Journal of Chromatography, 368 (1986) 283–289
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 866

COMPARISON OF REVERSED-PHASE COLUMN MATERIALS FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

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(Received June 9th, 1986)

SUMMARY

Nine reversed-phase materials with various bonded phases from different suppliers were studied for the separation of hydrophilic proteins with two solvent systems. Protein retention, resolution and recovery were not correlated with the nature of the hydrocarbonaceous ligand. Peak volumes increased with molecular weight, which led to broad, irregular peaks for the larger proteins on some columns.

Four columns that performed equally well were selected for the purification of hydrophobic Sendai virus membrane proteins. In this case, more distinct differences were found between columns. Recovery of the membrane proteins strongly depended on the combination of column and solvent systems.

INTRODUCTION

In the past five years several high-performance liquid chromatography (HPLC) modes (ion-exchange, size-exclusion, reversed-phase, hydrophobic interaction) have become important for the purification of proteins[1]. However, adaptation of these techniques to purification schemes of integral membrane proteins proceeds slowly since the methodology to handle these proteins is still in its infancy. Membrane proteins show a marked tendency to aggregate, which is not surprising in view of their natural environment, which is a bilayer of lipid molecules.

In our studies for the development of HPLC methods for the purification of integral membrane proteins, Sendai virus membrane proteins are used as model proteins. Sendai virus, a paramyxovirus of mice, contains three proteins that are associated with the lipid bilayer membrane, the matrix protein M (M_r = 38 000), the hemagglutinin-neuraminidase protein HN (M_r = 67 000) and the fusion protein F. The latter consists of two subunits, F_1 (M_r = 50 000) and F_2 (M_r = 14 000), connected by disulphide bonds. These proteins can be selectively extracted from purified virions with Triton X-100 in the presence of 1 *M* salt[2]. Previous studies have shown that Sendai virus membrane proteins can be purified by reversed-phase HPLC (RP-HPLC)[3,4]. However, the mass recovery of these proteins is generally poor.

0021-9673/86/$03.50

During the past two years, a wide variety of new column packings for RP-HPLC has become available. The present study was understaken to compare the suitability of these columns for the separation of water-soluble proteins in general, and the application to the purification of integral membrane proteins in particular.

EXPERIMENTAL

Reagents and materials

Bovine pancreatic ribonuclease and bovine erythrocyte carbonic anhydrase were from Sigma (St. Louis, MO, U.S.A.), horse heart cytochrome *c* from Boehringer (Mannheim, F.R.G.) and ovalbumin from Millipore (Freehold, NJ, U.S.A.). Acetonitrile (HPLC/Spectro grade) was from Alltech (Deerfield, IL, U.S.A.), ethanol, *n*-butanol (both LiChrosolv) and trifluoroacetic acid (TFA) were from E. Merck (Darmstadt, F.R.G.). Prior to analysis, Sendai virus membrane proteins extracted with Triton X-100 in the presence of 1 *M* sodium chloride were treated with Amberlite XAD-2 to remove detergent and with dithiothreitol to reduce protein disulphide bonds for 20 min at room temperature as described previously[3]. Crude samples of Sendai virus M protein were obtained after selective precipitation of this protein from extracts by removal of the salt by dialysis[2].

Chromatography

Chromatography was performed with a system consisting of an LKB 2150 pump, a Rheodyne 7125 injector and a Waters 441 detector (214 nm). Low-pressure gradients were generated by an Acorn Atom microcomputer (Acorn Computers, Cambridge, U.K.), interfaced with an LFYX three-way solenoid valve (Lee, Westbrook, CT, U.S.A.)[5].

All columns were of equal size (50 × 4.6 mm I.D.), and were packed in the laboratory[6] with the materials listed in Table I, except for the phenyl column, which was from Toyo Soda (Tokyo, Japan).

Standard proteins and viral membrane proteins were eluted with linear gradients of: (i) 0.1 or 0.05% TFA in water–acetonitrile and (ii) 12 m*M* hydrochloric acid in water–ethanol–*n*-butanol (4:1, v/v). With both solvent systems gradients of

TABLE I

COLUMN MATERIALS

Column	*Ligand*	*Particle size (μm)*	*Pore size (nm)*	*Packing*
I	C_{18}	10	10	Nucleosil 10 C_{18} (Macherey-Nagel)
II	C_{18}	5	30	Nucleosil 300 C_{18} (Macherey-Nagel)
III	C_{18}	10	30	Vydac 218 TP (The Separations Group)
IV	C_{18}	5	33	Octadecyl (Baker)
V	C_8	5	30	Hypersil WP-300 octyl (Shandon)
VI	C_4	5	30	Hypersil WP-300 butyl (Shandon)
VII	C_1	10	25	TMS-250 (Toyo Soda)
VIII	Phenyl	10	100	Phenyl 5PW-RP (Toyo Soda)
IX	Cyano	5	33	Cyano (Baker)

2.5% organic solvent per min at a flow-rate of 1 ml/min were employed. Further details are given in the appropriate legends.

