Journal of Chromatography, 523 (1990) 131–138
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 717

Hydrophobic interaction chromatography of proteins on Separon HEMA

II. Influence of sorbent modification on efficiency of separation

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(First received August 28th, 1989; revised manuscript received July 23rd, 1990)

ABSTRACT

Macroporous rigid hydrophilic Separon HEMA sorbents have been used in the hydrophobic interaction chromatography of proteins without any additional chemical modification but the efficiency of unmodified Separons is not very high. Simple chemical modification by reaction with benzoyl chloride and especially with 1,2-epoxy-3-phenoxypropane increases their efficiency and selectivity for the separation of proteins. As the concentrations of benzoyl and 2-hydroxy-3-phenoxypropyl groups on the surface of the sorbents were nearly identical, direct comparison of both materials was possible, showing that the best results were obtained with a spacer-containing derivatizing agent. The separation potential of this sorbent is demonstrated with the purification of β_2-microglobulin and immunoglobulin.

INTRODUCTION

Hydrophobic interaction chromatography (HIC) is widely used in the separation of proteins. The principle is based on hydrophobic interactions between the hydrophobic areas of proteins and isolated hydrophobic sites on the hydrophilic surface of sorbents. In comparison with reversed-phase high-performance liquid chromatography (RP-HPLC), operating on the same principle but with a much higher density of hydrophobic sites on the sorbent surface, milder elution conditions (usually a descending gradient of salt in buffer of pH *ca.* 7) are needed. This results in a higher mass recovery and also in a substantially higher recovery of biological activity of separated proteins in comparison with RP-HPLC [1–3].

Sorbents for HIC are usually based on hydrophilic organic matrices which are suitably hydrophobized [4–8] or on modified silica gels [2,3,9–16]. In addition to porous packings non-porous sorbents have also been described [17,18]. Silica gels and some organic gels are rigid enough for use in high-performance hydrophobic interaction chromatography (HP-HIC).

0021-9673/90/$03.50

Separon HEMA materials, which are copolymers of 2-hydroxyethyl methacrylate (HEMA) and ethylene dimethacrylate (EDMA), are rigid and hydrophilic products that can be commonly used under HPLC conditions. Whereas the organic sorbents usually applied in HP-HIC (TSKgel Phenyl of Superose Phenyl) are modified by chemical bonding of hydrocarboneous ligands, Separon HEMA materials have been used in HIC without any modification with interesting results [17–23]. In the case of Separon HEMA only the hydrophobic sites on the surface of proteins can interact with hydrophobic areas on the support of the sorbent whereas the hydrocarboneous ligands of the sorbents mentioned above are able to penetrate into the "pockets" in the structure of proteins. This results in the specific selectivity of Separon HEMA in some instances [20].

The drawback of unmodified Separon HEMA is a low efficiency in comparison with comercial organic materials even if small particles (10 μm) are used [23].

The aim of this work was to examine whether simple chemical modification of Separon HEMA (esterification [24] and reaction with an epoxy derivative [25]) can improve the efficiency of the separation of proteins by HIC on this material.

EXPERIMENTAL

Sorbents and chemicals

Separon HEMA 1000 H (10 μm) and Separon HEMA 1000 DEAE (10 μm) was obtained from Tessek (Prague, Czechoslovakia), Sephadex G-100 from Pharmacia (Uppsala, Sweden) and 1,2-epoxy-3-phenoxypropane (EPP) from Aldrich (Steinheim, F.R.G.). Boron trifluoride (BF_3) etherate (40%) and ammonium sulphate (for biochemical use) were purchased from Merck (Darmstadt, F.R.G.). Benzoyl chloride, disodium hydrogenphosphate, dioxane, toluene, acetone and methanol were from Lachema (Brno, Czechoslovakia).

Chicken egg-white lysozyme (LYS), horse skeletal muscle myoglobin (MYO), and bovine pancreas ribonuclease A (RNase) were purchased from Sigma (St. Louis, MO, U.S.A.) and bovine pancreas chymotrypsin (CHYT) from Reanal (Budapest, Hungary).

Chromatography

All experiments were performed with a Model 2150 HPLC pump, a low-pressure gradient mixer, a Model 2152 LC controller, a Model C6W-HC injector, a Model 2140 rapid spectral detector and a Data Print computer (Pharmacia–LKB, Bromma, Sweden).

Sorbents were packed into a stainless-steel column (50 × 4 mm I.D.) or a titanium column (50 × 8 mm I.D.). A 2 *M* solution of ammonium sulphate was used as the slurrying and packing solvent. A pressure of 2 MPa was maintained during the whole operation.

