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Fast protein separation by reversed-phase high-performance liquid chromatography on octadecylsilyl-bonded non-porous silica gel

Effect of particle size of column packing on column efficiency

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ABSTRACT

The effect of the particle size of column packings on column efficiency in the reversed-phase high-performance liquid chromatographic separation of proteins was studied. Fast protein separations on non-porous octadecylsilyl (C_{18}) spherical silica gels of three different particle diameters (2, 5 and 20 μm) were examined, and five standard proteins were completely resolved within 20 s using a steep gradient elution with 0.1% aqueous trifluoroacetic acid–acetonitrile in all instances. The column efficiencies were approximately the same for all particle sizes. The large-particle (20 μm), non-porous C_{18} column could be operated at room temperature for the fast protein separation. Rapid separations on conventional macroporous C_{18} silica particles of 7 and 20 μm were also examined.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (HPLC) has become a widely used technique for the separation of proteins and peptides. Many kinds of stationary phases have been investigated [1] and silica gel or organic polymer gel-based macroporous supports with hydrophobic surfaces have been developed. Since Unger and coworkers [2–6] presented a chemically bonded non-porous silica gel with particles of 1.5 μm for protein separation, it has been shown that small-particle non-porous supports are more suitable than porous supports for the separation of proteins [7,8]. More recently, fast protein separations within 10 or 20 s [9–12] have been achieved on reversed-phase non-porous packings with particles of less than 2 μm. However, these approaches are generally restricted by the concomitant decrease in column permeability due to excessive pressure. Therefore, the column temperature needs to be maintained at 70–80°C for fast separation.

The mechanisms of protein separation in the reversed-phase gradient elution mode have also been investigated [13–19]. It is thought that the proteins are retained at the column inlet until at some point in the gradient they are desorbed completely. They then move through the column without further in-

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teraction with the stationary phase [15,16]. Protein separation by reversed-phase gradient chromatography is therefore based on a characteristic elution mode which differs from the chromatographic separation of low-molecular-weight compounds. These results prompted the consideration that the particle size of the column packings may not be such an important parameter in the efficiency of the columns in the reversed-phase gradient chromatographic separation of proteins.

In the work reported here, fast protein separation was studied on octadecylsilyl-bonded non-porous silica gels with 2-, 5- and 20-μm particles. Five standard proteins were completely resolved within 20 s on the all column packings and the efficiencies of the columns were approximately the same. The column packed with the 20-μm gel could be operated at room temperature for fast protein separation as a result of its low back-pressure.

EXPERIMENTAL

Reagents

Protein samples, bovine serum albumin, carbonic anhydrase (bovine erythrocytes), cytochrome *c* (horse heart), insulin (bovine pancreas), insulin B chain (bovine insulin), α-lactalbumin (bovine milk) and lysozyme (chicken egg white) were purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile and trifluoroacetic acid (TFA) were from Wako (Osaka, Japan). The water was purified by passing through a Milli-R/Q system (Millipore, Bedford, MA, USA).

Non-porous silica gels (mean particle diameter 2, 5 and 20 μm) were manufactured by Nomura (Seto-City, Japan) and these materials were chemically bonded with *n*-octadecyldimethylchlorosilane and end-capped with trimethylchlorosilane by conventional methods [20]. The resulting octadecylsilyl-bonded phases were slurry-packed into 30 mm × 4.6 mm I.D. stainless-steel column tubes. For comparison with the separation on the non-porous silica column, the porous silica gels Develosil 300-ODS-7 and -20 (mean pore diameter 30 nm, particle sizes 7 and 20 μm, Nomura) were packed into the same size column tubes.

Instruments

A low-pressure gradient HPLC system consisting of an 880-PU HPLC pump (Jasco, Tokyo, Japan) equipped with an ERC-3510 degasser (Erma, Tokyo, Japan), an 880-SC system controller (Jasco), an 880-02 ternary gradient unit (Jasco), a Model 7125 injector (Rheodyne, Cotati, CA, USA) and an 860-CO column oven (Jasco) was used. The polypeptides were detected with a Shimadzu SPD-2A spectrophotometric detector. The chromatograms were processed by a Model LC100W/F PC workstation equipped with an analogue-to-digital converter (Yokogawa Electric, Tokyo, Japan).

Chromatographic conditions

The following eluent composition was used for gradient elution: A, 0.1% TFA in water; B, 0.1% TFA in 90% aqueous acetonitrile. The gradient programs and flow-rates are given in the text and figure legends. The column temperature was maintained at 75°C or room temperature. The column effluent was monitored at 220 nm.