The relative recovery of standard proteins was determined from the areas of the eluted peaks measured with a data processor Chromatopac C-R3A (Shimadzu, Kyoto, Japan). Amounts of 10 μg of each protein were injected. For each protein the data obtained from all columns and both solvent systems were grouped and expressed as a percentage of the largest observed area for that protein.

Resolution was quantitatively measured by use of the peak capacity, *i.e.* the number of peaks with a resolution of unity that can be fitted in a chromatogram if the linear gradient is completed (*i.e.* carried out to 100% organic solvent concentration). The peak capacity for each individual protein was obtained by division of the extrapolated gradient time by the peak volume. The latter was calculated as 1.7 times the peak width at half-height in time units[6,7].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of viral proteins was performed as previously described[3].

RESULTS AND DISCUSSION

Nine RP-HPLC column materials (Table I), which differed with respect to bonded ligands, pore size and particle size, were compared. Eight materials were silica-based and one (VIII) was a hydrophilic resin-based support. All materials used in this study have been developed specifically for protein purification, with the exception of conventional packing I, which was included for comparison with previous work and for comparison with materials specifically intended for protein RP-HPLC.

First, columns were evaluated for their suitability for protein purification. Standard proteins were eluted with gradients of: (S1) 0.1% TFA in water–acetonitrile; (S2) 12 m*M* hydrochloric acid in water–ethanol–*n*-butanol (4:1). The latter system was required for the separation of Sendai virus proteins on the RP-HPLC column materials used in earlier studies[3]. The former system, S1, was found more suitable for the purification of these and other proteins on some newly developed materials[4,8].

Retention time

With solvent system S1, no large differences in protein retention were found on the first six bonded phases from Table I. The octyl packing (V) appeared to be the most hydrophobic support. Thus, a reduction in length of the silica-bonded alkyl chain from C_{18} to C_4 was not accompanied by a reduction of the acetonitrile concentration needed for elution of all five standard proteins. However, lower concentrations (5–12%) were needed on the phenyl (VIII), C_1 (VII) and cyano (IX) columns (arranged in order of decreasing protein retention).

With solvent system S2, the cyano packing (IX) also appeared to be the least hydrophobic support, whereas proteins had the largest retention volumes on the octyl column (V), closely followed by the phenyl column (VIII). On the other columns the organic solvent concentration at which the first protein (ribonuclease) eluted showed no great differences. Retention of the other proteins, although more variable, was not related to the bonded phase.

Proteins chromatographed with S2 eluted at organic solvent concentrations up to 12% lower than with S1 owing to the stronger organic modifier present in solvent system S2.

Peak capacity

The resolution or peak capacity, summarized in Table II, was generally lower with solvent system S2 than with the low-viscosity acetonitrile–TFA system S1 (Fig. 1) the strong increase in peak width of ribonuclease with S2 can, however, be partially attributed to a difference in the protein denaturation effect of the organic solvents[9].

TABLE II

PEAK CAPACITY (SOLVENT SYSTEM S1 AND S2, RESPECTIVELY)

Column	*RNase*	*Cyt.c*	*BSA*	*Carb.an.*	*Ovalb.*
I	95, 42	104, 60	61, 58	69, 64	65, 53
II	100, 53	102, 84	57, 58	88, 74	61, 53
III	67, 43	75, 60	47, 44	59, 47	61, 50
IV	96, 57	102, 77	xx, xx*	93, 66	85, 51
V	66, xx	51, xx	xx, xx	46, xx	xx, xx
VI	89, 47	97, 74	68, 54	82, 44	61, 36
VII	78, 43	85, 65	63, 45	80, 50	72, 46
VIII	83, 30	100, 88	85, 65	88, 54	82, 52
IX	31, xx	79, 42	60, xx	86, 42	75, xx

* Not quantified owing to poor peak shape.