Preparation of Separon 1000 G benzoyl

A 5-g amount of Separon HEMA 1000 H, dried overnight at 348 K, was suspended in 50 ml of dry toluene, then 0.23 g of benzoyl chloride was added. The suspension was refluxed for 5 h under anhydrous conditions. The sorbent was filtered, washed thoroughly with methanol and acetone and dried at 348 K for 6 h. The

concentration of benzoyl ligands was calculated from the difference between the amount of hydroxyl groups presented on the surface of the sorbent before and after the modification [24]. This content was 0.3 mmol of benzoyl ligands per gram of dry sorbent in this instance.

Preparation of Separon HEMA 1000 H phenyl

A 2.5-g amount of Separon HEMA 1000 h (dried overnight at 348 K) was suspended in 10 ml of dry dioxane, then 0.15 g of BF_3 etherate and 0.188 g of EPP were added. The suspension was stirred at room temperature for 45 min under anhydrous conditions. The sorbent was filtered and washed thoroughly with dioxane, methanol and acetone and dried at 348 K for 6 h. The concentration of 2-hydroxy-3-phenoxypropyl ligands was determined by the photometric measurement of unreacted EPP at 270 nm, and was 0.25 mmol of ligand per gram of dry sorbent.

RESULTS AND DISCUSSION

Separon HEMA 1000 H (unmodified matrix) and its derivatives were compared. The concentrations of benzoyl and 2-hydroxy-3-phenoxypropyl ligands were 0.30 and 0.25 mmol/g, respectively. These substituent concentrations were found to be optimum from the point of view of the efficiency and selectivity of the separation of a model mixture of proteins (MYO, RNase, LYS, CHYT). Several concentrations of both ligands were prepared and tested (results not shown). In Figs. 1–3 the separations of the model mixture of proteins are demonstrated. Separon HEMA 1000 H benzoyl and phenyl were tested under the same conditions. With the unmodified matrix the starting concentration of salt was higher to compensate for the too high hydrophilicity of the sorbent. It can be clearly seen from Figs. 1–3 that a retention which should be proportional to the character of the hydrophobic interactions be-

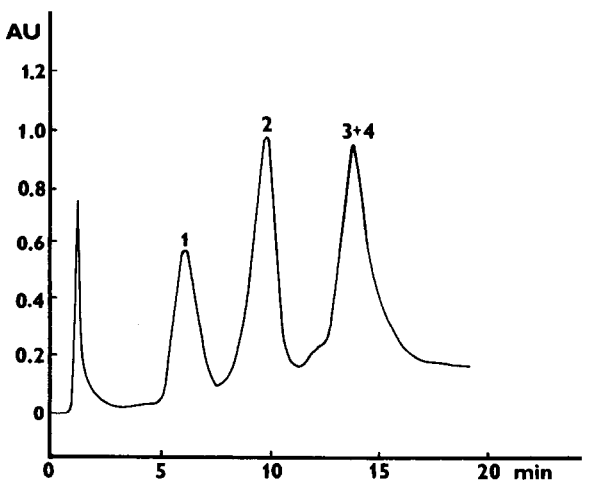

Fig. 1. Separation of model proteins on Separon HEMA 1000 H. Column: 50 × 4 mm I.D. Flow-rate: 0.5 ml/min. Gradient: from 2.4 to 0 *M* $(NH_4)_2SO_4$ in 0.1 *M* Na_2HPO_4 (pH 7.0) in 30 min. Proteins: 1 = MYO; 2 = RNase; 3 = LYS; 4 = CHYT.

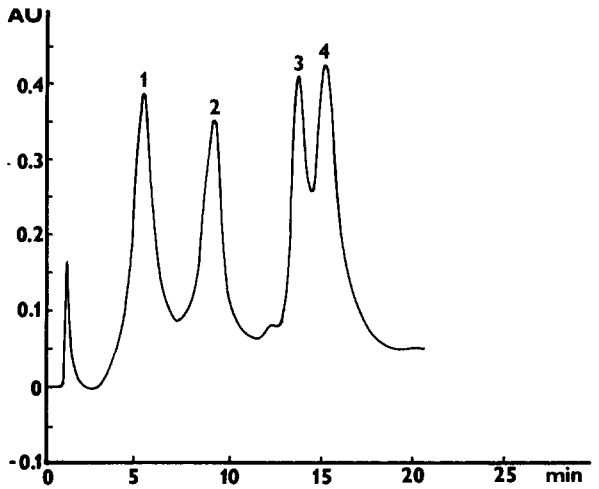

Fig. 2. Separation of model proteins on Separon HEMA 1000 H benzoyl. Conditions and proteins as in Fig. 1, except the gradient started from 2.2 *M* $(NH_4)_2SO_4$.

tween the sorbent and protein arose on going from the unmodified matrix to the phenyl derivative. The selectivity of the benzoylated sorbent is better than that of the the basic material, but the phenyl derivative showed the best selectivity of the three sorbents.

