Journal of Chromatography, 237 (1982) 407–416
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,478

COMPARISON OF THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PEPTIDES AND PROTEINS ON 100- AND 300-Å REVERSED-PHASE SUPPORTS

KENNETH J. WILSON*, ERIKA VAN WIERINGEN, STEFAN KLAUSER and MARTIN W. BERCHTOLD*

Biochemisches Institut der Universität Zürich, CH-8028 Zürich (Switzerland)

and

GRAHAM J. HUGHES

Laboratorium für Biochemie I der Eidgenössischen Technischen Hochschule, CH-8092 Zürich (Switzerland)

(First received July 21st, 1981; revised manuscript received October 17th, 1981)

SUMMARY

The chromatographic separations of peptides and proteins on commercially available 100- and 300-Å pore size reversed-phase columns have been compared using various buffer systems. The larger-pore-size packing exhibits a slightly more hydrophilic character while maintaining flow and back-pressure characteristics typical of 10-μm supports. In addition to equal or improved resolving capabilities for smaller amino acid derivatives and peptides, this column material is notably superior to the 100-Å supports for the chromatography of proteins with molecular weights exceeding *ca.* 15,000.

INTRODUCTION

Applications of the method referred to as high-performance liquid chromatography (HPLC) are being found in such widely diversified areas as forensic medicine and clinical, as well as analytical, chemistry. Recently, its uses as an analytical or preparative method in the field of biochemistry have become more obvious. Amino acid analysis of either the free acids[1–3] or derivatives[3–5] thereof, as well as the identification of amino acid derivatives from Edman degradations[6,7], have been reported. Peptides from natural sources[8–11] and those from enzymatic digestions[11–15] or from chemical fragmentations[15–18] can be isolated and efficiently desalted[19].

There have been fewer reports describing the chromatography of large peptide fragments[12,16,17–20], proteins[21–23] or enzymes[24–27]. The main reason for this limited use has been the lack of a suitable commercially available reversed-phase packing which does not experience non-specific adsorption, and thereby low recoveries and/or

* Permanent address: Institut für Pharmakologie und Biochemie, Tierspital, CH-8057, Zürich, Switzerland.

reduced resolution. Some researchers have circumvented this problem by synthesizing their own packings, *e.g.* C_8, diphenyl and cyanopropyl groups have been attached to 330- and 500-Å pore size (10 μm) materials[26] or C_8, C_{18} and adamantyl groups on a series of 5- and 10-μm packings ranging in pore sizes from 55 to 1000 Å[20]. Similarly, a commercially available 300-Å pore size C_{18} column has also been utilized[16]. In each of the above-mentioned cases the chromatographic results have suggested that protein recovery increases with increasing pore size.

In this report we compare the applicability of the 100- and 300-Å packings available from Brownlee Labs.; the results indicate that the larger-pore-sized material offers superior resolution and recovery of not only peptides and proteins but also Dns-amino acids[4].

EXPERIMENTAL

Apparatus

An HPLC unit consisting of two Altex Model 100 pumps, a Rheodyne Model 7125 injector (600-μl sample loop), a Kontron Model 200 microprocessor and an Unvikon 725 spectrophotometer equipped with an 8-μl flow-through cell was used for UV detection. Samples were chromatographed on 250 × 4.6 mm 10-μm particle size columns of LiChrosorb RP-18 (100 Å pore size) or Aquapore RP 300 (300 Å pore size) from Brownlee Labs. (Santa Clara, CA, U.S.A.). When necessary the column effluent was collected automatically using an LKB Ultrarac Model 7000 fraction collector. Portions were removed (10–15%), diluted into a scintillator and radioactivity measurements performed using a Packard Model PLD Tri-Carb liquid scintillation counter. No quenching of radioactivity by the buffers was observed under the experimental conditions used.