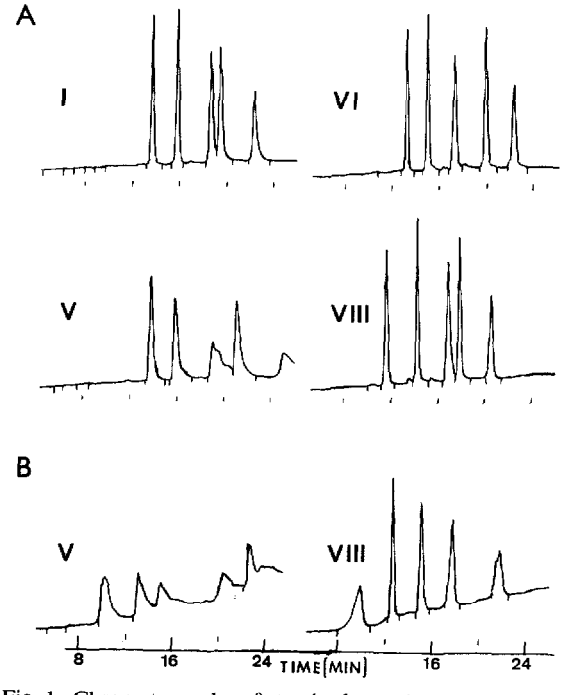

Fig. 1. Chromatography of standard proteins on columns I, V, VI and VIII (Table I). Proteins in order of elution: ribonuclease, cytochrome *c*, serum albumin, carbonic anhydrase and ovalbumin (10 μg each). Elution was performed with linear gradients of (A) 0–60% acetonitrile in 0.1% TFA (S1), or (B) 0–60% ethanol–*n*-butanol (4:1, v/v) in 12 m*M* hydrochloric acid (S2), in 24 min at 1 ml/min.

Generally, peak capacity was not related to the nature of the bonded ligand. However, comparison of the octyl (V) and butyl (VI) columns, which came from the same supplier and should only differ in the alkyl chain, showed that resolution decreased with increasing length of the ligand (Fig. 1A, V and VI).

On all columns and with both solvents, peak capacities tended to decrease with increasing molecular weight, as predicted by Stadalius *et al.*[10]. This effect differed for each column packing. On columns IV and V it resulted in broad, irregular, and tailing peaks for the larger proteins, serum albumin and ovalbumin. Therefore, these aspects were most noticeable for these proteins with solvent system S2, but on some columns, *e.g.* column V, very poor peak shapes were observed with S2 for all proteins (Fig. 1A and 1B, V).

For all proteins, the highest resolution was obtained with solvent system S1 on columns I, VI, VIII.

Recovery

For each protein only relative recoveries (Table III) were determined to study the effects of column type and solvent, although the absolute recoveries may vary for each protein. For one of the studied column materials (VIII), absolute recoveries of 88% and higher have been reported for the same set of proteins under conditions that differed only by a lower (0.05%) TFA concentration[8]. All columns except VII tended to have slightly higher recoveries with S1 than with S2. The combination of column VII with S2 was, in fact, among the better ones with respect to protein recovery.

Relatively low yields were obtained, or could not be determined at all owing to poor peak shape, with columns IV, V, and IX, especially for the larger proteins. No large differences between the yields on the other columns were observed for each protein, which means that the yields were independent of the characteristics of the supports concerned.

TABLE III

RELATIVE RECOVERY (SOLVENT SYSTEM S1 AND S2, RESPECTIVELY)

Relative recovery is expressed as percentage of the maximum area observed for the protein (average of three determinations) as described under Experimental.

Column	*RNase*	*Cyt.c.*	*BSA*	*Carb.an.*	*Ovalb.*
I	90, 95	90, 94	86, 87	93, 78	74, 72
II	96, 95	99, 96	95, 87	100, 85	78, 64
III	90, 94	95, 93	94, 90	90, 80	77, 83
IV	96, 94	98, 95	xx, xx*	89, 84	75, 78
V	94, xx	95, xx	xx, xx	80, xx	xx, xx
VI	93, 93	96, 89	91, 89	93, 84	87, 125**
VII	84, 100	87, 100	82, 99	84, 92	75, 100
VIII	94, 88	93, 89	100, 90	93, 85	78, 72
IX	65, xx	84, 87	94, xx	87, 80	64, xx

* Not quantified owing to poor peak shape.

** Too high value due to the presence of a solvent peak.

Membrane proteins

Columns I, VI, VII and VIII were selected for further evaluation with Sendai virus membrane proteins. Selection was based on criteria that included high recovery and resolution, especially with regard to the larger proteins, and diversity of the bonded phase.

