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Preparative high-performance liquid chromatographic separation of proteins with HyperD ion-exchange supports

Egisto Boschetti[a,*], Luc Guerrier[a], Pierre Girot[a], Judit Horvath[b]

[a]*BioSepra SA, 35 Avenue J. Jaurès, 92390 Villeneuve la Garenne, France*
[b]*Beckman Instruments Inc., 2500 Harbor Blvd., Fullerton, CA 92634, USA*

Abstract

HyperD ion-exchange media combine the mechanical strength of a rigid polystyrene–mineral composite skeleton with the high protein-binding capacity of a three-dimensional soft gel located inside the skeleton. The skeleton solid matrix is completely filled with functionalized, highly hydrophilic, chemically stable ion-exchange hydrogels. These materials gave very efficient columns for protein separation with superior dynamic capacity, high resolving power and excellent protein recovery. Various protein mixtures were used to study the chromatographic performance of these new stationary phases. Comparisons between different particle size packing materials demonstrated the potential of this ion-exchange material for use on a large scale.

1. Introduction

The purification of proteins from crude extracts implies the use of various separation techniques, the most popular and efficient of which is liquid chromatography. Although this technique suffers from a number of difficulties related to highly complex differential mechanisms of molecular recognition and discrimination, it is the only available technique for the preparative separation of proteins with good yield and the possibility of automation.

The existence of numerous commercially available stationary phases [1] reflects the efforts made in the past and still in progress to improve the performance of chromatographic packings. Stationary phases based on polysaccharides, hydrophilic synthetic polymers, polystyrene or modified silica are offered with a variety of functionality, particle sizes, chemical and physical stabilities and pore diameters.

In addition to the range of physical properties, the correct combinations of high sorption capacity, high flow-rates and high separation efficiency are important features of these materials for producers of highly pure biologicals on a preparative scale.

In the early stages of HPLC, a major concern was how to increase the separation efficiency at high speed for analytical purposes. The particle size was thus the parameter to study, with the expected results of higher efficiency for smaller beads. However, because very small particles produce high flow resistance and back-pressure,

* Corresponding author.

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incompressible material such as silica was recognized as offering good solutions for preparing solid phases.

Today in preparative HPLC, another important parameter has been added to the list of desired characteristics: high sorption capacity. Productivity improvement requires high capacity and high speeds. This is not easy to achieve knowing that the sorption capacity, separation efficiency and back-pressure are generally degraded with increase in flow-rate. All these factors were at the basis of the development of defined chemical composition and behaviour of HyperD material, where a rigid skeleton based on porous polystyrene–mineral composite material sustains a functionalized soft gel homogeneously distributed in the pore volume [2,3].

In this paper, specific applications of HyperD ion exchangers are reported; some of them demonstrate the ability of this material to perform rapid protein separations, others the possibility of changing scale using material of different particle sizes. Studies were also carried out to establish the stability of these materials over many cycles and under severe operation conditions (e.g., acidic and alkaline regenerations).

2. Experimental

2.1. Chemicals

Q- and S-HyperD ion-exchange supports of 10-μm particle size were obtained from BioSepra (Marlborough, MA, USA.). Mono Q prepacked columns were purchased from Pharmacia–LKB Biotechnology (Uppsala, Sweden).

All pure proteins such as human transferrin, human and bovine albumin, trypsin, cytochrome *c*, lysozyme, β-lactoglobulin and formate dehydrogenase were purchased from Sigma (St. Louis, MO, USA). All pure chemicals and solvents used for the preparation of the liquid phases were purchased from Aldrich (Brussels, Belgium).

2.2. Determination of basic behaviour of Q and S-HyperD

Measurements of the sorption capacity of the resins for standard proteins were based on breakthrough curves and calculations made at 10% and 50% breakthrough. For this purpose, 5 cm × 5 mm I.D. columns were prepared and equilibrated with 50 m*M* Tris–HCl buffer (pH 8.6) for Q-HyperD and 50 m*M* acetate buffer (pH 4.5) for S-HyperD. Determinations were made at various linear velocities of up to several meters per hour. In the same experiments the back-pressure was also measured at different linear velocities.

