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REVERSED-PHASE SUPPORTS FOR THE RESOLUTION OF LARGE DENATURED PROTEIN FRAGMENTS*

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SUMMARY

Large pore ($\geqslant$ 300 Å), spherical partical silica stationary phases possessing either C_{18} or C_8 hydrocarbon ligands out-performed small pore (60–100 Å), irregular shaped silicas for the purification of large denatured peptides. Since columns 5 cm in length appeared to be as effective in separating peptides as columns 5 times longer, it is likely that large peptides absorb to the matrix rather than partition between the stationary and mobile phases.

INTRODUCTION

Several chromatographic techniques for separating peptide mixtures are severely limited in terms of the size of the peptides that can be successfully chromatographed. Large denatured fragments of proteins are usually highly insoluble in aqueous solutions, especially near neutral pH, and solubilizing agents (such as organic acids or alcohols, urea or guanidine) or strong bases are often necessary to effect solution of the peptides. Ion-exchange chromatography of such fragments is usually unwieldy, requiring high concentrations of urea. Gel filtration in high concentrations of organic acids or in fairly strong bases (*e.g.*, 0.1 *M* NH_3) is practical, but another separation technique based on something other than molecular size is highly desirable as a complement to gel filtration.

Application of reversed-phase high-pressure liquid chromatography (RP-HPLC) for the isolation and purification of peptides has been demonstrated. Reports have detailed the use of RP-HPLC for the purification of both synthetic[1,2] and naturally occurring[3] peptides, as an analytical tool to follow the progress of protein digestion[4–7], as a procedure to detect protein variants via peptide mapping[8–11] and as an indirect method of determining gene activity during cellular differentiation[12]. Until recently, RP-HPLC, like chromatography on classical supports, suffered from a lack of solvents suitable for dissolving and chromatographing large denatured peptides. Consequently, most studies on the materials and conditions suitable for peptide

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separations by RP-HPLC have focused on small peptides or on native water-soluble proteins[3]. With the introduction of mobile phases capable of dissolving large denatured peptides[13], reversed-phase supports could be developed to optimize both resolution and yield. We have used peptides with 13 to 78 amino acid residues, prepared by cleaving fetal globin (predominantly HbF) with cyanogen bromide to examine the effects of several support variables.

MATERIALS

LiChrosorb and LiChrospher silicas were purchased from E. Merck (Darmstadt, G.F.R.). A LiChrosorb C_8 (5 μm) column (0.46 × 25 cm) was obtained from Brownlee Labs. (Santa Clara, CA, U.S.A.). Silica 2362 was obtained from SynChrom (Linden, IN, U.S.A.)*. Zorbax CN and C_{18} (5 μm) columns (0.46 × 25 cm) were obtained from DuPont (Wilmington, DE, U.S.A.). Trichlorosilanes were purchased from Petrarch Systems (Levittown, PA, U.S.A.). Trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL, U.S.A.) and purified by refluxing for several hours with CrO_3 and then distilled through a 30-cm Snyder column; only the constant-boiling fraction (b.p. 71–72°C) was collected. 1-Propanol and 2-propanol were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

EXPERIMENTAL

Support preparation

Trichlorosilanes were bonded to silica via siloxane formation, then encapped with trimethylchlorosilane using the methanolysis technique of Evans *et al.*[14].

Column packing

Silicas of pore diameter 50, 100 and 300 Å were slurry-packed into columns of the indicated length in 2-propanol at 7800 p.s.i. by means of a pneumatic pump (Haskell, Burbank, CA, U.S.A.); silicas of larger pore diameters (500 and 1000 Å) were packed at 2500 p.s.i.

Globin preparation

The globin from several approximately 20-week-old human fetuses (crown–rump length 181–190 mm) was prepared by acid-acetone precipitation[15]. After reduction and S-pyridylethylation[16], the lyophilized protein was dissolved in 70% formic acid and cleaved at methionyl residues with cyanogen bromide[17]. After recovery of the resulting peptides by lyophilization, the mixture was dissolved in 0.013 *M* TFA in water[13] to a concentration of 2 mg per ml.

Designation of fragments

The cyanogen bromide fragments were numbered consecutively from those containing the amino-terminal sequence to those containing the carboxyl-terminal sequence of the α- and γ-chains. Three fragments of each chain were produced ranging in size from 13 residues to 78 residues. Thus, the three α-chain fragments were

* C_8-coated silica 2362 is now marketed by SynChrom in columns or in bulk as stationary phase RP-P.

designated αCB-1 (residues 1–32), αCB-2 (residues 33–76) and αCB-3 (residues 77–141). The three γ-chain fragments were designated γCB-1 (residues 1–55), γCB-2 (residues 56–133) and γCB-3 (residues 134–146).