Large differences were observed when the extracted Sendai virus membrane proteins HN, F1, and F2 were separated on these columns. Elution was performed with a slightly modified solvent system S1 (0.05% TFA instead of 0.1%), which resulted in higher recoveries for the F1 protein on the phenyl column (VIII) although it made no difference in the recovery of the other proteins. Except for the F2 protein, which eluted as a sharp peak with a high recovery on all four columns, the elution of the other proteins depended strongly on column type, as was confirmed by SDS-PAGE. The recoveries of F1 and HN on columns I and VI were very poor, but much higher yields (60–80%) were obtained with columns VII and VIII.

Fig. 2 shows the separation of crude samples of Sendai virus M protein with both solvent systems. The elution position of this protein, indicated with an asterisk,

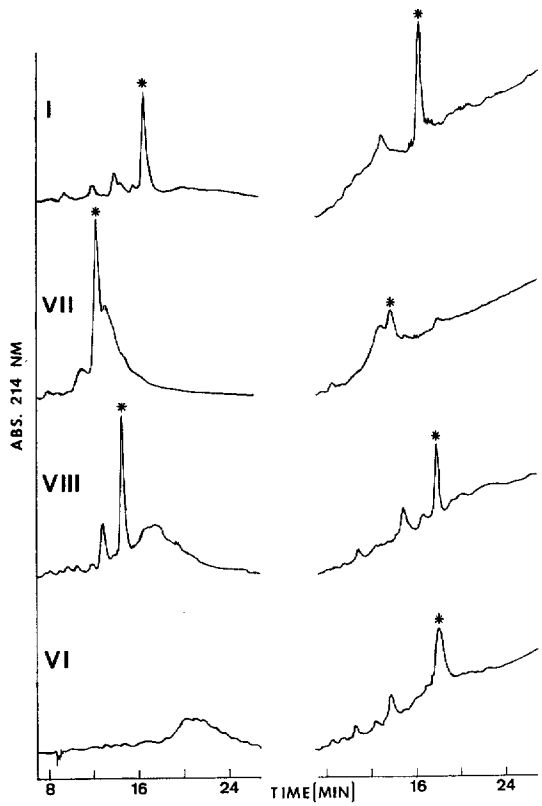

Fig. 2. Chromatography of crude samples of Sendai virus M protein on columns I, VII, VIII, and VI (Table I). Elution was performed with linear gradients of 15–75% acetonitrile in 0.05% TFA (S1, left), and with 10–70% ethanol–*n*-butanol (4:1, v/v) in 12 m*M* hydrochloric acid (S2, right), in 24 min at 1 ml/min. The elution position of the M protein as determined with SDS–PAGE is marked with an asterisk.

was confirmed by SDS-PAGE. The presence of some broad peaks in the chromatograms was caused by residual detergent and solvent impurities. The M protein was recovered only in trace amounts from column VI with solvent system S1, whereas a 35% yield was obtained when it was eluted with S2. This solvent effect on the recovery of the M protein was the reverse of that with column VII. In contrast to the results with the standard proteins, the recovery of the M protein in this case was much higher with solvent system S1 than with S2 (40–65% and 15–25%, respectively). Such a strongly selective solvent effect was not observed for columns I and VIII; more similar yields on these columns were obtained with boith solvent systems (column I 35% and 40%, column VIII 40% and 25%, for S1 and S2, respectively).

CONCLUSIONS

The first part of this study shows that the TFA–acetonitrile system is to be preferred for the elution of simple water-soluble proteins since it resulted in a higher resolution. This system did not result in higher recoveries, however, and a drawback might be the relatively high organic solvent concentrations needed for elution, with increased denaturating effects[9].

No correlation was observed between resolution and the specific type of the hydrocarbonaceous ligand, although some of the packings showed a poor shape for the larger proteins. Resolution therefore seems to be more dependent on other chemical characteristics of the packing material.

Protein recoveries were relatively independent of the bonded ligand as well. Interestingly, the results obtained with one of the C_{18} packings (II), which is marketed specially for protein purification, were almost the same as the results with a narrow-pore-size conventional C_{18} support (I) from the same supplier.

This study shows that there is no simple correlation between performance of the packings with the water-soluble proteins and with the membrane proteins. Two out of four packings with relatively high recoveries for the hydrophilic proteins had a low performance with the membrane proteins, but selection of the solvent may drastically influence the outcome of the separation.

In conclusion, the proper solvent depends on both the column packing and the protein to be purified.

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