Under similar conditions, resolution determinations were also made using mixtures of pure compounds, such as human transferrin and human albumin for Q-HyperD and β-lactoglobulin and cytochrome *c* for S-HyperD.

2.3. Separation properties

Separations of various protein mixtures were performed with 50 mm × 5 mm I.D., and 100 mm × 4.3 mm I.D., HPLC columns. Separations were typically performed using 50 m*M* Tris–HCl buffer (pH 8.2–8.6) for Q-HyperD or 50 m*M* acetate buffer (pH 4.5) for S-HyperD, followed by a sodium chloride linear elution gradient. The separation velocities varied from 80 to 700 cm/h.

2.4. Long-term properties under intensive use

This study was carried out using the same column for a large number of separations alternated or not with 2*M* sodium hydroxide washings (25% of the column volume). The separation conditions were as described above; drifts in resolution and in back-pressure were measured for both Q- and S-HyperD.

3. Results and discussion

Q- and S-HyperD ion exchangers are recently developed packings for the separation of pro-

teins using liquid chromatography. They are described as unique stationary phases [2–4] constituted of two parts, each of them playing a particular independent role. A solid skeleton made of a mixture of mineral oxides and polystyrene with large pores allows the operation of stable beds under a wide range of flow-rates, generating pressures that can exceed 150 bar without shrinking. The moiety responsible for the adsorption–desorption mechanism is constituted of a soft three-dimensional cross-linked hydrogel located within the pores of the rigid structure. This gel carries the ionic groups for the adsorption of any molecule of opposite electrical charge. This material has evolved from the dextran gel-filled porous silica described several years ago [5,6] and has improved properties such as sorption capacity, selectivity and chemical stability. The hydrogel alone possesses a very high reversible sorption capacity for proteins under classical conditions. However, because of its softness, it is not usable in chromatographic columns where it collapses rapidly under low pressure, generating a total column shut-off. In contrast, when trapped in a strong solid porous network, it becomes perfectly suited to the HPLC of proteins.

The initial binding capacity for medium-sized proteins such as serum albumin was found to be particularly high (around 175 mg/ml) with a slow decrease when high linear velocities are used. The productivity factor, which is proportional to both dynamic binding capacity and flow-rate, increased almost linearly with increase in linear velocity (see Fig. 1). The flow-rate increase, however, diminishes the resolution factor because it affects the column efficiency [7] (see also Fig. 1). This means that when the resolution is high it becomes possible to increase the flow-rate and achieve very rapid separations. However, care must be taken with difficult separations where the resolution factor is close to unity. Modification of the efficiency factor when modifying the flow-rate is limited with supports with very large pores where convective flow is present or predominant [8].

Flow-rate is not the only factor influencing the productivity of a preparative column. High sample loads can also be used to separate large amounts of material in a single run. Here again the resolution can be diminished according to the nature of the sorbent. With HyperD ion exchangers the resolution with increasing loads did not seem significantly diminished, at least within the studied range. Using human transferrin and human albumin as a mixture to be separated on a column of Q-HyperD under the above-mentioned conditions, the resolution was 5.5 with a sample load of 80 μg of proteins per ml of resin. The resolution decreased to about 5 with a load of 1200 μg (15 times higher) for the same amount of resin at the same linear velocity of about 370 cm/h.

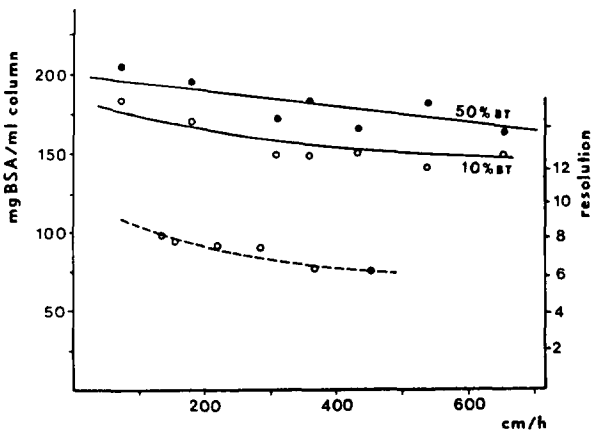

Fig. 1. Effect of linear velocity on dynamic binding capacity for bovine serum albumin (solid lines) and on resolution between human transferrin and human albumin (broken line). Dynamic capacities were measured by breakthrough curves and calculated at 50% and 10% breakthrough (BT). Bovine serum albumin concentration was 10 mg/ml in 50 m*M* Tris–HCl buffer (pH 8.6). Resolution was determined for a 50 × 5 mm I.D. and the same buffer; elution was performed using a linear gradient of sodium chloride up to 0.4 *M*.

