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Short Communication

Very high speed separation of proteins with a 20-μm reversed-phase sorbent

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ABSTRACT

A five-protein mixture was successfully separated in less than 15 s by reversed-phase gradient elution on a 20-μm polymeric flow-through type chromatographic packing material (POROS R/M). The same mixture could also be separated with far better resolution in less than 60 s on the same column by simply extending the gradient volume. Pressure drop across the column was less than 130 bar, despite a superficial linear velocity of nearly 9000 cm/h. Frontal chromatography at different flow-rates was used to demonstrate that the ability to separate at high speed was due to greatly enhanced mass transport within the particles due to the flow-through (perfusion chromatography) effect.

INTRODUCTION

It has been recognized for half a century that stagnant mobile phase mass transfer is a dominant limitation in liquid chromatography [1]. Although the development of microparticulate, macroporous supports substantially diminished this problem, resolution was still seriously compromised at high mobile phase velocity [2–4].

Recent work has shown that the stagnant mobile phase mass transfer problem in porous sorbents may be dealt with in several ways. One approach is to eliminate the pores. Through the use of 1–2 μm non-porous particles it has been possible to carry out protein separations an order of magnitude faster, albeit at the expense of diminished loading capacity and high operating pressure [5–7]. A second alternative is to cause liquid to flow or perfuse through the particles. An initial report indicates that 6000–8000 Å diameter, particle-transecting pores allow convective transport into the interior of supports [8,9]. Because convective transport is much more rapid than that achieved by diffusion, these materials can also be used at an order of magnitude

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higher mobile phase velocity in protein separations. This raises the question of whether the facilitation of transport provided by intraparticle convective flow will allow the use of larger particles in rapid separations.

Although 20 μm porous particles are easy to prepare and pack, provide high loading capacity, operate at low pressure and are generally less expensive to produce than microparticulate materials, they are never considered for high speed protein separations because of the mass transfer restrictions outlined above. This note reports the behavior of 20 μm perfusable reversed-phase sorbent at 30–60 times greater mobile phase velocity than is conventional.

MATERIALS AND METHODS

Reagents

All proteins used in this work were obtained from Sigma (St. Louis, MO, USA).

Chromatography

Gradient separations were carried out on an HP 1090 liquid chromatograph (Hewlett-Packard, Avondale, PA, USA) equipped with a diode array detector operated at 220 nm. The column used was a 30 × 2.1 mm POROS R/M (PerSeptive Biosystems, Cambridge, MA, USA). This packing consists of highly cross-linked styrene–divinyl benzene with no surface derivatization (nominal particle diameter, 20 μm).

The protein mixture used in the gradient separation consisted of ribonuclease A (6 mg/ml), cytochrome *c* (4 mg/ml), lysozyme (3 mg/ml) β-lactoglobulin (4 mg/ml) and ovalbumin (6 mg/ml) dissolved in 0.1% trifluoroacetic acid (TFA). A sample volume of 2 μl and flow-rate of 5 ml/min were used in all cases. Elution was achieved with a linear gradient from 0.1% TFA in 20% aqueous acetonitrile (ACN) to 0.1% TFA in 50% aqueous ACN. Gradient times are specified in the captions.

Frontal uptake studies were carried out on a Waters Delta Prep System (Waters Associates, Milford, MA, USA) equipped with a UV–VIS detector operated at 220 nm. The same column was used as in the gradient separation study.

The sample for the frontal uptake consisted of lysozyme (1 mg/ml) dissolved in 0.1% TFA in 1% aqueous ACN.

The column was equilibrated in 0.1% TFA in 1% aqueous ACN. A 2.0-ml injection was made at 0.1, 1.0 and 4.0 ml/min, which fully saturated the column. Following the injection, the column was washed with 0.1% TFA in ACN to remove all bound protein and reequilibrated in the starting mobile phase.

The superficial linear velocity (u in ml/h · cm^2 or cm/h) of mobile phase through the columns was calculated using the equation $u = 60\ vL/V$, where v is the flow-rate in ml/min, L is the column length in cm and V is the column volume in ml (0.104 ml for the column used in this study). Superficial linear velocity (flow-rate divided by column cross-sectional area) is often used because it is a simple method of comparing columns without regard to the type of packing material or packing density. It should be noted that true linear velocity is a function of both packing density and the specific interstitial volume of the sorbent.

RESULTS AND DISCUSSION

A separation of five proteins in 12 s on a POROS R/M column packed with 20 μm particles and operated at a superficial mobile phase linear velocity of 8700 cm/h is shown in Fig. 1. Peaks 1–5 in this figure are ribonuclease A, cytochrome *c*, lysozyme, β-lactoglobulin and ovalbumin, respectively. Since the pore volume is approximately 45% of the particle volume and the interstitial volume is approximately 38% of the column volume, it may be calculated that the liquid volume in the column is about 69 μl. With a flow-rate of 5 ml/min (8700 cm/h) the entire 69 μl liquid volume is displaced in 0.82 s. The five proteins standards were eluted within 15 column liquid volumes of the 30 column liquid volume gradient. The column pressure drop in these runs averaged around 120 bar.