Each protein was dissolved in eluent A at a concentration of 1 mg/ml and each solution was mixed as an equivalent volume prior to injection. An aliquot of the mixture (5 μl) was injected into the HPLC system. Sample injections coincided with the commencement of the gradient and the actual gradient delay was previously determined by a tracer technique with an acetone-containing eluent. The sample loading capacity of the 20-μm non-porous column, which is the lowest of the columns examined here, was about 10 μg of carbonic anhydrase.

RESULTS AND DISCUSSION

In a preliminary examination, standard proteins were separated on the non-porous C_{18} silica column under conventional gradient elution conditions and the separation was compared with that on a macroporous C_{18} silica column under the same elution conditions. Fig. 1 shows the chromatograms of proteins on the non-porous and macroporous columns. Although the surface areas of the non-porous and macroporous silica were approximately 0.6 and 100 m^2/g, respectively, the retention times on the non-porous silica column were approximately the same as those on the macroporous column, in spite of their greatly different surface areas. This result is consistent with previous studies on protein separation mechanisms such as the on–off mecha-

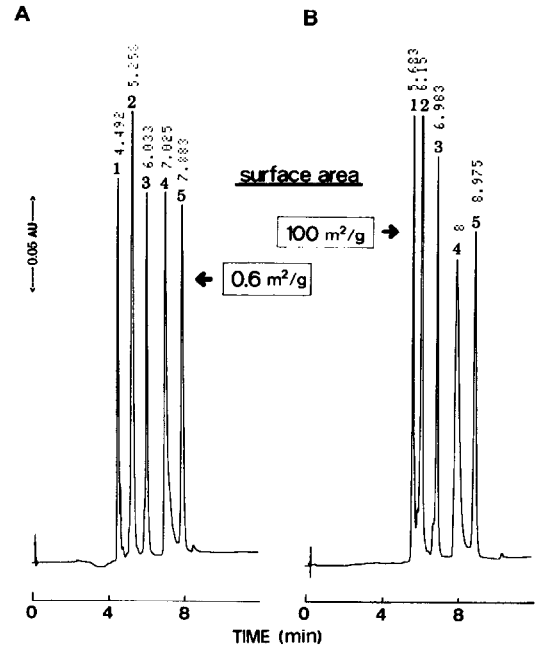

Fig. 1. Chromatograms of proteins on octadecylsilyl (C_{18}-bonded non-porous (NP-C_{18}-5, particle size 5 μm) (A) and macroporous (MP-C_{18}-7, particle size 7 μm) (B) silica columns under conventional gradient elution conditions. Conditions: flow-rate, 2 ml/min; linear gradient, from 10 to 100% eluent B in 20 min; column temperature, ambient. Peaks: 1 = insulin; 2 = cytochrome *c*; 3 = lysozyme; 4 = bovine serum albumin; 5 = carbonic anhydrase.

nism presented by Regnier and co-workers [13,16]. Proteins in the reversed-phase gradient separation would not be repeatedly partitioned on the stationary phase. This suggests that diffusion of the chromatographic peak by repeated adsorption and desorption between the stationary phase and the mobile phase is negligible. Therefore the particle size of the column packings, which is the most important factor in plate theory, would not be such a significant parameter in determining the column efficiency in the reversed-phase gradient chromatographic separation of proteins.

Fig. 2 shows chromatograms of the rapid separation of proteins obtained using columns packed with non-porous C_{18} silica gels of different particle sizes (2, 5 and 20 μm) at a constant flow-rate of 4.0 ml/min. Under this elution condition, as the 2- and 5-μm silica columns showed high back-pressures (over 200 kg/cm^2), all the columns were operated at 75°C.

In all instances the five standard proteins were completely resolved within 20 s and the column efficiencies were determined with the peak widths and resolutions approximately the same. The efficiency achieved by the 20-μm silica column was slightly poorer than those achieved by 2- and 5-μm silica columns. The peak width of carbonic anhydrase on the 20-μm silica column was 1.2 times that on the columns with smaller particles. However, the extent of the decrease of the 20-μm silica column efficiency was negligible small. These results were expected, for the following reasons. Solutes in gradient elution generally disperse not only in a process of repeating partition, but also in a process of non-retention followed by desorption. Especially in protein

