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COMPARISON OF POROUS SILICA PACKING MATERIALS FOR PREPARATIVE ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

Although analytical high-performance liquid chromatographic columns have been successfully used for purification of milligram amounts of proteins, they do not appear to be ideal for preparing gram or kilogram quantities because of cost and load capacities. In this paper the development of preparative weak anion-exchange materials is described. These materials possess similar chromatographic characteristics to analytical 5–10-μm materials, yet also have high load capacities. A number of inorganic packings were examined to determine which had the best combination of high load capacity, good resolution, stability, and low cost. When appropriate flow-rates and gradient shapes were used, 30–50-μm materials produced resolution of components of a commercial ovalbumin sample that was comparable to that achieved on a 6-μm material. An amount of 3 g of a protein could be loaded onto a 250 × 21 mm-I.D. column with adequate resolution to separate it from some of its impurities.

INTRODUCTION

In the past decade, protein purification has undergone a virtual revolution in terms of speed and resolution by the application of high-performance liquid chromatographic (HPLC) materials that have capacities and resolution equal to or better than their soft gel predecessors. Because the HPLC packings are rigid, and stable to pressure and solvent changes, recycling can be accomplished quickly, and analyses can frequently be carried out at ambient temperatures without detrimental effects on the proteins. HPLC on an analytical scale has become an important means of purifying proteins in quantities of less than 1 g[1]. Because of the success of analytical procedures, similar packing materials or columns that would allow the preparation of gram or kilogram quantities of proteins have been sought[2–5]. Two methods for scale-up are the use of larger columns of microparticulate packings or of larger-particle packings that are similar to the analytical materials. The 5–10-μm HPLC materials used in the analytical laboratory may not be ideal for large-scale preparative purposes for two major reasons: (1) silica of this grade is very expensive (US $ 25–35 per g) and (2) microparticulate packings are difficult to handle and to pack effectively into very large columns. The second problem could possibly be solved by

hardware developments, but the first requires a search for alternative packing materials.

This paper describes the development of macroparticulate, weak anion-exchange materials that are designed specifically for large-scale protein purification. Numerous materials were prepared by forming a weak anion-exchange layer on silica samples, including some that were not primarily designed for HPLC. Columns containing these packings were evaluated for resolution and load capacity of proteins. Analytical techniques for implementing preparative protein purification were also developed in the course of this study.

EXPERIMENTAL

Chemicals

Tris(hydroxymethyl)aminomethane (Tris) was purchased from Aldrich (Milwaukee, WI, U.S.A.). Ovalbumin, bovine serum albumin (BSA), and sodium chloride were purchased from Sigma (St. Louis, MO, U.S.A.).

Apparatus

SynChropak AX300 columns, (250 × 4.1 mm I.D.) and SynChroprep AX300 were obtained from SynChrom (Linden, IN, U.S.A.). Silica samples were obtained from eleven vendors for evaluation. A System 42 preparative chromatograph with a 50-ml head and a Gradient Manager 702 software package from Gilson Medical Electronics (Middleton, WI, U.S.A.) were interfaced with an Apple IIe computer (Apple, Cupertino, CA, U.S.A.), a Model UA-5 spectrophotometer and recorder from ISCO (Lincoln, NE, U.S.A.), and a Model 7161 injector from Rheodyne (Cotati, CA, U.S.A.). Also, a Varian Model 5000 gradient high-performance liquid chromatograph with a Valco Model CV-6-UHPa-N-60 injection valve (Varian, Walnut Creek, CA, U.S.A.) and a Chem Research Model 2020 UV spectrophotometer (ISCO) with a Model 1200 recorder (Linear Instruments, Irvine, CA, U.S.A.) were used.

Methods

The buffers were prepared by adding the appropriate amount of sodium chloride to 0.02 M Tris in deionized water. Hydrochloric acid was used to adjust the pH. Standards were dissolved in the initial buffer.

Dynamic column capacity is defined as the sample size that causes decreased retention time and loss of peak shape when normal gradient operating conditions are used[6-8]. From previous studies, the dynamic load capacity of a 250 × 10 mm I.D. column of 30-μm SynChroprep AX300 was determined to be much higher than 40 mg. Because the peak width had not changed at levels below 40 mg, this was selected as the initial sample size for determining the dynamic column capacity. Increasing amounts of BSA were injected until the width of the peak at half height was 70% greater than that of a 40-mg sample.