High-performance liquid chromatography

Analyses by HPLC were carried out by using a Varian 5000 instrument equipped as previously described[13]. Peptide elution was accomplished by the application of a binary gradient in which the primary mobile phase was 0.013 *M* (0.1%, v/v) TFA in water and the secondary phase was either 1- or 2-propanol[13].

RESULTS AND DISCUSSION

The cyanogen bromide fragments of fetal globin were separated by gel filtration in order to demonstrate the relative composition and size distribution of this peptide mixture (Fig. 1). An initial study on the effects of various RP-HPLC supports on the resolution and recoveries of the peptides was carried out with commercial columns. Significant differences in the peptide-elution profiles were observed when identical

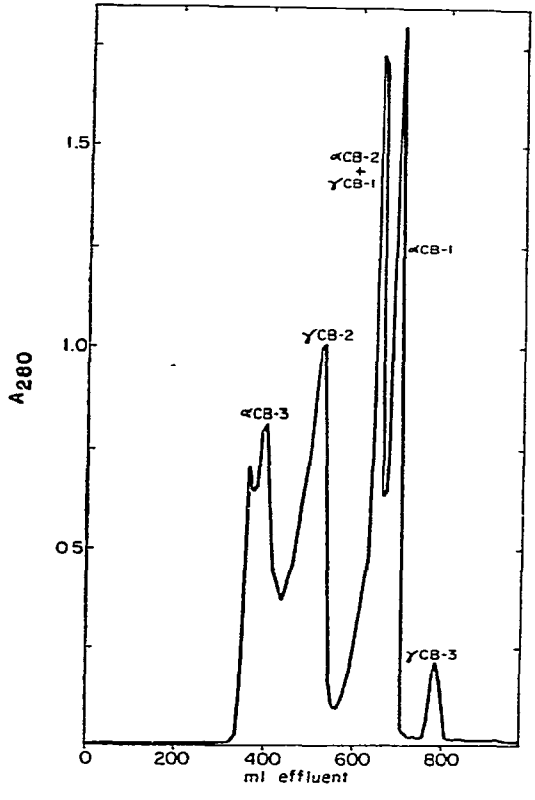

Fig. 1. Elution profile of cyanogen bromide peptides of human fetal globin (200 mg) on a column (2.0 × 195 cm) of Sephadex G-50 (fine) equilibrated and developed with 1% formic acid: flow-rate 20 ml/h.