Chemicals

Acetonitrile and 2-propanol (HPLC quality) were purchased from J. T. Baker (Gross Gerau, G.F.R.), water was quartz bi-distilled. All other chemicals were of reagent-grade quality from Fluka (Buchs, Switzerland) or E. Merck (Darmstadt, G.F.R.) and used without further purification. Obtained from Sigma (St. Louis, MO, U.S.A.) were cytochrome *c* (horse heart), carbonic anhydrase (bovine erythrocytes) serum albumin (chicken) and myoglobin (whale) which was further converted into the apo-form by extraction with methyl ethyl ketone[28]. Alcohol dehydrogenase (yeast), maleic dehydrogenase (pig heart mitochondria), creatine kinase (rabbit muscle) and 3-phosphoglycerate kinase (yeast) were from Boehringer (Mannheim, G.F.R.).

Ovalbumin (egg white) was purchased from Worthington (Freehold, NY, U.S.A.). Parvalbumin (rat muscle) was prepared as described for the isolation of the same protein from chicken muscle[29]. Human serum albumin was isolated and further purified as described[30]. The bovine pancreatic trypsin inhibitor (Trasylol®; Bayer, Leverkusen, G.F.R.) and lysozyme (chicken, Sigma) were used as their carboxymethylated[31] derivatives.

Methods

Prior to reversed-phase chromatography the proteins were labelled by reduc-

tive methylation[32] using sodium cyanoborohydride and [^{14}C]formaldehyde (Radiochemical Centre, Amersham, Great Britain), followed by desalting over Sephadex G-25 and lyophilization. Each sample was then dissolved in water, or in some cases 1.0% phosphoric acid, diluted into the starting buffer for chromatography and injected at concentrations between 5 and 600 μg/μl. Unless otherwise stated, the amounts of protein applied onto the column ranged between 20 and 150 μg. Following effluent collection the percent recovery was determined by liquid scintillation.

Peptides of rat muscle parvalbumin were prepared as described[33] by tryptic digestion (TPCK–trypsin, Worthington) and recovered by lyophilization. Chromatography was carried out on 30–60-μg amounts of the peptide material.

The buffer systems used for chromatography were (I) buffer A, 0.01 *M* sodium perchlorate in 0.1% phosphoric acid, pH 2.1, and B, as A except 60% (v/v) in acetonitrile; or (II) as in (I) except 60% (v/v) in 2-propanol. All chromatography was carried out using a linear gradient of B buffer (2.22% per min) at room temperature (*ca.* 22°C) with a flow rate of 1.0 ml/min. For both the 100- and 300-Å packings the column back pressures increased during gradient development from 500 to 800 p.s.i. with buffer system I and from 725 to 2200 p.s.i. with system II. For a higher pH phosphate system, A buffer of system I (see above) was titrated with 2 *M* sodium hydroxide to pH 7.0. Similarly, the aqueous part of B buffer (containing the appropriate amounts of phosphoric acid and sodium perchlorate to yield the correct concentrations upon final dilution with the organic phase) was adjusted to pH 7.0 prior to addition of 60% (v/v) acetonitrile. The Tris buffer system used consisted of 50 m*M* Tris-HCl, pH 7.5, as A buffer and the same containing 60% (v/v) acetonitrile as B buffer.

RESULTS AND DISCUSSION

The chromatography of peptides and smaller protein fragments by reversed-phase HPLC has been carried out predominantly on either C_8 or C_{18} hydrocarbon ligands bonded to 5- or 10-μm particle size packings with nominal pore sizes of <100 Å. Fig. 1 illustrates that the more hydrophobic character of a C_{18} hydrocarbon ligand on such packings (Fig. 1B) results in a higher resolution of the chromatographed peptides, ranging in length between 3 and 15 residues[33], than the cyanopropylmoeity (Fig. 1A). The yields from the peptide separation on the C_{18} support (Fig. 1B) averaged 66.9%. That the amino acid compositions of the peptides are in excellent agreement with their known sequences[33,34] supports minimal cross-contamination between closely eluting peaks.

Chromatography of the same peptide mixture on 100- and 300-Å supports at low pH, conditions identical to those in Fig. 1, are illustrated in Fig. 2. The more hydrophilic nature of the 300-Å support is indicated by the quicker elution of the peptides. Peak resolution is not identical on both supports since the two peptides indicated in Fig. 2A (by arrows) were not found to separate on the 300-Å column (Fig. 2B). The similar peak areas from both columns indicate that the 300-Å support can be conveniently used for peptide mapping with yields approximating those from the 100-Å packing (see above).

