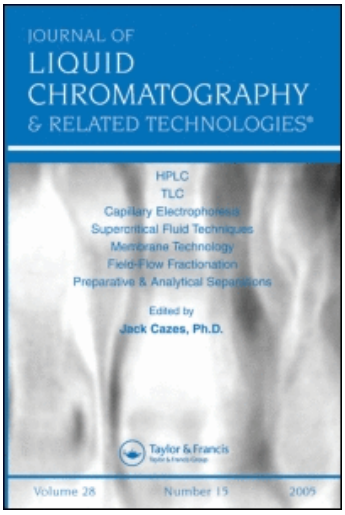

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Preparative Chromatography of Proteins

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PREPARATIVE CHROMATOGRAPHY OF PROTEINS

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ABSTRACT

High performance liquid chromatographic methods that have been developed for microparticulate supports can be adapted to 30u supports, such as the SynChroprep series, for large scale purification. Loading capacity and resolution of the ion exchange and reversed phase macroparticulate supports are examined. One gram of protein may be loaded on the ion exchange support in a 250x10 mm I.D. column.

INTRODUCTION

Over the past five years, HPLC has been seen to be an effective way to analyze protein mixtures by a variety of mechanisms - steric-exclusion, ion-exchange, reversed-phase and hydrophobic-interaction chromatography (1). The use of rigid supports has allowed milligram amounts of proteins to be purified within minutes. As the use of this methodology became more widespread, the scale-up to columns of 1 cm I. D. was used to prepare hundreds of milligrams of proteins (2). Unfortunately, when pilot plant levels were desired, the extremely high cost of the HPLC grade silica was realized and possible alternatives were sought (3).

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One of the first problems in preparative HPLC is to define the goals of the analysis. A chromatographer wants to separate a mixture into the maximum number of components with the best resolution possible. The engineer in a pilot plant wants to separate his compound from its impurities and load it in the highest possible quantity. These differences in goals produce two definitions of "loading capacity." To a chromatographer, loading capacity is the amount of solute which can be applied to a column before resolution is decreased. To an engineer in a pilot plant, loading capacity means the absolute capacity of the support for the solute. These differences in definitions suggest that there may also be differences in support requirements.

This report examines the capacity and resolution of a series of 30u macroparticulate supports to see whether methods which have been developed on analytical columns can be adapted for preparative purposes.

EXPERIMENTAL

Chemicals

Tris(hydroxymethyl)aminomethane (TRIS) and sodium acetate were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Sodium chloride and Moni-Trol I.X were from American Scientific Products (McGaw Park, IL, U.S.A.). Propanol-2 was purchased from The Anspec Co., (Ann Arbor, MI, U.S.A.) and trifluoroacetic acid from Pierce Chemical Co. (Rockford, IL, U.S.A.). Lysozyme, ribonuclease A, α-chymotrypsin, chymotrypsinogen A, and bovine serum albumin (BSA) were all obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Apparatus

The 30u SynChroprep CM300, SynChroprep AX300, and SynChroprep RP-P, 250x10 mm I.D. and 6.5u SynChropak AX300, 250x4.1 mm I.D. columns were obtained from SynChrom, Inc.

(Linden, IN, U.S.A.). The 100x25 mm I.D. column was from HP Chemicals (St. Louis, MO. U.S.A.). A Varian Model 5000 gradient high-performance liquid chromatograph with a Valco Model CV-6-UHPA-N-60 injection valve (Varian Instrument, Walnut Creek, CA, U.S.A.) and Chem Research Model 2020 UV detector (Instrumentation Specialties Co., Lincoln, NE, U.S.A.) with a Linear Model 1200 recorder (Linear Instruments, Irvine, CA, U.S.A) were used for analyses.

METHODS

Because general guidelines have been established in previous analytical investigations, solvent selection for ion-exchange and reversed phase chromatography was not investigated (1,4,5). 0.02M TRIS buffers with sodium acetate or sodium chloride gradients from 0-0.5M were used for ion exchange, and propanol-2 gradients in .1% trifluoroacetic acid were used for reversed phase. The gradient times and flow rates used are given in the Tables and Figures.