The important factor is the difference in the peak widths (W). W was calculated from well known relationship [26] $W = 1.7\ W_{1/2}$, where $W_{1/2}$ is the peak width at half-height). Results are given in Table I. It is evident that the efficiency of the phenyl derivative is substantially better the that of the two other materials.

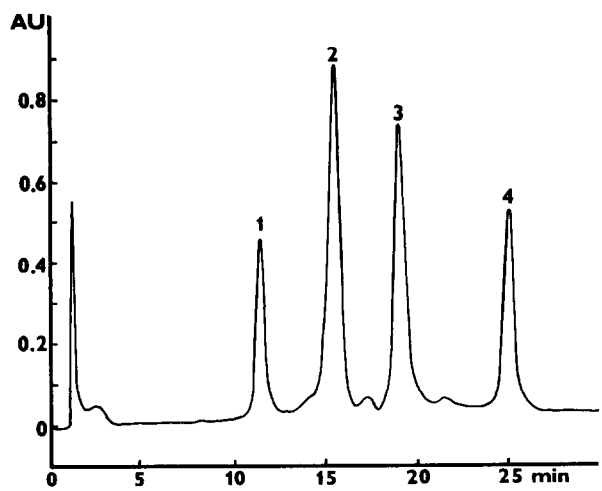

Fig. 3. Separation of model proteins on Separon HEMA 1000 H phenyl. Conditions and proteins as in Fig. 2.

TABLE I

PEAK WIDTHS (W) OF MODEL PROTEINS ON DIFFERENT SEPARON HEMA MATERIALS

For conditions, see Figs. 1–3

Sorbent	W (ml)			
	MYO	RNase	LYS	CHYT
Separon HEMA 1000 H	1.14	1.22	1.58	–
Separon HEMA 1000 H benzoyl	1.05	1.08	1.00	1.20
Separon HEMA 1000 H phenyl	0.47	0.62	0.60	0.58

The separation potential of Separon HEMA 1000 H phenyl is demonstrated on two examples. The first is the purification of β_2-microglobulin (β_2-m). A complex mixture of proteins from the urine of patients with kidney disfunction was obtained by precipitation with ammonium sulphate. This mixture was preseparated by size-exclusion chromatography on Sephadex G-100 and ion-exchange chromatography on Separon HEMA 1000 DEAE (see Fig. 4). Two fractions were obtained containing β_2-m which were separated by HIC on Separon HEMA 1000 H phenyl. The pertinent chromatograms are demonstrated in Figs. 5 and 6. The retention time of β_2-m was 26.5 min and both separations gave a product of high purity which was monitored by sodium dodecyl sulphate–polyacylamide gel electrophoresis (SDS-Page) (results not shown).

The second example is the purification of monoclonal antibody against prolactin. Mouse ascitic fluid was preseparated on Separon HEMA 1000 DEAE and the immunoglobulin G (IgG) containing fraction was then separated on Separon HEMA

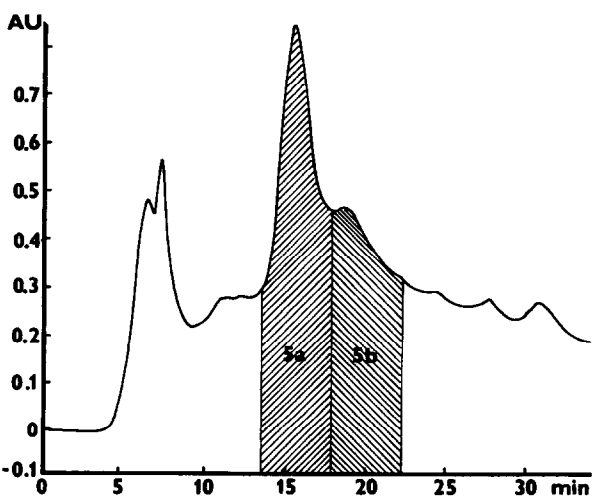

Fig. 4. Preparative separation of β_2-microglobulin fraction on Separon HEMA 1000 DEAE (in the marked area the presence of β_2-m was indicated by an ELISA test) Column: 100 × 8 mm I.D. Flow-rate: 0.5 ml/min. Gradient: from 0 to 1 M NaCl in 0.01 M Tris–HCl (pH 7.4) in 60 min.