Productivity increase in preparative HPLC for proteins is thus a result of the high intrinsic dynamic sorption capacity of the resin (chemical nature, availability of ionic groups, three-dimensional-based adsorption mechanisms) and the possibility of increasing the sample load and the possibility of increasing the linear velocity.

The separations illustrated in Figs. 2 and 3 show that ion-exchange-based separation is very similar to that observed with a well known resin

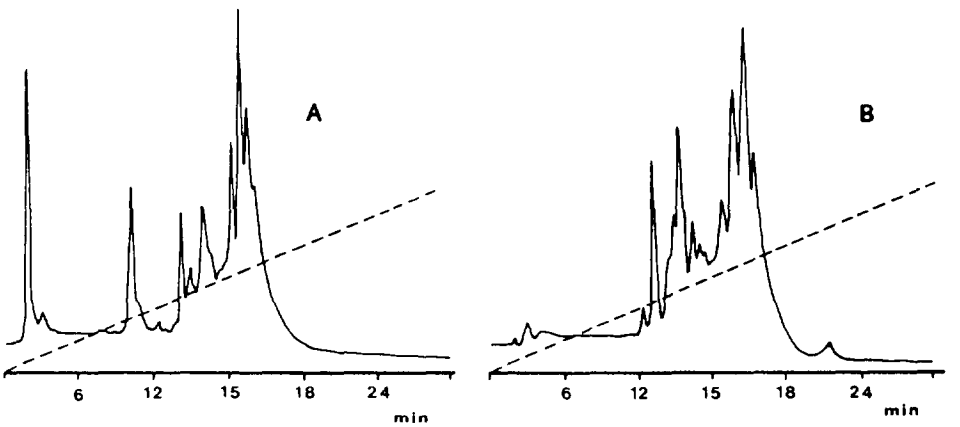

Fig. 2. Chromatographic separation of formate dehydrogenase (290-mg loading) on (A) Q-HyperD and (B) Mono Q. Column, 50 × 5 mm I.D.; 50 m*M* Tris–HCl buffer (pH 8.6); linear salt gradient up to 1 *M*; flow-rate, 1.0 ml/min.

such as Mono Q (Fig. 2). The separation efficiency was similar as a result of a similar particle size (10 μm bead diameter for both resins); the separation selectivity was different, however owing to the difference in the chemical nature of the ion-exchange polymers. In the particular case of the formate dehydrogenase separation, protein components of the first part of the chromatogram were better separated on HyperD than on Mono Q, but this is not neces-

