ELSEVIER

Journal of Chromatography A, 702 (1995) 157–162

JOURNAL OF CHROMATOGRAPHY A

Effect of stationary phase on preparative protein separation in reversed-phase chromatography

Dauh-Rurng Wu*, Hellen C. Greenblatt

BTR Separations, Concord Plaza, Quillen Building, 3521 Silverside Road, Wilmington, DE 19810, USA

Abstract

A matrix of Zorbax Pro-10 (10 μm) packing materials with differing alkyl chain lengths (C_3, C_8, C_{18}) and pore sizes (60 Å, 150 Å, 300 Å) was utilized to determine the influence of chain length and pore size on chromatographic performance (i.e., resolution) and column capacity. Proteins ranging in size from M_r 5700 to 68 000 were used as model compounds. For each category of packing material, retention times of the proteins decrease and peak widths increase with increasing sample loading. Among the matrix materials, C_3/300 Å and C_8/300 Å provide the highest resolution of a mixture of proteins with total protein loading from 60 μg to 6 mg on analytical columns (25 × 0.46 cm).

The influence of alkyl chain length and pore size on column capacity was investigated by frontal chromatography. For a fixed chain length, materials with larger pore size provide greater column capacity of the model proteins. In addition, for materials of the same pore size, the longer the alkyl chain, the greater the column capacity. Column capacity for a given alkyl chain length and pore size is indirectly correlated to protein size; the greater column capacity is found for the smaller tested proteins.

1. Introduction

Reversed-phase high-performance liquid chromatography (HPLC) has become an important and powerful technique in the isolation and purification of large biomolecules [1–3]. The selection of packing material with appropriate characteristics for reversed-phase HPLC is critical. The importance of the surface (i.e., pore size and surface area) properties of the stationary phase in ion-exchange [4,5] and reversed-phase [6–9] HPLC has been identified. In general, material with larger pores is suitable for separation of large biomolecules. However, there is little comprehensive data on the influence of pore size and alkyl chain length on resolution and loading capacity of large biomolecules in a reversed-phase mode.

In this paper, we examine the influence of pore size and alkyl chain length on resolution and loading capacity of proteins. This information will provide a better understanding of the effect of these two parameters on resolution and

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(94)01139-7

loading capacity. Additionally, the effect of protein size on capacity is also explored.

2. Experimental

2.1. Material

Bonded Zorbax 10 μm silica-based materials were obtained from BTR Separations (Wilmington, DE, USA). Bovine serum albumin (BSA, M_r 68 000), lysozyme (M_r 14 400), ribonuclease A (M_r 13 700), insulin (M_r 5700) and trifluoroacetic acid (TFA) were from Sigma (St. Louis, MO, USA). Acetonitrile was purchased from Fisher Scientific (Malvern, PA, USA). Series 410 LC pump, LC-95 UV–Vis detector and Nelson 2700 chromatography software were obtained from Perkin-Elmer (Cupertino, CA, USA).

2.2. Methods

Separation of ribonuclease A, lysozyme and BSA was conducted in 25 × 0.46 cm columns packed with a variety of bonded Zorbax phases with pore sizes of 60 and 300 Å. The separation conditions were 10 to 70% acetonitrile with 0.1% TFA in 20 min at a flow-rate of 2 ml/min, a wavelength of 280 nm, and room temperature. The total sample loading was from 60 μg to 6 mg for each packing material. Resolution was calculated by:

$$R_s = 2(t_{R2} - t_{R1})/(W_2 + W_1)$$

where t_{R2} and t_{R1} are retention times of two adjacent peaks; W_2 and W_1 are peak widths at the baseline.

Columns of 5 × 0.46 cm were packed with a variety of bonded Zorbax phases in pore sizes of 60, 150 and 300 Å to study the effect of pore size and alkyl chain length on protein capacity using frontal chromatography. BSA, lysozyme and insulin were employed as model proteins in this capacity study. Protein solutions of 1 mg/ml in 0.1% aqueous TFA were continuously passed through columns at 1 ml/min until breakthrough occurred and absorbance became constant. The time at the half heights of breakthrough was measured and utilized to calculate protein column capacities.