RESULTS AND DISCUSSION

LOADING CAPACITY

Dynamic column capacity, which fits the needs of the chromatographer, may be described as the sample size that causes decreased retention time and loss of peak shape using normal gradient operating conditions (6). This column capacity was seen to vary according to the nature of the protein, its molecular weight, and its retention characteristics. The loss in peak shape with increased loading is evident in the analysis of bovine serum albumin in Fig. 1. Table I shows that the capacity of a weak anion-exchange support (SynChroprep AX 300) for BSA is approximately 6 times greater than that of a reversed-phase material (SynChroprep RP-P) for the same protein. When using a weak cation-exchanger (SynChroprep CM300), the capacity for a small protein, ribonuclease-A was about 5 times that of a protein twice its size, chymotrypsinogen-A.

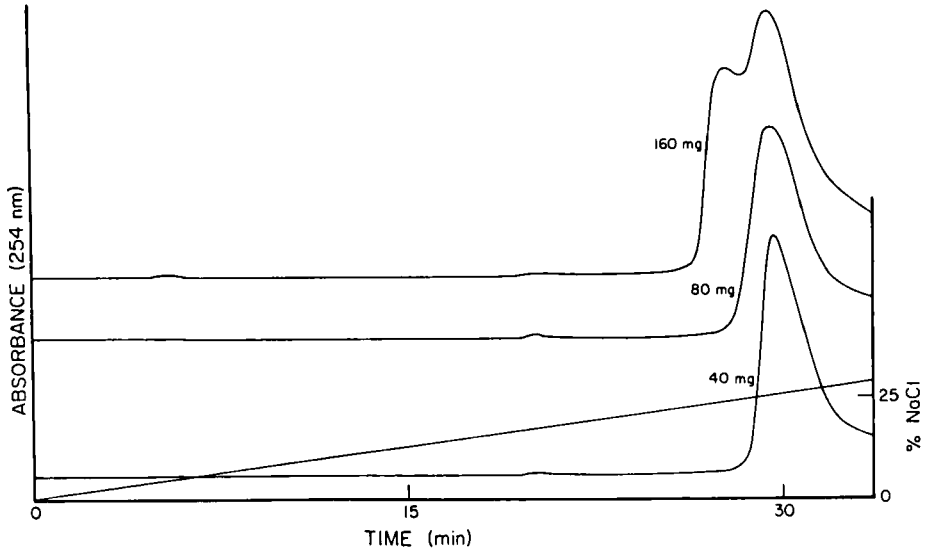

Fig. 1 Effect of sample loading on the resolution of a bovine serum albumin sample using a semi-preparative column of 30u support. Column: SynChroprep AX300, 250x10 mm I.D. Flow-rate: 1.5 ml/min. Buffer: 0.02M TRIS, pH7, 120 min. linear gradient from 0 to 0.5M CH_3CO_2Na.

TABLE I

Loading Capacity of 30u SynChroprep Supports

	Probe	Gradient Time (min)	Dynamic Capacity	Absolute Capacity
AX300	BSA	120	160 mg	1 g
RPP	BSA	40	25 mg	400 mg
CM300	α-chymotrypsin	120	80 mg	1 g

Mobile phase as in Methods. Flow-rate: 1.5 ml/m
Column: 250x10 mm I.D.

Such variance is probably due to the available surface area of the support.

Absolute column capacity may best be described as mass overload (7) utilizing the displacement mode (8) of chromatography. To utilize this technique, the column was equilibrated with a mobile phase that had a low affinity for the stationary phase. The sample was introduced and adsorbed on the column until it was completely loaded. Under these conditions, impurities with less retention eluted from the column during the sample application process. For example, the early eluting impurities of ∝chymotrypsin were eluted with each injection on a SynChroprep CM300 column while ∝chymotrypsin itself was retained. When absolute capacity was reached, the sample was released from the column by running a step gradient with a mobile phase having a stronger affinity for the stationary phase than the sample. By suitably choosing the mobile phase used in the "step", later eluting impurities could be retained on the column and dumped after the purification was finished. Again, different observations were made for various combinations of proteins and columns. While loading BSA on a SynChroprep RP-P column, a "breakthrough" impurity peak that had less column affinity than BSA was observed from almost the first injection. Sample was injected onto the column until a constant integrated area of the "breakthrough" peak was achieved. While loading ∝-chymotrypsin onto a SynChroprep CM300 support, an impurity "breakthrough" peak, that did not change in size was observed from the initial injection. ∝Chymotrypsin did not "breakthrough" until absolute capacity was reached.