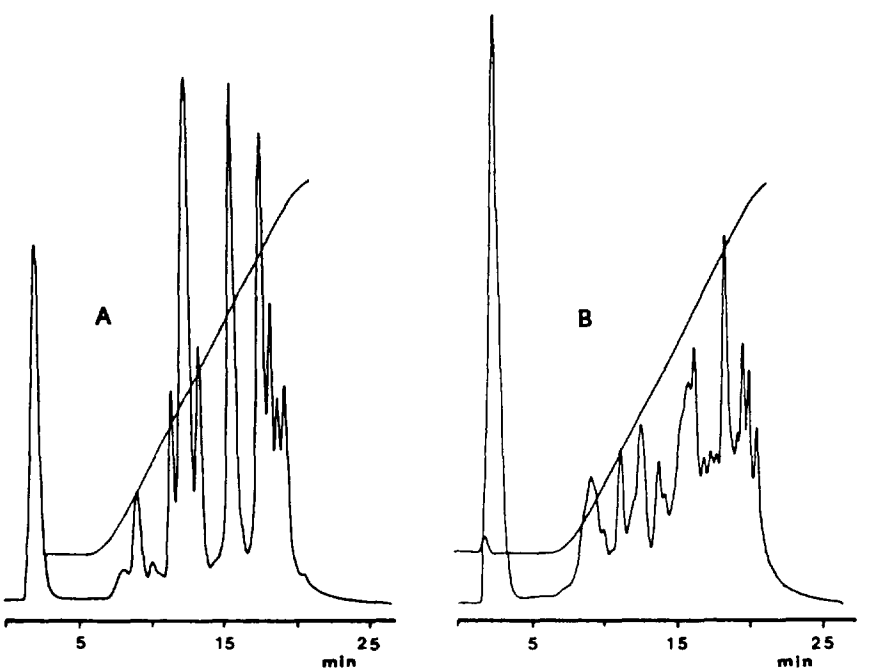

Fig. 3. Separation of protein fragments on Q-HyperD obtained by trypsin hydrolysis of (A) β-lactoglobulin and (B) human albumin. Column, 100 × 4.3 mm I.D.; 25 m*M* Tris–HCl buffer (pH 8.2); elution gradient from 0 to 0.4 *M* sodium chloride; flow-rate 0.4 ml/min; sample load, 200 μl.

sarily a general rule for other protein mixtures and can depend on the nature of the proteins to be separated and the separation conditions. Many other separations of protein mixtures have been effected with 10-μm particles of Q- and S-HyperD which are not shown here. However, we illustrate here the ability of this material to fractionate protein hydrolysis components from trypsin treatment obtained after digestion of human albumin and β-lactoglobulin using trypsin at 37°C overnight. Protein fragments were then separated on a column of Q-HyperD as shown in Fig. 3. It appears that numerous fragments could be easily separated in a very short period of time for subsequent analysis and sequence studies. In spite of the complexity of these polypeptide mixtures, the resolution efficiency remained particularly high.

Being designed for preparative protein separation, HyperD ion exchangers have to preserve their separation performance over a number of cycles This was studied by repeated injections of