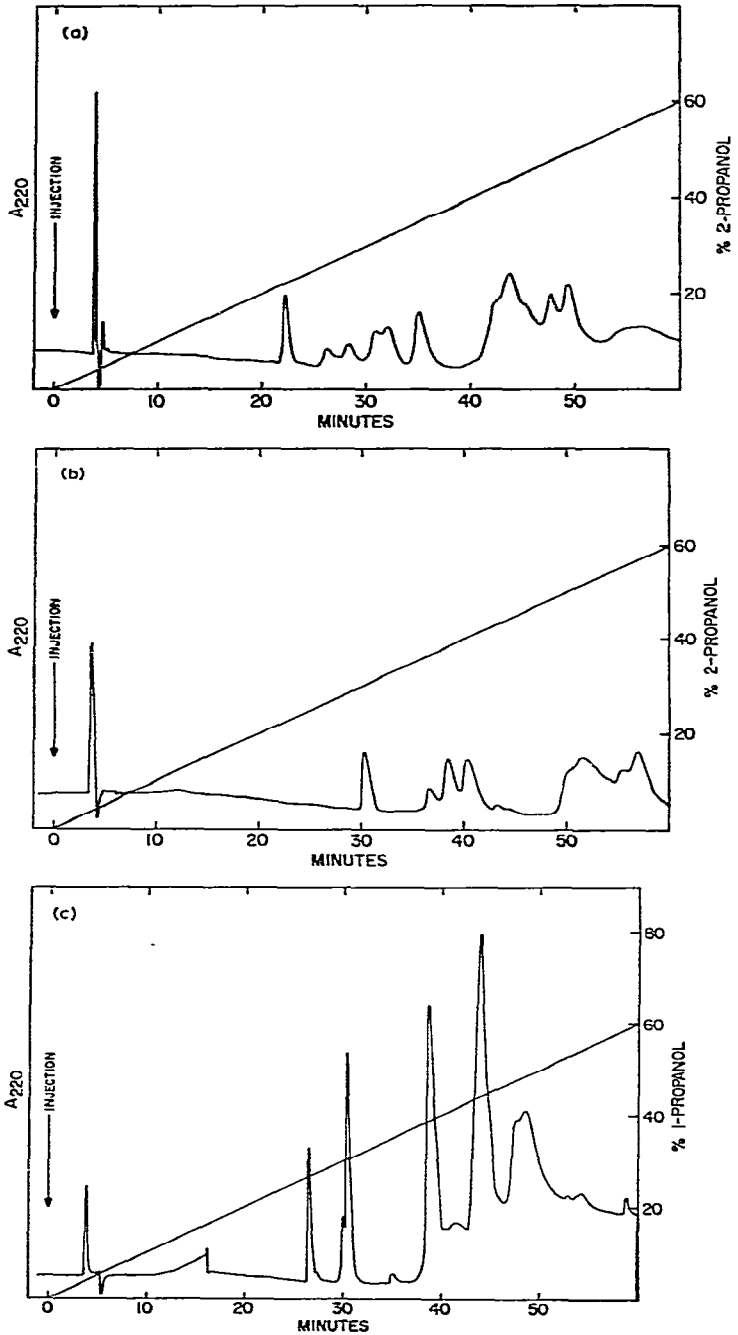

Fig. 2. Separation of cyanogen bromide fragments of fetal globin by HPLC. (a) The globin fragments (200 μg) were applied to a Zorbax CN column (0.46 × 25 cm) and eluted with a 60-min linear gradient from 0 to 60% of 2-propanol in 0.1% (v/v) TFA: temperature, ambient; flow-rate 0.7 ml/min. (b) As in (a), but with a Zorbax C_{18} column. (c) As in (a), but with 1-propanol as eluent and a Brownlee C_8 column. The major peaks correspond to the peptides identified in Fig. 3.

separation conditions were used on the various columns (Fig. 2). Both sample recovery and resolution were poor on Zorbax CN and C_{18} columns, and no attempt was made to identify components in the resulting chromatograms. Peptide resolution and yield were better with columns of LiChrosorb C_8 (Fig. 2c), although in all instances a progressive drop in the recoveries of peptides was correlated with increasing hydrophobic character of the peptides as judged by increases in retention times and by amino acid compositions[13].

It has been suggested that the structure of the hydrocarbon ligand in the support phase[18], the method in which the bonded phase is applied to the support[19] and the presence of residual silanol groups all contribute to retention in RP-HPLC[20]. The proprietary nature of commercial supports precluded identification of those variables in the column packing material that contribute to performance. To examine further support variables for the resolution of protein fragments, several different support materials were prepared (Table I). The adamantylethylsilane (AES) bonded phase was used to study the effect of matrix particle geometry on both column selectivity and solute recovery. Variation in individual components and in the contributions of bonded phase, pore diameter and silica type to the relative recoveries of the peptides were examined.

TABLE I

TEST COLUMNS

*Column No.**	*Silica*	*Pore diameter (Å)*	*Particle diameter (μm)*	*Coating*	*Integrated area of peaks under the specified regions*			*Total area (mm²)*
					A (mm²)	*B (mm²)*	*C (mm²)*	
1	Partisil	55	10	AES	715	922	3798	5435
2	LiChrosorb**	100	10	AES	1048	925	1772	3745
3	LiChrospher	100	10	AES	765	805	3857	5427
4	Silica 2362	300	10	AES	988	914	3887	5789
5	LiChrospher	500	10	AES	905	1066	4264	6235
6	LiChrospher	1000	10	AES	936	1091	4425	6452
7	Silica 2362	300	5	C_{18}	1108	1687	5484	8279
8	Silica 2362	300	5	C_8	1179	1532	5080	7791
9	Brownlee C_8**,***	100	5	C_8	1189	1214	3283	5686

* Columns 1–8 were packed in tubes 0.41 × 5 cm.

** Irregular shaped silica.

*** Commercially bonded C_8 packed in a column 0.46 × 25 cm.

The results in Table I were generated by chromatographing the denatured globin fragments under identical instrument and elution conditions. As complete resolution of all components was not achieved, and elution of these peptides was in the order of increasing hydrophobic character, the chromatograms were divided into three regions and the total area for each region was reported. Region A contained the small, less hydrophobic peptides; region B contained peptides of intermediate hydrophobicity; and region C had the most hydrophobic peptides. When the relative recoveries of peptides from Silica 2362 with AES, C_8 and C_{18} bonded phases were examined (Table I), C_8 and C_{18} supports did not differ significantly, but AES was

slightly inferior. The chromatographic resolution achieved using the C_8 support (Fig. 3a) appeared to be slightly better than C_{18} (Fig. 3b) in this application. This was most apparent with the higher-weight peptides eluted from 35 to 45 min into the gradient; again, the AES support was slightly inferior.