Table I compares the dynamic and absolute column capacities of three modes of chromatography supports - weak anion-exchange(AX300), weak cation-exchange(CM300) and reversed phase(RP-P), run in 250x10 mm I.D. columns. The dynamic capacity was calculated as the amount of sample that increased the width at peak half height by approximately 70%. If the resolution of the sample components is great, the actual dynamic capacity could be higher than these values (9). As would be expected, for fairly pure proteins,

TABLE II

BAND-SPREADING FOR 30u SUPPORTS

F_r (ml/min)	Gradient Time (min)	SynChroprep AX 300 Bovine Serum Albumin t_r (min)	SynChroprep AX 300 Bovine Serum Albumin t_r/t_w	SynChroprep CM 300 αChymotrypsin t_r (min)	SynChroprep CM 300 αChymotrypsin t_r/t_w
6.0	30			17.9	17.9
3.0	60	32	30.5	35.4	16.85
2.0	90	48	32	54.0	19.56
1.5	120	64	34.6	75.0	20.1
1.0	180	99	38.1	108.6	21.5
0.5	360			215	26.88

F_r (ml/min)	Gradient Time (min)	SynChroprep RP-P Bovine Serum Albumin t_r (min)	SynChroprep RP-P Bovine Serum Albumin t_r/t_w
3.0	20	13.5	38.5
2.0	30	20.4	42.7
1.5	40	27.1	43.3
1.0	60	40.5	40.5
0.5	120	82	45.5

Mobile phase as in Methods
Column: 250x10 mm I.D.

the absolute capacity was significantly greater than the dynamic capacity.

FLOWRATES

One easy way to increase resolution is to change the mobile-phase flowrate. Because proteins normally must be eluted with a gradient, ordinary plate height measurements cannot be used to determine the effect of flowrate velocity on band broadening. Therefore,the ratio of the retention time/peak width was used to indicate protein peak spreading.

In this method, larger numbers indicate better resolution. Flow-rates were varied from 0.5-3.0 ml/min while holding the gradient volume constant. The results are listed in Table II. In most cases, a decrease in flow-rate with a constant gradient volume resulted in both higher resolution and longer retention times. Due to the inaccuracy of some HPLC equipment at slow flowrates and low pressures, and the disadvantages of long retention times, flow-rates must be selected on these bases as well as that of resolution.

GRADIENT SHAPE

Solvent gradients can also be used to optimize separations with respect to both time and resolution. For example, on the SynChroprep CM300 column, a 90 minute linear gradient at 1.5 ml/min gave a very good separation of ribonuclease-A and lysozyme. However, it was necessary to use at least a 120 minute gradient at the same flowrate to separate chymotrypsinogen-A from the ribonuclease-A and lysozyme peaks as seen in Fig. 2. Similarly, on SynChroprep AX300, impurities in ovalbumin were not adequately separated until a 120 min. gradient at 1.5 ml/min. was used.

COLUMN DIAMETER

The scale-up of a chromatographic process from the analytical laboratory level to the pilot plant is accomplished by increasing the width of the column rather than the height. In this way, the parameters that have been worked out, such as the linear flow rates and gradient times would remain about the same. Because the capacity of a column roughly corresponds to the amount of packing material in it, the sample loading would be increased proportionately to the volume. Fig. 3 shows the same 3-component sample separation that was seen in Fig. 2 using a 2.5 cm I.D. column for 1.3 g of total protein instead of 75 mg.