3. Results and discussion

3.1. Effect of sample loading on retention time and resolution

Fig. 1 shows the result of separation of ribonuclease A, lysozyme and BSA at different sample loadings (from 20 μg to 2 mg of each protein) on Zorbax Pro-10 C_3/300 Å material. The retention

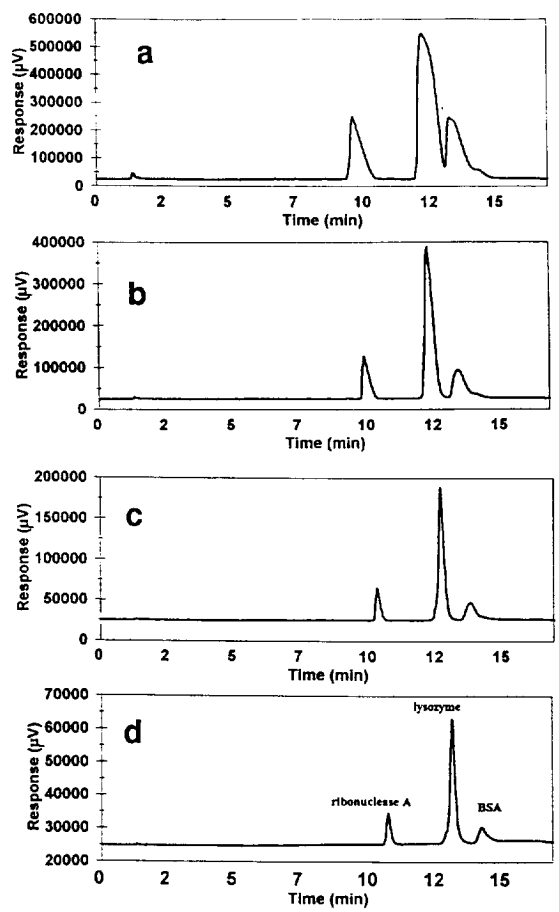

Fig. 1. Separation of ribonuclease A, lysozyme and BSA at sample loadings of total protein from 60 μg to 6 mg on Zorbax Pro-10 C_3/300 Å material (individual protein loading from 20 μg to 2 mg). (a) 6 mg; (b) 1.2 mg; (c) 0.3 mg; (d) 0.06 mg.

time of each protein decreases, and peak width increases, with increasing sample loading. This indicates that these proteins have Langmuir-like isotherms (concave down). Resolution decreases with increasing sample loading due to decreasing retention time and increasing peak width. A similar trend of retention time and resolution is observed for other Zorbax packing materials with different pore sizes and alkyl chain lengths.

3.2. Effect of pore size on retention time and resolution

Fig. 2 presents separations of ribonuclease A, lysozyme and BSA on Zorbax C_8/60 Å and C_8/300 Å at a total sample loading of 0.3 mg. The retention time of each protein decreases as the pore size decreases from 300 to 60 Å. Although 60 Å material has a higher surface area than 300 Å material, this phenomenon may be due to a size-exclusion effect with the 60 Å material. Due to size exclusion, the majority of large molecules may not be able to penetrate into the pores, resulting in less accessible surface area for the large molecules. The influence of pore size on retention time of BSA in C_8 and C_{18} at different sample loading is presented in Fig. 3a and b, respectively. Within the range of sample loading, 300 Å materials provide higher retention times than 60 Å ones. However, the difference in retention time is reduced with increasing protein loading.

The effect of C_8 pore size on the resolution of BSA and lysozyme is shown in Fig. 4a at different sample loadings from 20 μg to 2 mg. The 300 Å material offers higher resolution than the 60 Å material. The lower resolution of the 60 Å material is primarily due to the lower selectivity, shorter retention times (size-exclusion effect) and lower saturation capacity of the material. However, differences in resolution are reduced with increasing sample loading. The results presented on Fig. 4b indicate that C_{18}/60 Å material provides a higher resolution than C_{18}/300 Å. [C_8 results were reversed, i.e., C_8/300 Å resulted in