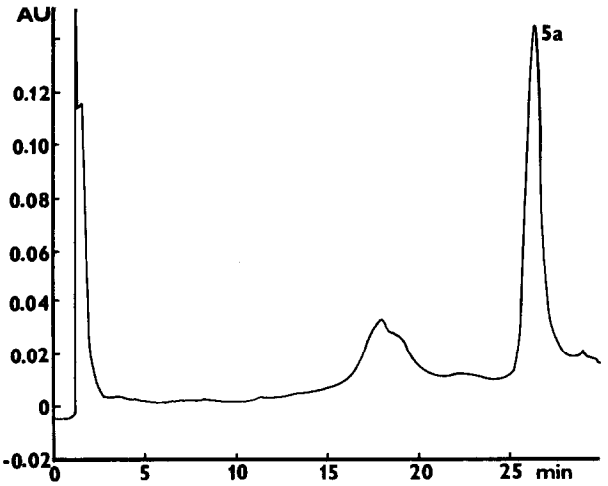

Fig. 5. Preparative separation of fraction 5a (Fig. 4) on Separon HEMA 1000 H phenyl. Conditions as in Fig. 1, except the gradient started from 2.0 *M* $(NH_4)_2SO_4$.

1000 H phenyl (Fig. 7). The complete separation of transferrin and IgG was achieved and the high purity of both proteins was verified by SDS-PAGE. The recovery of imunoactivity of IgG was determined to be 80% by a radioimmunoassay test with ^{125}I-labelled prolactine.

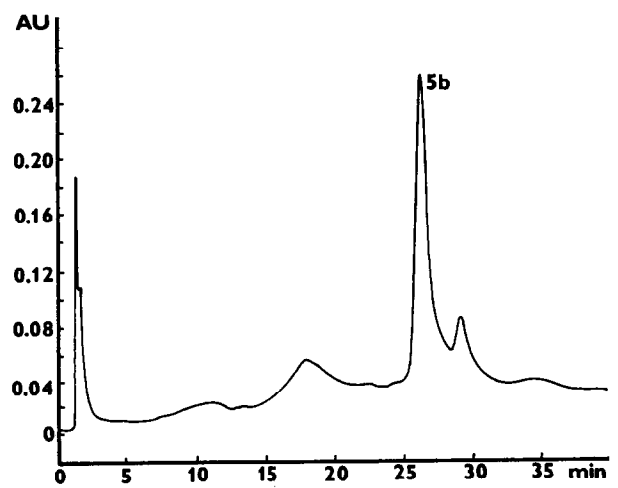

Fig. 6. Preparative separation of fraction 5b (Fig. 4) on Separon HEMA 1000 H phenyl. Conditions as in Fig. 5.

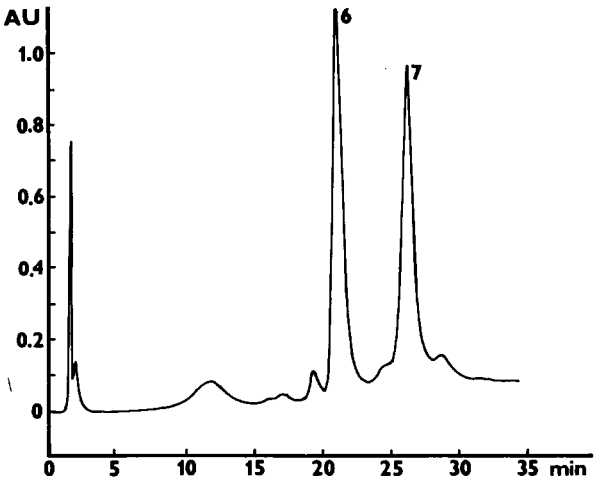

Fig. 7. Separation of IgG fraction (obtained from ascites fluid by gradient separation on Separon HEMA 1000 DEAE) on Separon HEMA 1000 H phenyl. Column: 50 × 8 mm I.D. Flow-rate: 0.5 ml/min. Gradient: from 1.8 to 0 *M* $(NH_4)_2SO_4$ in 0.1 *M* Na_2HPO_4 (pH 7.0) in 25 min.

CONCLUSIONS

The results support the hypothesis given in the Introduction that chemical modification of Separon HEMA can improve its selectivity and efficiency in the HIC of proteins. The comparison of benzoyl and 2-hydroxy-3-phenoxypropyl derivatives showed that a phenoxy group bonded via a 2-hydroxypropyl spacer gives the better results than a benzoyl group anchored directly on the sorbent surface. The small distance between the phenyl group and the sorbent surface with the benzoyl derivative is the probable cause of the unfavourable interactions between hydrophobic sites on the sorbent surface and the protein. The evidence for is the pour chromatographic characteristics of the unmodified matrix. On the other hand, the 2-hydroxypropyl spacer seems to be sufficient to prevent or minimize these unfavourable interactions.

The separation potential of EPP-derivatized Separon HEMA 1000 H was demonstrated by the purification of two proteins, β_2-microglobulin and immunoglobulin, routinely used in our department. The results obtained show that this sorbent is comparable to commercially available sorbents for HP-HIC.

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