RESOLUTION ON 30u VS. 6.5u

Once the operational parameters such as flow rate,

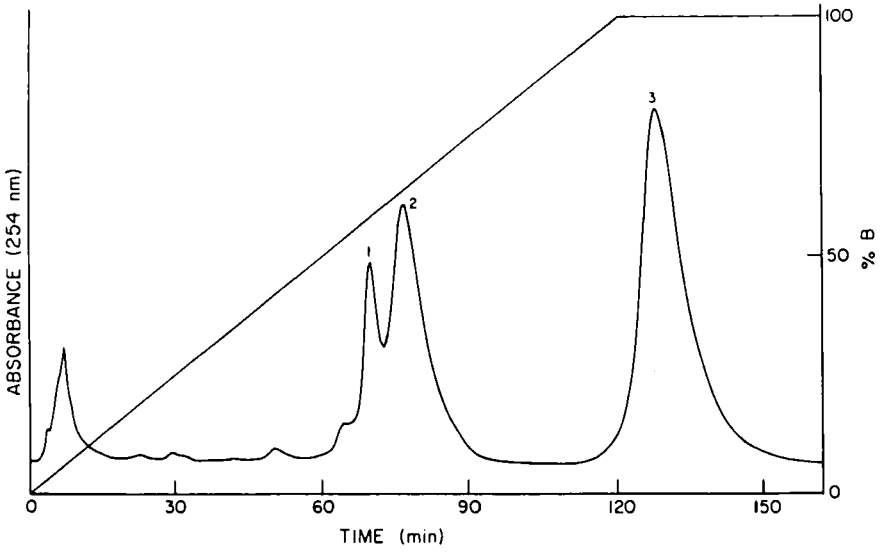

Fig. 2 Resolution of 45 mg ribonuclease-A (1), 15 mg chymotrypsingen-A (2), 15 mg lysozyme (3), using a semi-preparative column of 30u support. Column: SynChroprep CM300, 250x10 mm I.D. Flow-rate: 1.5 ml/min. Buffer: 0.02 M TRIS , pH7, 120 min linear gradient from 0 to 0.5M CH_3CO_2Na.

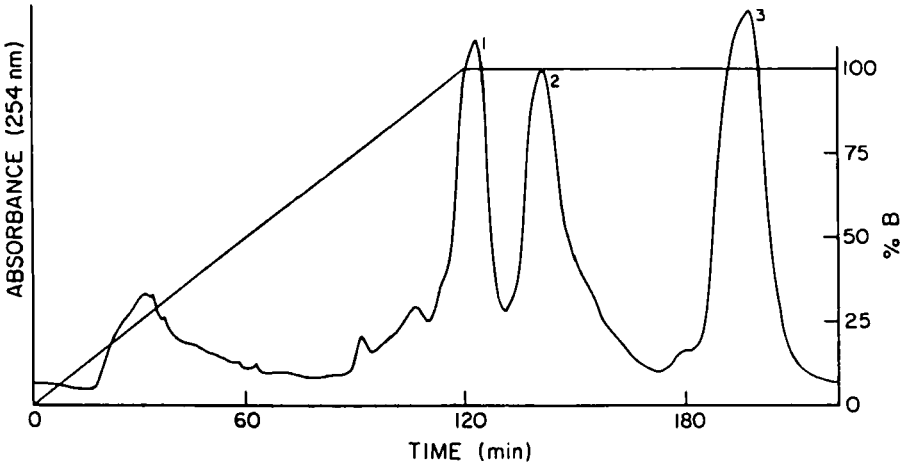

Fig. 3 Resolution of 750 mg ribonuclease-A (1), 130 mg chymotrypsinogen-A (2), and 150 mg lysozyme (3) using a preparative column (250x25 mm I.D.) of 30u support. Conditions as in Fig. 2.