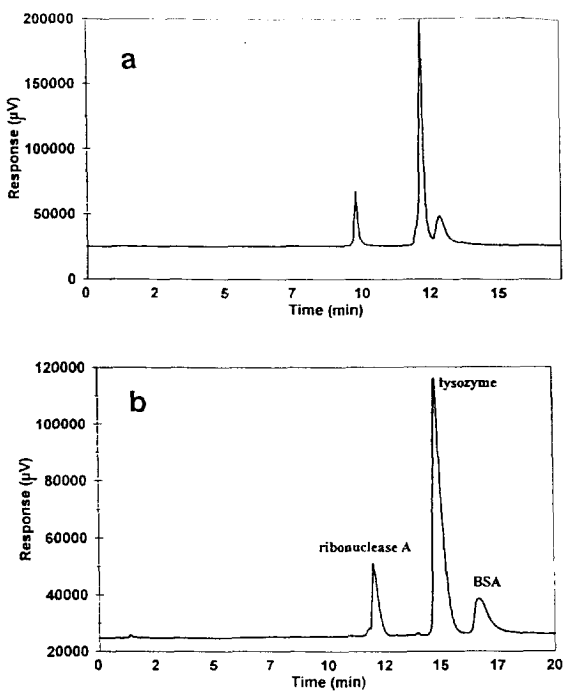

Fig. 2. Separation of ribonuclease A, lysozyme and BSA on Zorbax C_8/60 Å (a) and C_8/300 Å (b) materials at a total protein loading of 0.3 mg.

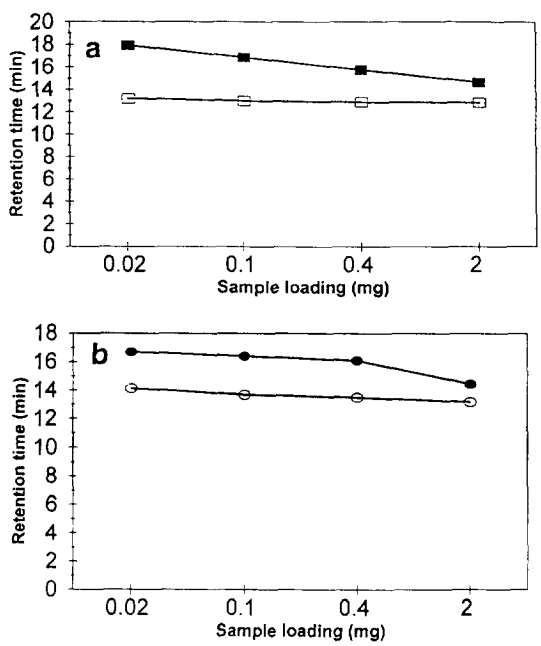

Fig. 3. Effect of pore size on retention time of BSA at different sample loadings on (a) Zorbax C_8/60 Å (□) and C_8/300 Å (■) and (b) Zorbax C_{18}/60 Å (○) and C_{18}/300 Å (●).

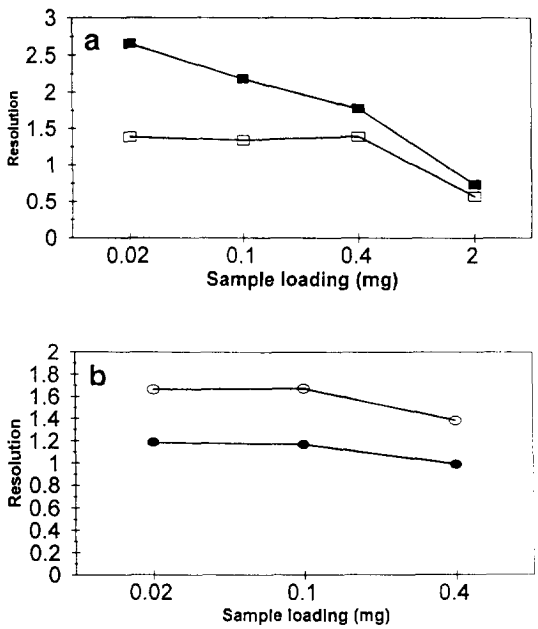

Fig. 4. Effect of pore size on resolution of BSA and lysozyme at different sample loadings on (a) Zorbax C_8/60 Å (□) and C_8/300 Å (■) and (b) Zorbax C_{18}/60 Å (○) and C_{18}/300 Å (●).

better resolution than C_8/60 Å (Fig. 4a)]. The lesser resolution of C_{18}/300 Å is due to peak tailing, which may be controlled by slow kinetic desorption and/or intra-particle mass transfer. Since the majority of the larger molecules are restricted to the exterior of the 60 Å particle, diffusion distance is significantly reduced and therefore the intra-particle mass transfer effect on resolution may not be significant.

