flow-rates.

2.8. Influence of the bed height on the separation pattern

Microcolumns with an inner diameter of 0.32 mm and bed heights from 2.5 to 20 cm were used for the separation of proteins at a flow-rate of 6 μl/min. The chromatograms are shown in Fig. 5, which demonstrates that the proteins can be separated rapidly even on a bed as short as 5 cm without loss in resolution. However, the resolution obtained on the column with a bed height of only 2.5 cm is acceptable in some cases.

2.9. The influence of residence time on the separation pattern

A microcolumn with dimensions of 12 cm×0.32 mm I.D. was equilibrated with buffer B. After the sample (4 μl) was injected, the elution was performed under the same conditions as described above. A similar experiment with the same sample was then performed on the same column, but 100 μl of starting buffer was pumped into the column after application of the sample. After a residence time of 4 h on the column, the sample was eluted by the salt gradient.

As shown in Fig. 6, the two chromatograms obtained are very similar, which indicates that the 4-h adsorption of the proteins on the column did not

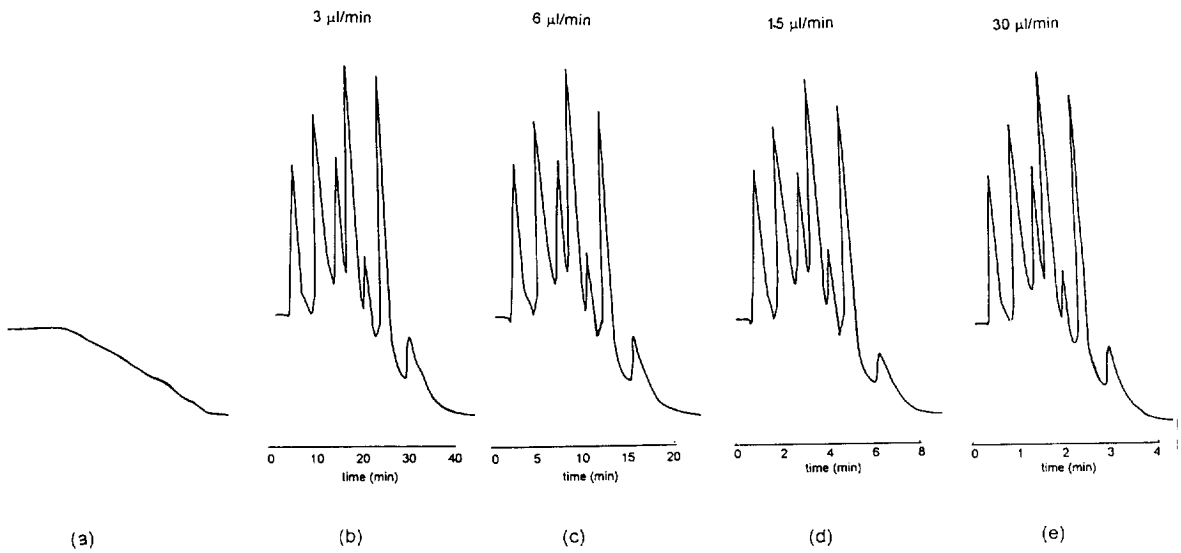

Fig. 4. Hydrophobic-interaction chromatography at various flow-rates at constant gradient volume (120 μl). Bed dimensions: 12 cm×0.32 mm I.D.. Flow-rate: (a) 6 μl/min, blank gradient, (b) 3 μl/min, (c) 6 μl/min, (d) 15 μl/min and (e) 30 μl/min. For other experimental conditions, see the text. Comparision of the chromatograms shows that the resolution is independent of the flow-rate.