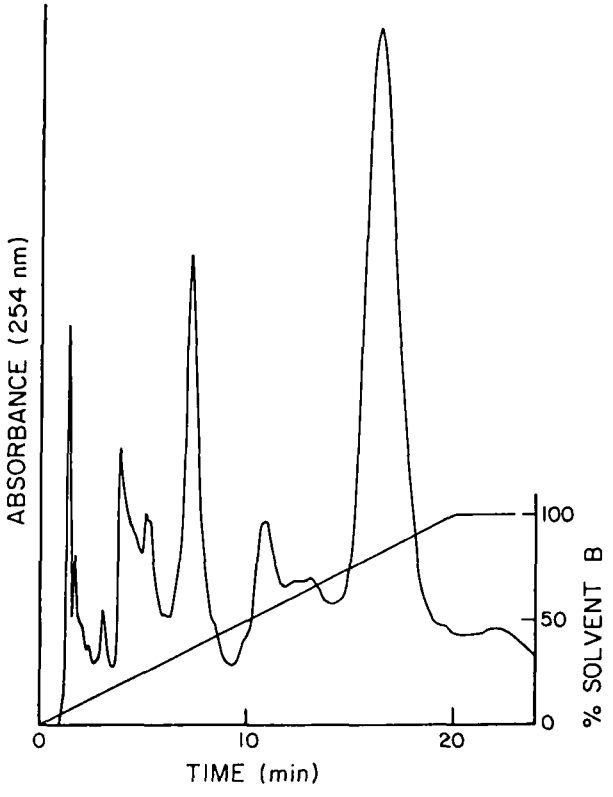

Fig. 4 Analysis of human adult serum using an analytical column of 6.5u support. Column: SynChropak AX300, 250x4.1 mm I.D. Flow-rate: 2 ml/min. Buffer: 0.02M TRIS, pH8. 20 min linear gradient from 0 to 0.5M CH_3CO_2Na.

gradient time, and component separation have been investigated for 30u supports, the chromatographer and engineer must know how the resolution of the 30u preparative material compares with that of the 6.5u supports used in the analytical columns. Fig. 4 and 5 compare the analysis of human adult serum on a 250x4.1 mm I.D. column packed with 6.5u SynChropak AX300 with the same analysis on a 100x25 mm I.D. column packed with SynChroprep AX300. There is a good

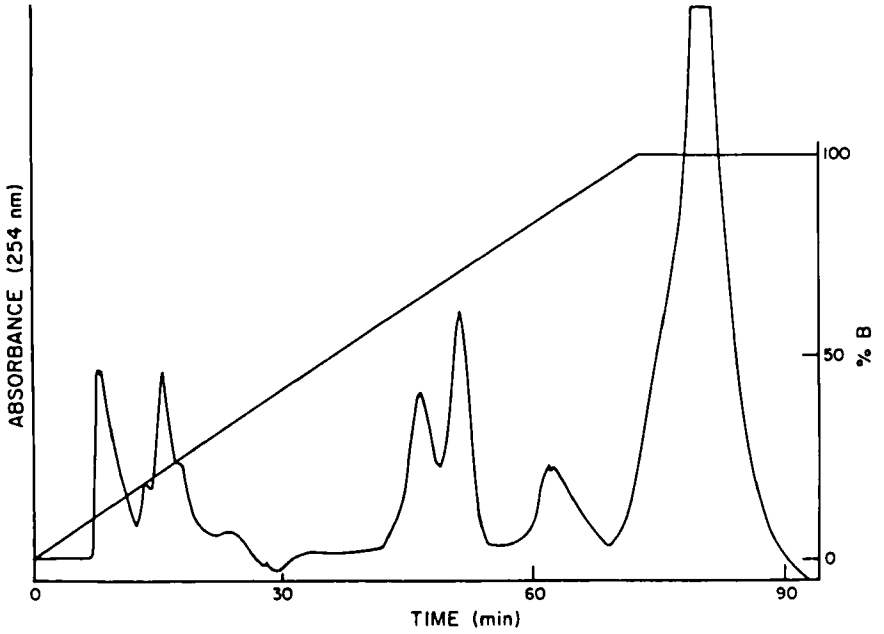

Fig. 5 Analysis of human adult serum using a preparative column of 30u support. Column: SynChroprep AX300, 100x25 mm I.D. Flow-rate: 3.2 ml/min. Buffer: 0.02M TRIS, pH7. 72 min. linear gradient from 0 to 0.5M NaCl.

correlation between the chromatograms, and the major components, and some minor ones, are sufficiently resolved on the 30u support to satisfy the engineers needs for purification.

CONCLUSIONS

This study shows that HPLC methods that have been developed on microparticulate supports can be adapted to 30u supports for large scale purification. Absolute loading of a gram of protein are possible on ion-exchange columns of 250x10 mm I.D. dimensions. Adequate resolution can be obtained by adjusting the flowrate and gradient shape of the analysis.

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