Absolute column capacity is the total amount of sample that can be bound on a column of a given size[7,8]. It can be described as mass overload[9], utilizing the displacement mode[10] of chromatography. For this technique, the column was equilibrated with a mobile phase that had low affinity for the stationary phase (0.02 M Tris, pH 7). Samples (40 mg) were adsorbed on a 250 × 10 mm I.D. column until

a "breakthrough" peak of constant integrated area was achieved. The sample was then released from the column with a step gradient to a mobile phase having a stronger affinity for the stationary phase than the sample (0.02 M Tris, 0.5 M sodium chloride, pH 7).

RESULTS AND DISCUSSION

Selection of silica and operational parameters

Conditions for the evaluation of macroparticulate HPLC packings were established with 30-μm SynChroprep AX300 materials[7]. These packings are spherical, with 300-Å pores and a polyamine coating, and, except for the particle size, they are chemically identical to the 6.5-μm SynChropak materials used for HPLC. This consistent quality of silica guaranteed that the operating parameters to be established would be based on the 30–40-μm particle size and not on the pore structure or chemical composition. A fourfold decrease in mobile phase velocity and an identical increase in gradient length gave resolution of proteins on 30-μm columns that was similar to that obtained on 6.5-μm columns.

Columns with dimensions of 250 × 10 mm I.D. were chosen for this study, because they were wide enough for proper dry-packing and for loading gram quantities of proteins, yet small enough for analysis times of less than 2 h. BSA and ovalbumin were used as solutes for the operational studies because their chromatographic behavior on anion-exchangers is known[11]. Sodium chloride was substituted for sodium acetate in the salt gradient, because it yielded sharper peaks, and it is cheaper and non-toxic. Fig. 1 illustrates the analysis of ovalbumin on 6.5-μm and 30-μm columns. The separation of the major ovalbumin peak from its minor com-
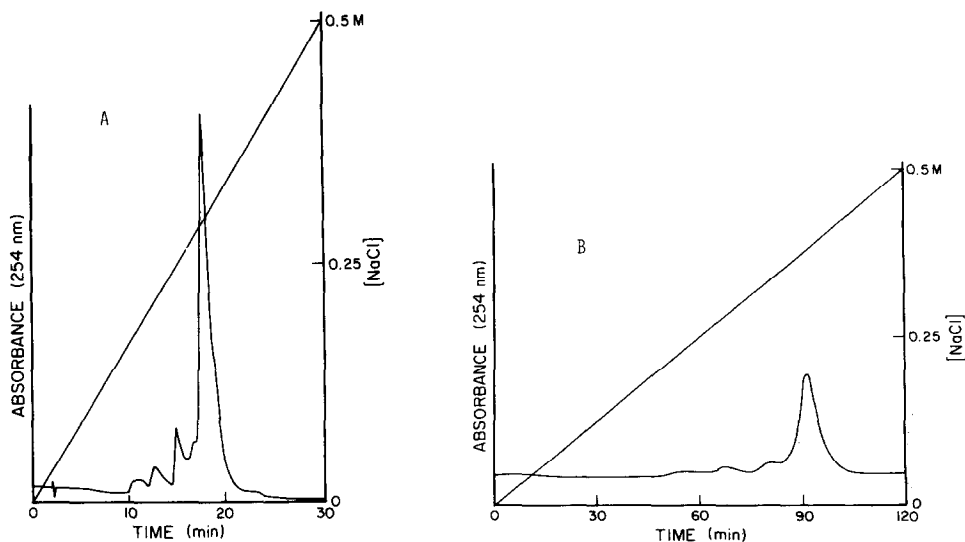

Fig. 1. Analysis of ovalbumin by anion-exchange chromatography. (A) Column, 6.5-μm SynChropak AX300, 250 × 4.1 mm I.D.; buffer, 0.02 M Tris (pH 7); 30-min gradient from 0 to 0.5 M sodium chloride; flow-rate, 1 ml/min. (B) Column, 30-μm SynChroprep AX300, 250 × 10 mm I.D.; buffer, 0.02 M Tris (pH 7); 120-min gradient from 0 to 0.5 M sodium chloride; flow-rate, 1.5 ml/min.

ponents is similar on the two columns. The differences in ionic strength at the elution times are probably due to the linear velocity and column volume discrepancies.

Silica samples had to meet several criteria before they were selected. Pore diameters had to be 150–300 Å to allow maximum penetration and interaction of protein molecules. Particle diameters of 30–60 μm were preferred, because they can be easily dry-packed. Low cost and ready availability were also major considerations. The silica was then derivatized with a polyamine coating to yield the weak anion-exchanger.

Capacity

Each of the packings was evaluated for its dynamic and absolute capacity for binding BSA under actual chromatographic conditions, as described in *Methods*. Table I lists the dynamic load capacities of all the columns tested. It can be seen that six of the columns have dynamic capacities exceeding 20 mg of BSA per ml of packing material. In actual use, the dynamic load capacity would be higher than this arbitrary value if the peaks were well resolved, or it would be less, if the peaks were very close together.