3.3. Effect of alkyl chain length on retention time and resolution

Fig. 5 represents the separation of ribonuclease A, lysozyme and BSA on 300 Å material with differing alkyl chain lengths (C_3, C_8 and C_{18}) at a total sample loading of 1.2 mg. Both C_8 and C_{18} have greater retention than C_3 resulting from the stronger interaction between these two stationary phases and the model compounds. The influence of alkyl chain length on retention

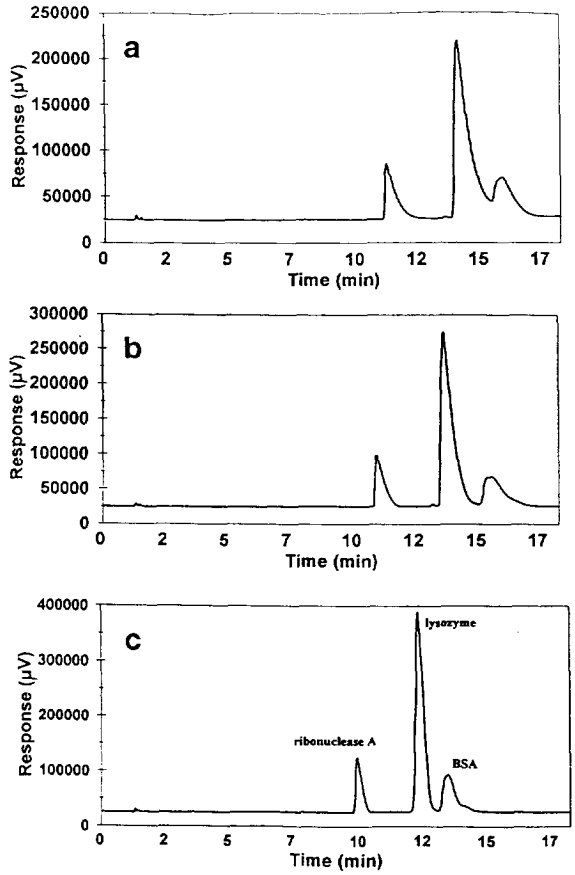

Fig. 5. Separation of ribonuclease A, lysozyme and BSA on Zorbax 300 Å material with differing alkyl chain lengths [C_3 (c), C_8 (b)and C_{18} (a)] at a total sample loading of 1.2 mg.

time specifically of BSA at different sample loadings is presented in Fig. 6.

Columns of three different alkyl chain lengths

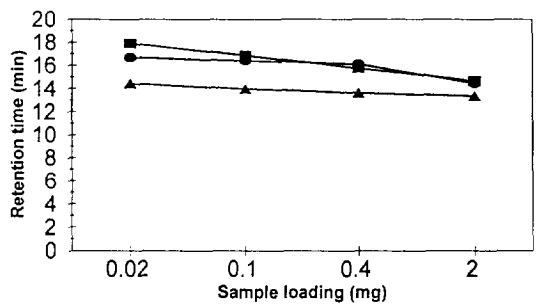

Fig. 6. Effect of alkyl chain length on retention time of BSA at different protein loadings on Zorbax C_3/300 Å (▲), C_8/300 Å (■) and C_{18}/300 Å (●).

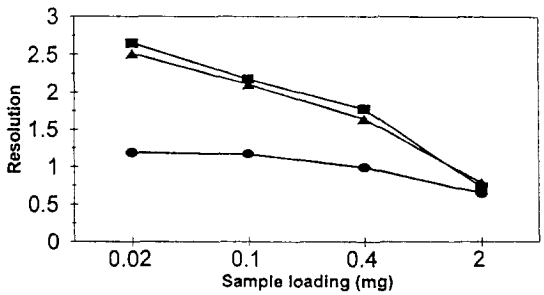

Fig. 7. Effect of alkyl chain length on resolution of BSA and lysozyme at different protein loadings on Zorbax C_3/300 Å (▲), C_8/300 Å (■) and C_{18}/300 Å (●).

(C_3, C_8 and C_{18}) bonded to 300 Å Zorbax were individually tested using 20 μg to 2 mg of each protein. C_3/300 Å and C_8/300 Å were best able to resolve BSA from lysozyme (Fig. 7). The lower resolution of C_{18} is due to peak tailing. Since C_8 and C_{18} have surface coverage values of 3.44 μmol/m^2 and 3.14 μmol/m^2, respectively and both are end-capped, the more severe peak tailing in C_{18} is mainly caused by slower kinetic desorption between the stationary phase and the proteins. C_3, due to its polymeric bonding (i.e., trifunctional silanes), has a higher surface coverage than C_8 and C_{18}. Therefore, the sharper peak shapes in C_3 than in C_8 and C_{18} are likely due to the combination of higher surface coverage and faster kinetic desorption.