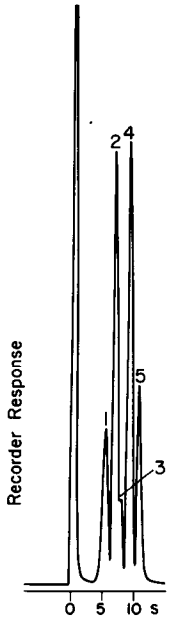

Fig. 1. A high speed reversed-phase separation of proteins with a steep gradient. Sample, operating parameters and peak identification were as specified in the text. Elution was achieved with a 24-s gradient.

In an effort to improve the resolution, the gradient time was extended to 1.5 min (110 column liquid volumes). It will be seen in Fig. 2 that this five-fold decrease in gradient slope substantially increased the resolution. (Peak identification is the same as in Fig. 1). All major protein species were fully resolved in less than 60 s (73 column liquid volumes). In addition, β-lactoglobulin (peak 4) was partially resolved into its A and B variants.

Because the mobile phase velocity in these separations is approximately 30–60 times higher than that generally used in high-performance liquid chromatography (HPLC), especially with larger particles, it could be argued that the proteins do not actually penetrate into the pores of the sorbent. This possibility was examined by

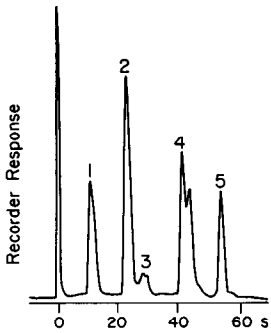

Fig. 2. A high speed reversed-phase separation of proteins with a shallow gradient. Sample, operating parameters and peak identification were as specified in the text. Elution was achieved with a 90-s gradient.

measuring the loading capacity of the column at 170, 1700 and 7000 cm/h superficial linear velocity (0.1, 1.0 and 4.0 ml/min). The results are shown in Fig. 3. Frontal loading curves at these low and high velocities show that the loading capacity (approximately 7 mg/ml for lysozyme under these conditions) is independent of the flow-rate. Thus the proteins actually can penetrate into the particles, even at these very short residence times.

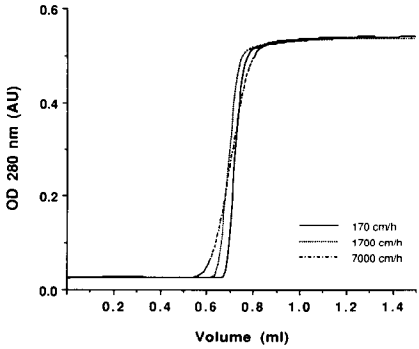

Fig. 3. Frontal loading of the column as a function of mobile phase superficial linear velocity. Study carried out with lysozyme on the 30 × 2.1 mm POROS R/M column as described in the text.

Based on the data presented above with a 20-μm perfusable reversed-phase sorbent it may be concluded that: (i) rapid separations of macromolecules may be achieved with 20-μm perfusable sorbents at very high mobile phase velocity; (ii) proteins have complete access to the interior of larger perfusable packings at superficial mobile phase velocities up to at least 7000 cm/h; and (iii) the ability of these materials to retain resolution at 30–60 times greater mobile phase velocity than normal in HPLC is the result of large particle transecting pores that allow convective transport within the particles.

The obvious advantages of 20-μm perfusable particles over 1–3 μm sorbents are that they are easier to pack, more difficult to plug and operate at much lower pressure. Similar high resolution analytical performance has been obtained using perfusable supports with other surface chemistries including ion exchange, hydrophobic interaction and affinity [10].

REFERENCES

1 A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, 35 (1941) 1358.
2 R. E. Majors, *Anal. Chem.*, 44 (1972) 1722.
3 K. K. Unger, R. Kern, M. C. Ninov and K.-F. Krebs, *J. Chromatogr.*, 99 (1974) 435.
4 F. D. Antia and Cs. Horváth, *J. Chromatogr.*, 435 (1988) 1.
5 K. K. Unger, G. Jilge, J. N. Kinkel and M. T. W. Hearn, *J. Chromatogr.*, 359 (1986) 61.
6 K. Kalghatgi and Cs. Horváth, *J. Chromatogr.*, 398 (1987) 335.
7 K. Kalghatgi and Cs. Horváth, *J. Chromatogr.*, 443 (1988) 343.
8 N. B. Afeyan, N. F. Gordon, I. Mazsaroff, L. Varady, S. P. Fulton, Y. B. Yang and F. E. Regnier, *J. Chromatogr.*, 519 (1990)1.
9 N. B. Afeyan, S. P. Fulton and F. E. Regnier, *J. Chromatogr.*, 544 (1991) 267.
10 N. B. Afeyan, S. P. Fulton, N. F. Gordon, L. Varady and F. E. Regnier, presented at *Anabiotech 1990, Burlingame, CA, October 1990.*