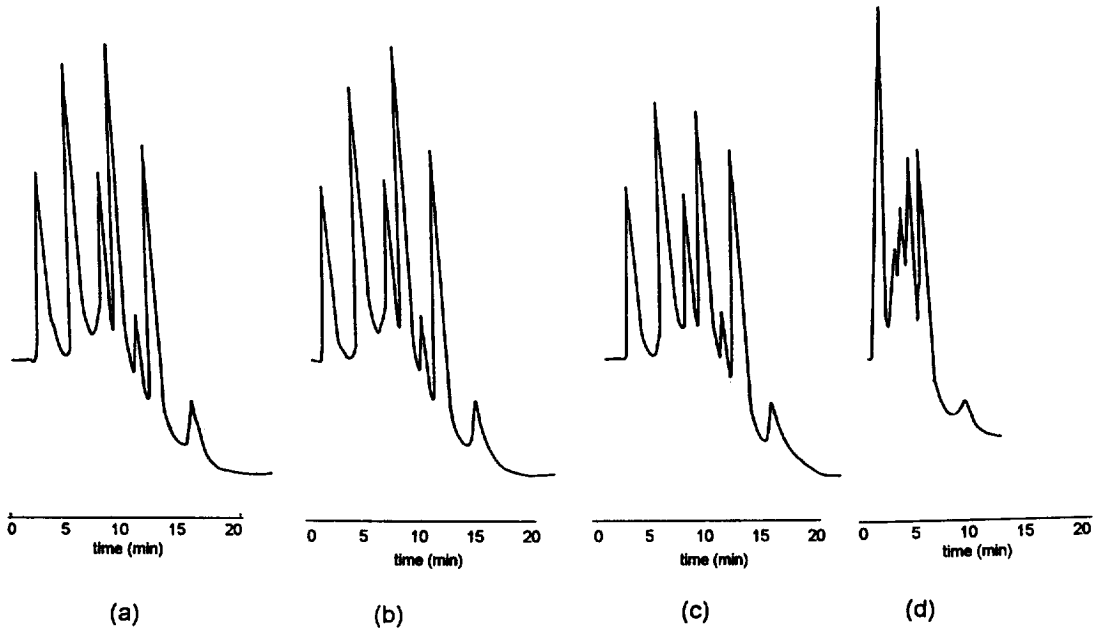

Fig. 5. Influence of bed height on the separation of model proteins on the HIC gel. I.D. of the column: 0.32 mm. Bed heights: (a) 20 cm, (b) 10 cm, (c) 5 cm and (d) 2.5 cm. Gradient volume: 120 μl. For other experimental conditions, see the legend to Fig. 1.

affect their interaction with the support in the presence of 2.2 *M* ammonium sulfate (cytochrome *c* was not adsorbed).

2.10. Influence of temperature on the adsorption

About 0.5 ml of the sedimented gel (prepared from 0.062 g PDA, 0.037g MA and 0.050 g IPA dissolved in 0.5 ml 50 m*M* sodium phosphate, pH 7.0) was equilibrated at room temperature with 10 m*M* sodium phosphate, pH 7.0 (buffer A) containing 1.2 *M* ammonium sulfate. As the sample we chose a colored protein, phycoerythrin, which has an absorption maximum at 540 nm. By measuring the absorption at this wavelength instead of in the UV region we could eliminate disturbances from contaminants having UV absorption. The phycoerythrin solution was dialyzed against the above phosphate buffer and then centrifuged. The supernatant was mixed with the gel in a centrifuge tube. Following gentle agitation for 10 min the excess non-adsorbed phycoerythrin was withdrawn after a centrifugation. The gel was then washed with the buffer and centrifuged. This washing procedure was repeated until the supernatant became colorless. The temperature of the gel in the centrifuge tube was then lowered from room temperature to 0°C by immersing it in an ice–water bath. After 5 min the gel was repeatedly washed with the buffer cooled to 0°C. This procedure released 39±2% of the phycoerythrin adsorbed at room temperature (Table 1).

3. Discussion

We have previously described the preparation of standard and microbore columns based on continuous beds for anion [3], cation [4], hydrophobic-interaction [3], bio-affinity [6], reversed-phase [7], chiral-recognition [8], dye-ligand [9] and liposome [10] chromatography and chromatofocusing [11]. The disadvantage of the 'old' HIC bed with butyl ligands described in Ref. [2] is that the crosslinker used (N,N′-methylenebisacrylamide) is susceptible to hydrolysis even at pH 5.5 and 9 [5]. The pH-stabilities of the 'new' and the 'old' columns are compared in Ref. [5]. In addition, the back pressure is relatively high and the ligand density is low