TABLE I

LOADING OF BSA

Columns: 250 × 10 mm I.D. (20 ml); mobile phase: 0.02 *M* Tris (pH 7); gradient: 120 min from 0 to 0.5 *M* sodium chloride; flow-rate: 1.5 ml/min.

Column No.	Pore diameter (Å)	Particle diameter (μm)	Load	
			Dynamic (mg)	Absolute (g)
1	175	37–74	520	2.1
2	300	15–20	200	0.96
3	200	30	360	2.7
4	150	>74	800	0.96
5	200	37–74	400	1.2
6	300	30	200	2.2
7	150	35–70	320	0.58
8	270	40	240	1.8
9	270	40	400	2.1
10	200	30	800	3.6
11	300	35–70	400	2.3

The total amount of protein that could be bound to the sorbent contained in a column was denoted as absolute load. The absolute load capacity, as described in *Methods*, was reproducible in duplicate analyses of the same sample size, as well as, with sample sizes in the range 20–200 mg. The absolute capacity was inversely affected by flow-rate, as seen in Fig. 2. This decrease in capacity with increase in flow-rate was probably due to slow diffusion into the pores and kinetic phenomena. The large BSA molecule could not enter or interact as effectively with the cations within the pores when the flow-rate was high. The absolute capacities obtained for this series of packings are also listed in Table I. Six of the packings have absolute capacities for BSA exceeding 100 mg/ml packing material.
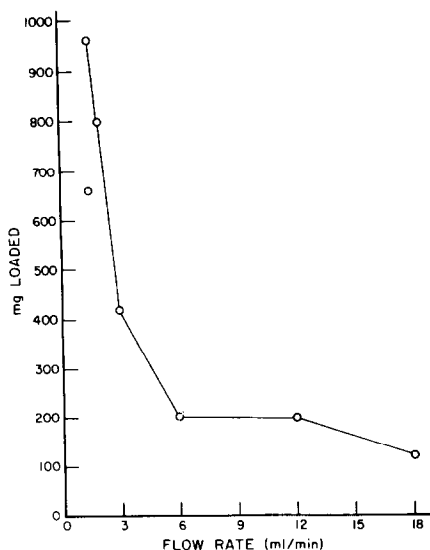
Fig. 2. Effect of flow-rate on the absolute load capacity of BSA. Column, AX Silica No. 4, 250 × 10 mm I.D. Conditions as in Fig. 1B.

Resolution

Resolution is determined by two parameters: the selectivity of a support for specific molecules and the efficiency or amount of bandspreading. An evaluation of these two parameters for this series of packings was made by using ovalbumin and BSA as solutes. The degree of bandspreading or efficiency produced by the various packing materials has been indicated by the ratio of the retention time (t_R) to the peak-width at half-height ($w_{1/2}$). The latter was selected, because it was less affected by shoulders than the peak-width at the baseline. Higher values for the ratio indicate narrower peaks and, therefore, potentially better resolution. The lower ratios for BSA are probably due to the frequent presence of shoulders, due to the protein multiplicity. The data are shown in Table II. The selectivities of these columns for ovalbumin and BSA can be ascertained by the number of component peaks separated by chromatography and are seen to differ greatly among the various packings. Table II also shows that the selectivity was independent of the column efficiency, because there is no apparent correlation between the two. Fig. 3 illustrates an example of the selectivity of one of the columns for ovalbumin components.

Stability and physical suitability

During both synthesis and column operation, several parameters indicated the physical suitability of each of the silica samples as HPLC materials. Table III lists these physical properties. The particle size distribution, in particular, the presence of fines or submicron particles, was an undesirable characteristic, because a high number of fines could plug the outlet of the column, causing high pressure and diminishing its lifetime. Most of the fines are removed during synthesis, but this process is time-consuming and adds significantly to the cost of manufacturing.

TABLE II

RESOLUTION

Columns: 250 × 10 mm I.D.; mobile phase: 0.02 M Tris (pH 7); gradient: 120 min from 0 to 0.5 M sodium chloride; flow-rate: 1.5 ml/min. For column properties, see Table III.