3.4. Effect of pore size, alkyl chain length and protein size on column capacity

Table 1 compares the column capacities for BSA (M_r 68 000) of packing materials with

Table 1
Effect of pore size and alkyl chain length on BSA column capacity

Chain length	Column capacity (mg)		
	300 Å	150 Å	60 Å
C_3	2.3	N/A[a]	N/A
C_8	7.0	1.0	0.7
C_{18}	9.0	2.8	1.41

[a] Not available.

Table 2
Effect of pore size and alkyl chain length on lysozyme column capacity

Chain length	Column capacity (mg)		
	300 Å	150 Å	60 Å
C_3	15.7	N/A	N/A
C_8	23.0	5.5	0.6
C_{18}	29.0	23.3	10.0

different pore sizes and alkyl chain lengths. The data indicates that for the same alkyl chain length, it is the larger pore size which provides the greatest column capacity. Additionally, there is a greater difference in column capacity when comparing the 150 Å to 300 Å pore size, than comparing the 150 Å to the smallest pore size. This phenomenon may be due to a size-exclusion effect. For the same pore size, a longer alkyl chain length also results in a higher column capacity possibly due to stronger interactions between the stationary phases and BSA. Column capacity for lysozyme with different pore size and alkyl chain length show a similar trend (Table 2).

The effect of protein size on column capacity for BSA, lysozyme and insulin using C_8 materials with different pore sizes are shown in Fig. 8. For a given pore size and ligand length, proteins with larger molecular masses bind with lower column capacity (possibly due to steric hindrance). The pore size effect becomes less pronounced with decreasing protein size.

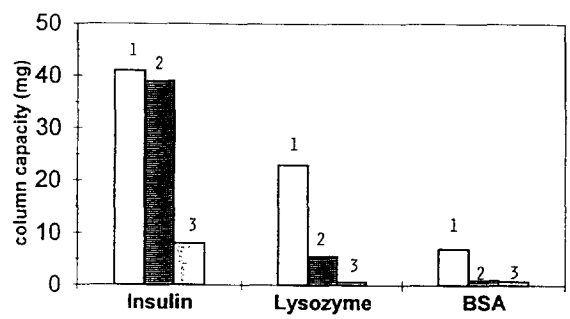

Fig. 8. Effect of protein size on column capacity on Zorbax C_8/300 Å (1), C_8/150 Å (2) and C_8/60 Å (3).

4. Conclusions

When testing a matrix of various packing materials, $C_3/300$ Å and $C_8/300$ Å provide the highest resolution of a mixture of model proteins with a total protein loading of 60 μg to 6 mg [onto analytical columns (25×0.46 cm)]. For any fixed chain length, material with a larger pore size provides greater column capacity. In addition, for materials with the same pore size, the longer the alkyl chain, the greater the column capacity. Column capacity for a given alkyl chain length and pore size is indirectly correlated to protein size. In this study, the higher column capacity is found for the smaller proteins.

References

[1] E.P. Kroeff, R.A. Owens, E.L. Campbell, R.D. Johnson and H.I. Marks, *J. Chromatogr.*, 461 (1989) 45.

[2] S. Linde and B.S.Welinder, in C.T. Mant and R.S. Hodges (Editors), *High-Performance Liquid Chromatography of Peptides and Proteins*, CRC Press, Boca Raton, FL, 1991, p. 351.

[3] P.H. Corran, in R.W.A. Oliver (Editor), *HPLC of Macromolecules: a Practical Approach*, IRL Press, Oxford, 1989, p. 127.

[4] M.P. Nowlan and K.M. Gooding, in C.T. Mant and R.S. Hodges (Editors), *High-Performance Liquid Chromatography of Peptides and Proteins*, CRC Press, Boca Raton, FL, 1991, p. 203.

[5] W. Kopaciewicz, M.A. Rounds and F.E. Regnier, *J. Chromatogr.*, 318 (1985) 157.

[6] Y.S. Kim, B.W. Sands and J.L. Bass, *J. Liq. Chromatogr.* 10 (1987) 839.

[7] B.W. Sands, Y.S. Kim and J.L. Bass, *J. Chromatogr.*, 360 (1986) 353.

[8] G.B. Cox, L.R. Snyder and J.W. Dolan, *J. Chromatogr.*, 484 (1989) 409.

[9] W. Kopaciewicz and F.E. Regnier, *J. Chromatogr.*, 358 (1986) 119.