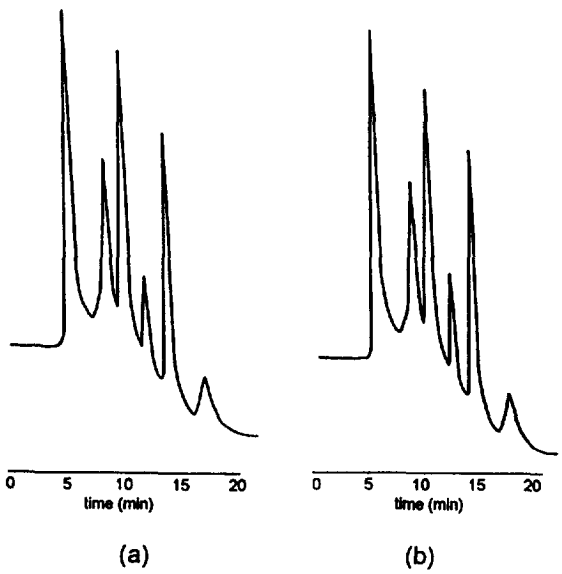

Fig. 6. Influence of the residence time of proteins on the separation pattern. Bed dimensions: 12 cm×0.032 mm I.D.. The experimental conditions were the same as those described in the legend to Fig. 1, except that the elution was started immediately (a) and 4 h after the application of sample (b). The similarity between the chromatograms indicates that the residence time has no significant influence on the strength of the adsorption of proteins, i.e. the duration of an experiment will not affect the reproducibility.

because butylacrylate, the ligand monomer, has a limited solubility in water. The new HIC gel described herein was designed to overcome these problems. It is synthesized from a mixture of PDA, MA and IPA and can be attached covalently to the inner wall of the fused-silica capillary, which makes it suitable also for the preparation of HIC columns with inner diameters as small as 5 μm [12]. The continuous bed is formed simply by polymerization of the monomer solution directly in the column tube and no frit is required to support the bed; for further details on the preparation of microcolumns, see Ref. [4].

Table 1
The effect of temperature on the hydrophobic interaction between a protein, phycoerythrin, and a continuous bed derivatized with isopropyl groups

Sample no.	Phycoerythrin released (%) upon a temperature decrease from 22 to 0°C
1	38.6
2	41.7
3	36.5
4	37.1
5	38.9
Mean±S.D.	38.6±2.0

The experimental procedure was the same as that described in Ref. [17] except that the sample was buffered at pH 7.0 with 10 m*M* sodium phosphate (buffer A) containing 1.2 *M* ammonium sulfate.

From Fig. 2 one can conclude that the minimum flow resistance is obtained for a support prepared with a PDA/MA ratio of 1.74, which is higher than the value found (1.27) when no isopropylacrylamide is added [5].

Based on the data presented in Fig. 3a and Fig. 3b, we chose an isopropylacrylamide concentration of 0.100 g/ml and a total monomer concentration $T=$ 30% (w/v) and a cross-linking concentration $C=$ 41.7% (w/w) for preparing an HIC gel which provided good resolution and an acceptable flow resistance.

The HIC gel prepared as described herein shares with all gels based on continuous beds the important property that the resolution is roughly independent of the flow-rate [1,2]. The columns packed with non-porous agarose beads have the same attractive feature [13,14].

The high ligand (isopropyl) density affords strong hydrophobic interactions with proteins, but the recovery is nevertheless high (91–99%). The hydrophilic nature of the matrix contributes to this absence of irreversible non-specific interaction and time-dependent adsorption (Fig. 6). The basic requirements for reproducible experiments are thus fulfilled. We have tried to use butylacrylamide for co-polymerization with PDA and MA, but the result was not satisfactory because of the poor solubility of butylacrylamide in aqueous media, which precludes the possibility to obtain a gel with high ligand density.

Continuous HIC beds can be prepared also in organic solvents from non-water soluble monomers, although the risk for disturbing non-specific hydrophobic interactions with the non-polar matrix cannot be excluded [15,16].

The difficulty to detect proteins on-tube when the inside diameter of the column is only 0.015 μm or less is obvious (Fig. 1d). We have, therefore, developed a more sensitive method for such experi-

ments: as the proteins leave the column chromatographically, they become adsorbed onto a rotating blotting membrane and are then stained by standard techniques [12].

A criterion of hydrophobic interaction is that it decreases upon lowering the temperature, in contrast to electrostatic interaction. Table 1 shows that a temperature drop from room temperature (about 22°C) to 0°C caused 39% of the adsorbed protein to be released. The same procedure was used as in an earlier experiment with the same protein adsorbed to pentyl-Sepharose 4B [17], where the corresponding figure was lower (25–30%). The rough surface of the particles making up the continuous bed accordingly affords a high capacity despite the fact that the particles are not permeable to proteins [9].

Acknowledgments

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