When the effect of pore diameter was examined, it was observed that supports of smaller pore diameter (55–100 Å) gave lower relative recoveries of peptides. With

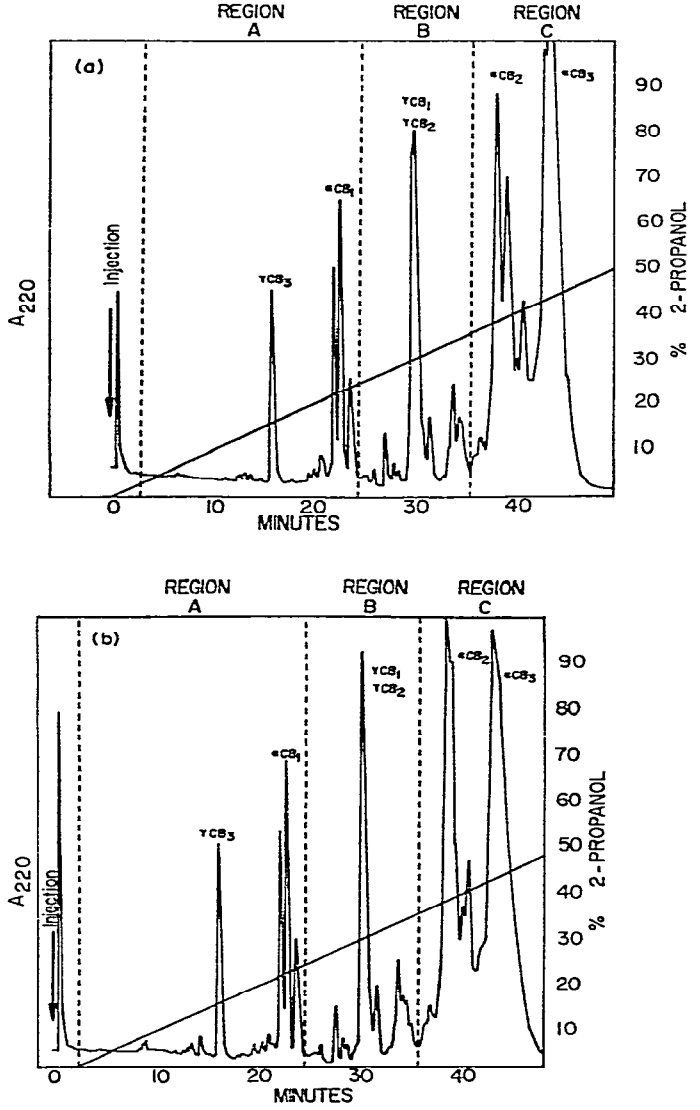

Fig. 3. Elution profile of cyanogen bromide fragments of fetal globin as described in Fig. 2. (a) Chromatogram produced by using a column (0.41 × 5 cm) of Silica 2362 coated with C_8. (b) As in (a), but with a coating of C_{18}. Regions identified as A, B, and C, which correspond to aeras of increasing hydrophobicity (see Table I), were chosen arbitrarily for purposes of peptide quantitation.

the AES coating, relative recovery of total peptides from the LiChrospher support of pore diameter 1000 Å was higher by a factor of 1.7 than that from the 100-Å LiChrosorb. This same trend was apparent when a Brownlee C_8 column (100 Å) was compared with a Silica 2362 (300 Å) column having a C_8 coating. The silica of larger pore diameter produced a 1.5-fold greater recovery of all peptides. It should be noted here that the Brownlee C_8 column was packed with Merck LiChrosorb RP-8, which is widely used for peptide separations.

The influence of pore size, although important in peptide recovery and resolution, may not totally account for all the differences observed (Table I). LiChrosorb (100 Å) with either the AES coating or the C_8 coating (in the Brownlee column) gave high recoveries of the low-molecular-weight (region A) peptides and low recoveries of the higher-molecular-weight (regions B and C) peptides. On the other hand, a silica of different geometrical shape, LiChrospher (100 Å), with the AES coating showed a higher recovery of region-C peptides.

An additional observation made during this study was that columns 5 cm in length used to evaluate supports were as efficient as 25-cm commercial columns in gradient-elution separations (Figs. 2 and 3); short columns had the additional advantage of being operable at lower back pressures. However, the effect of column length on loading capacity and resolution was not quantitatively evaluated.

In general, supports of larger pore diameter (>100 Å) and *n*-alkyl-bonded phases produce superior resolution and sample recoveries. The nature of the silica surface is also correlated with column performance. Finally, we have found that an RP-HPLC column of 0.41 × 5 cm is sufficiently long to permit resolution of a wide range of protein fragments.

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