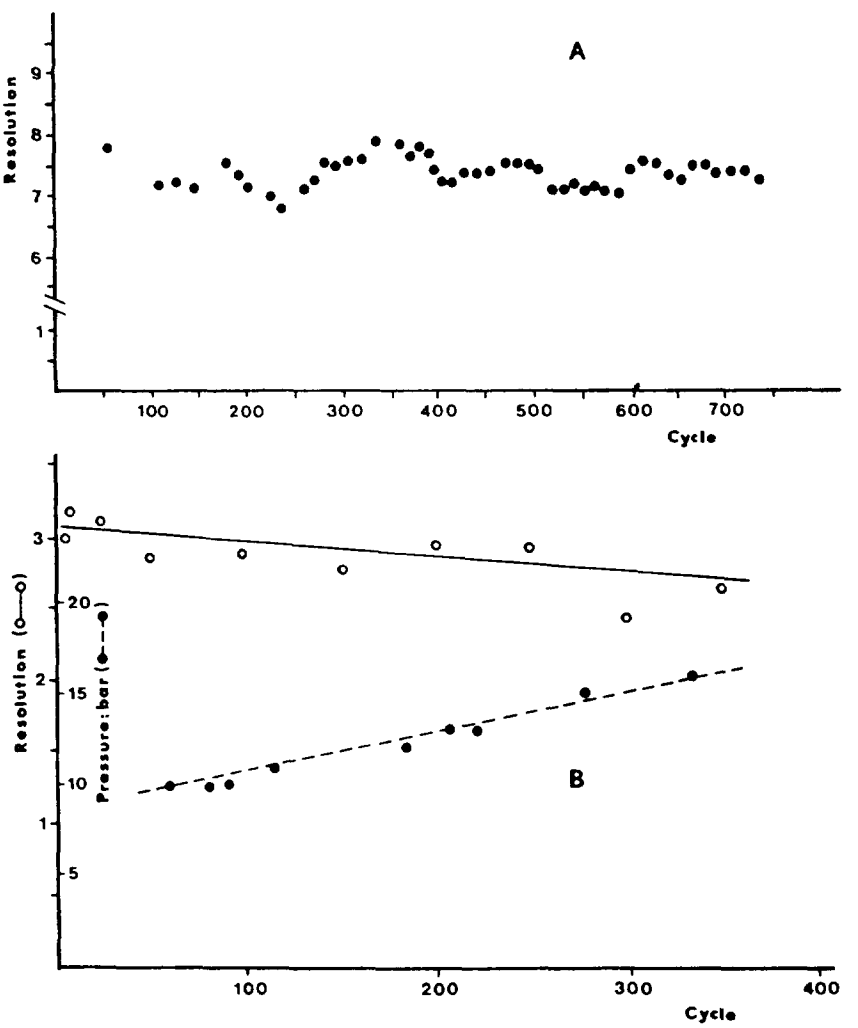

Fig. 4. Lifetimes of (A) Q-HyperD and (B) S-HyperD. Q-HyperD was repeatedly injected with a mixture of human transferrin and human albumin (375 mg of protein per loading); proteins were eluted by a salt gradient up to 0.4 *M* in 8 min in 50 m*M* Tris–HCl buffer (pH 8.6). S-HyperD was repeatedly injected with a mixture of β-lactoglobulin and lysozyme (200 μg of protein per load); proteins were eluted with a salt gradient up to 1 *M* in 25 min; after each separation the column was cleaned with 250 μl of 2 *M* sodium hydroxide and re-equilibrated in 50 m*M* acetate buffer (pH 4.5). Flow-rate (both columns), 1.0 ml/min.

protein mixtures into a single column while resolution and back-pressure were measured. Two experiments are reported: the first was a repeated injection (up to 700 runs) of a mixture of human transferrin and human albumin on a Q-HyperD column and the second was a repeated injection and elution of a mixture of β-lactoglobulin and lysozyme on an S-HyperD column. Each separation run in the second experiment involved an alternating protein separation step and cleaning cycle using 2*M* sodium hydroxide over 350 cycles. Fig. 4 shows that the column properties were almost unchanged for both the Q- and S-HyperD columns. A small drift of the back-pressure of S-HyperD was observed for an unidentified reason. Both experiments, however, demonstrate clearly that these sorbents can be used repeatedly for a long time with good preservation of initial chromatographic properties.

Scaling up in industrial preparative chromatography frequently implies a change from small to large particles to reduce operating back-pressures to acceptable levels. As a preliminary experiment, we tried to measure the effect of the particle size of Q-HyperD on the separation of a protein mixture under the same conditions (ovalbumin, β-lactoglobulin and cytochrome *c*). Table 1 shows that the protein peak positioning (sodium chloride molarity at the top of each peak) remained constant for both β-lactoglobulin and cytochrome *c*, while the resolution and the back-pressure were different, as expected. This experiment demonstrates the possibility of changing the HyperD particles size without modifying the column selectivity, a necessary condition for successful scaling up of operations.

4. Conclusions

HyperD ion exchangers of 10-μm bead diameter permit the separation of complex protein mixtures which can be easily transposed to preparative and large scales. The results of capacity versus flow-rate, resolution versus loadability and elution molarity versus particle size should allow the user to make the optimum choice of separation conditions. When the amount of protein to be separated is limited, small particle size packing materials (e.g., 10 μm) are recommended in laboratory columns with an acceptable back-pressure of a few bar. Separation under these conditions can be achieved in only a few minutes. When the amount of protein to be separated is much higher, larger columns with larger particle size packing materials can be advantageously used.

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Table 1
Comparative separation data using S-HyperD of different particle sizes for a mixture of ovalbumin, β-lactoglobulin and cytochrome *C*

Particle size (μm)	Elution molarity (NaCl)[a]			Resolution	Back-pressure at 10 m/h (bar)
	Ovalbumin	β-Lactoglobulin	Cytochrome *c*		
10	FT[b]	0.22	0.46	7.29	80
20	FT	0.21	0.44	6.01	12
35	FT	0.21	0.42	2.97	1

[a] Separation conditions: 100 × 4.3 mm I.D. column; 50 m*M* acetate buffer (pH 4.5); sodium chloride elution gradient from 0 to 1.5 *M*; flow-rate, 0.42 ml/min; sample load, 0.5 mg.
[b] FT = flow-through (non-retained compounds).

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