Column No.	Ovalbumin				BSA			
	t_R	$w_{1/2}$	$t_R/w_{1/2}$	Number of peaks	t_R	$w_{1/2}$	$t_R/w_{1/2}$	Number of peaks
1	55	7.9	7	4	60	8.7	6.9	1
2	87.5	5.4	16.2	7	84	7	12	2
3	87	8.2	10.6	2	90.6	13.2	6.9	2
4	74	12	6.2	3	74	16	4.6	1
5	57.3	4	14.3	3	69	9	7.7	1
6	91.5	6	15.3	5	90	9.2	9.8	2
7	79.2	12	6.6	3	66	10	6.6	1
8	73.5	9	8.2	3	65	6.7	9.7	2
9	61	5	12.2	4	63	7.5	8.4	1
10	66.8	6.8	9.8	4	70	9	7.8	2
11	58.5	7.2	8.1	4	66	8.5	7.8	1

Stability, or retention of physical integrity, is obviously necessary in the synthetic procedures and column operation. Typically, at least 400 column volumes were passed through each column during this study. During analyses, recycling was accomplished by flushing with ten column volumes of the high-ionic-strength buffer and ten column volumes of the low-ionic-strength buffer at flow-rates of 6 ml/min. About midway during the battery of experiments and after they were finished, the inlets of the columns were examined for settling of the beds. If the beds were not flush with the top, more of the same silica was added to fill the column. Slight settling could have been caused by difficulties in the dry packing process. Because silica does dissolve slightly in water, it was remarkable that only one silica sample showed a
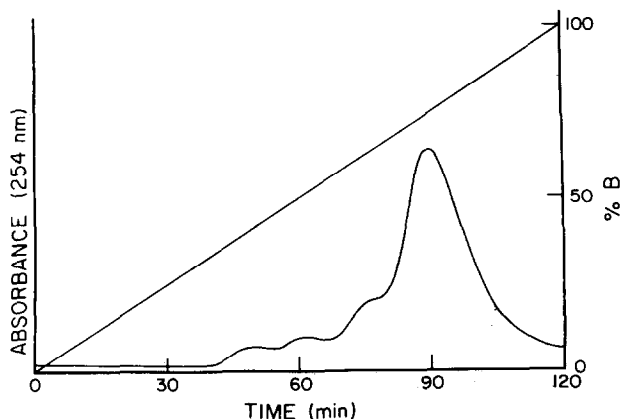

Fig. 3. Selectivity of AX Silica No. 1 for ovalbumin components. Conditions as in Fig. 1B.

TABLE III

PHYSICAL PROPERTIES OF PACKING MATERIALS

Column No.	Pore diameter ($\mathring{A}$)	Particle diameter (μm)	Stability	Fines*
1	175	37–74	+	0
2	300	15–20	+	+
3	200	30	–	+ +
4	150	> 74	+	+
5	200	37–74	+	+
6	300	30	+	+
7	150	35–70	+	+
8	270	40	+	+ +
9	270	40	+	+ + +
10	200	30	+	+
11	300	35–70	+	+

* The number of plus signs indicates the quantity (0 = none; + = few; + + = many; + + + = very many).

substantial loss in bed volume both times it was examined, and that the remainder of the column beds maintained their integrity. It was concluded, therefore, that the great loss in bed volume of silica No. 3 was due to the silica sample and not to the procedure for filling the columns. This problem was indicated with a " – " in the *Stability* column of Table III. The silica samples which had not settled are designated as " + " in Table III.

CONCLUSIONS

The goal of this research was to ascertain whether it was possible to prepare inexpensive preparative chromatography materials which would approximate analytical HPLC columns in their efficiency of protein purification and still possess high loading capacities. Our study has shown that several of the experimental materials had excellent loading characteristics and stability, in addition to yielding a resolution adequate for most protein purifications. Fig. 4 illustrates the ability of one of these
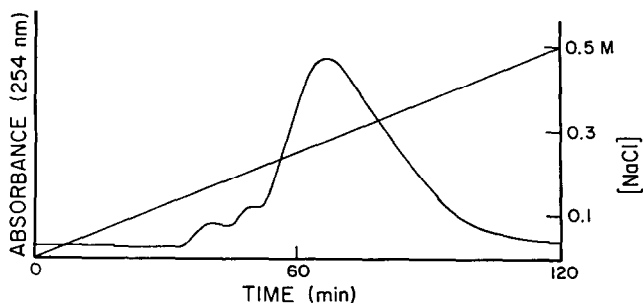

Fig. 4. Purification of 3 g of ovalbumin on a macroparticulate anion-exchange column. Column, Syn-Chroprep AX200, 250 × 21 mm I.D.; buffer and gradient as in Fig. 1B; flow-rate, 9 ml/min.

preparative supports to purify 3 g of ovalbumin with enough resolution to separate two impurities.

Since anion-exchange is one of the most popular means of large-scale protein purification, the commercial availability of this type of preparative HPLC material should soon provide a better means of producing large quantities of proteins from both biotechnological and natural sources.

ACKNOWLEDGEMENT

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