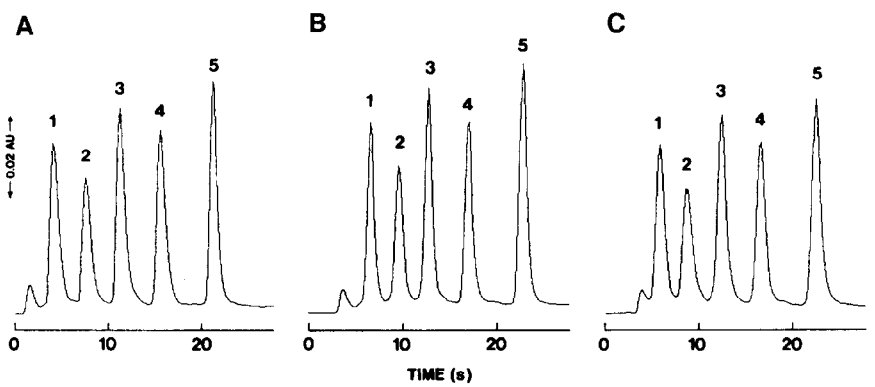

Fig. 2. Fast protein separations on C_{18}-bonded non-porous silica particles of diameter 2 μm (A), 5 μm (B) and 20 μm (C) performed at 75°C. Conditions: flow-rate, 4 ml/min; linear gradient, from 22 to 100% eluent B in 48 s; column temperature, 75°C. Peaks: 1 = insulin B chain; 2 = insulin; 3 = lysozyme; 4 = α-lactalbumin; 5 = carbonic anhydrase.

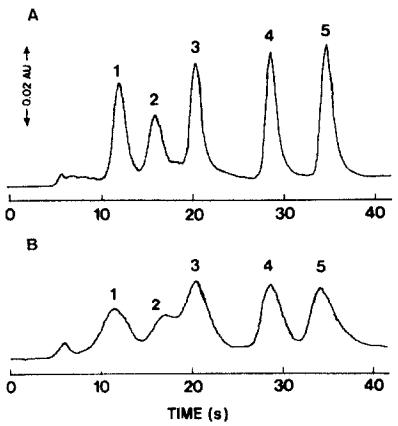

Fig. 3. Fast protein separations on C_{18}-bonded macroporous silica particles of diameter 7 μm (A) and 20 μm (B) performed at 75°C. Conditions: linear gradient, from 34 to 100% eluent B in 48 s. Other conditions and peaks as in Fig. 2.

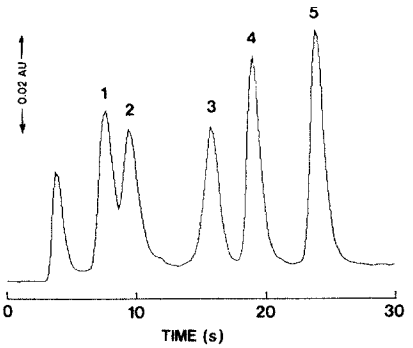

Fig. 4. Fast protein separation at room temperature on non-porous C_{18} particles of diameter 20 μm (NP-C_{18}-20). All conditions as in Fig. 2, except column temperature.

separation, diffusion during the partition process can be neglected as the adsorption-to-desorption transition of proteins [13,16] may only occur once by minute increases in the concentration of the organic solvent in the mobile phase. The proteins then move through the column without further interaction with the bonded phase. Although the dispersion of the unretained solute depends on the particle size of the packing material, this is not a significant factor if the column is small. Slight differences in the peak width dependent on the difference in particle size seem to be caused by such a diffusion factor in these data.

Fig. 3 shows chromatograms of the rapid separation of proteins on the 7- and 20-μm macroporous C_{18} silica particles performed at 75°C. Five proteins were separated within 40 s. The separations were influenced by the particle size. However, the column efficiency should also generally be affected by the distribution of pore size and decreases such as those shown here could be a result of both pore size and particle size distribution.

From these investigations it can been seen that non-porous supports might be favourable for rapid separations with a steep gradient elution because the stream of mobile phase will not become stagnant in pores as is observed on porous supports. Furthermore, a large-particle (20 μm), non-porous silica column can be operated at room temperature because of its low back-pressure. Fig. 4 shows the rapid separation of proteins on the 20-μm non-porous C_{18} column at room temperature. Proteins were satisfactorily separated within 30 s. An unknown peak was observed at the solvent front. This peak might be one of conformers of lysozyme, which is known to unfold reversibly in solution. The conformer concentration increased as the column temperature decreased and with an increasing concentration of acetonitrile at the start of the gradient. As shown previously by Benedek *et al.* [21], lysozyme gave two peaks in the reversed-phase separation; the native peak was more weakly retained than the denatured peak on the bonded phase surface. Therefore the first peak in Fig. 4 might be lysozyme without denaturation; the denatured peak, which was observed at about 15 s, decreased as the native peak increased.

The biological activities of the enzyme protein could often be regained after reversed-phase separation combined with an appropriate treatment such as dilution with a buffer solution [5]. A more rapid and lower temperature operation of the separation therefore allows a higher recovery of the biological activities of proteins. This suggests that the 20-μm non-porous support column may be very useful in the biochemical field.

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