When the pH of the chromatographic buffers were increased, the peptides generally became more hydrophilic owing to deprotonation and were thus eluted

TABLE I

EFFECT OF MOBILE PHASE pH ON PEPTIDE RETENTION

*Peak number**	*Peptide sequences***	*Retention times (min)*		*Peptide charge****		*Peptide hydrophobicities†*		
		pH 2.2 (Fig. 1B)	*pH 7.0 (Fig. 3A)*	*pH 1.9*	*pH 6.5*	*Wilson et al.[††]*	*Meek and Rossetti[†††], pH 2.1*	*Meek[†], pH 7.4*
1	ETK	5.1	2.0	+2	0	− 5.8	− 2.9	−14.1
2	SADDVKK	12.2	2.5	+3	0	− 5.0	− 3.2	−11.8
3	DLSAK	13.8	5.8	+2	0	+ 0.7	+ 5.4	+ 2.4
4	GFSSDAR	17.1	8.3	+2	0	+ 4.7	+ 5.9	+ 8.7
5	TLMAAGDKDGDGK	18.7	9.2	+3	−1	− 1.5	+ 7.7	− 7.1
6	VFHILDKDK	26.0	20.8	+4	+0.5	+13.0	+31.1	+18.9
7	AIGAFTAADSFDHKK	27.3	17.9	+4	+0.5	+ 5.2	+26.9	+26.5
8	Ac-SMTDLLSAEDIKK	29.0	16.2	+2	−2	−	+29.2	+14.4
9	IGVEEFSTLVAES	29.7	17.2	+1	−3	+27.7	+39.6	− 3.8
10	FFQMVGLK	30.5	22.9	+2	+1	+27.4	+44.1	+38.0
11	SGFIEEDELGSILK	31.1	16.2	+2	−3	+25.5	+44.2	+ 2.2

* Refers to those numbered peaks in Figs. 1B and 3A which were identified by amino acid analysis.

** From ref. 33.

*** Calculated according to Offord[35] and assuming histidine to have a charge of +0.5 at pH 6.5.

† Calculated by summation of the hydrophobic values for each amino acid side chain within the peptide; lists of amino acid hydrophobicities are given in the indicated refs.

†† Ref. 36.

††† Ref. 37.

† Ref. 38.

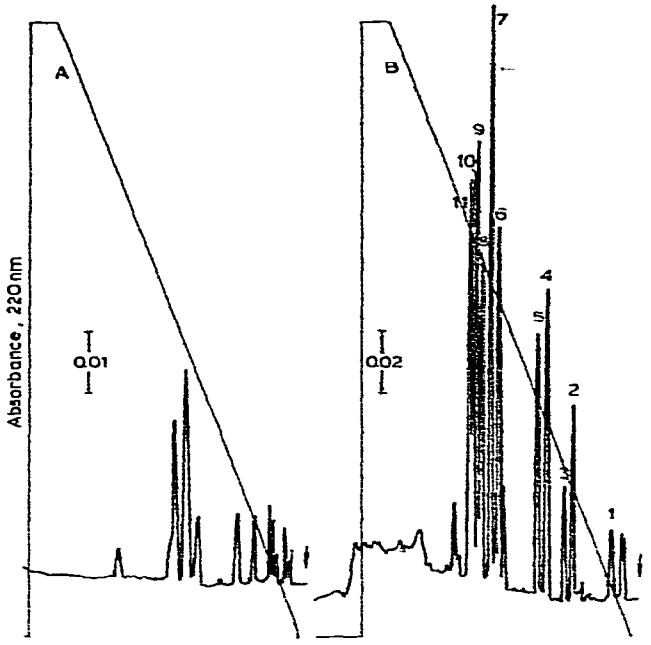

Fig. 1. Chromatography of the tryptic peptides from rat muscle parvalbumin on (A) 70-Å (6 μm) cyanopropyl- and (B) 100-Å (10 μm) C_{18} packings. The peptides were dissolved in 100 μl of A buffer [2.7 nmol and 5.0 nmol for (A) and (B), respectively] and chromatographed with an acetonitrile gradient (buffer system I at pH 2.1, see Experimental). The arrows indicate the points of injection. The peaks in (B) are numbered so that comparisons can be made with Fig. 3A; see Table I.

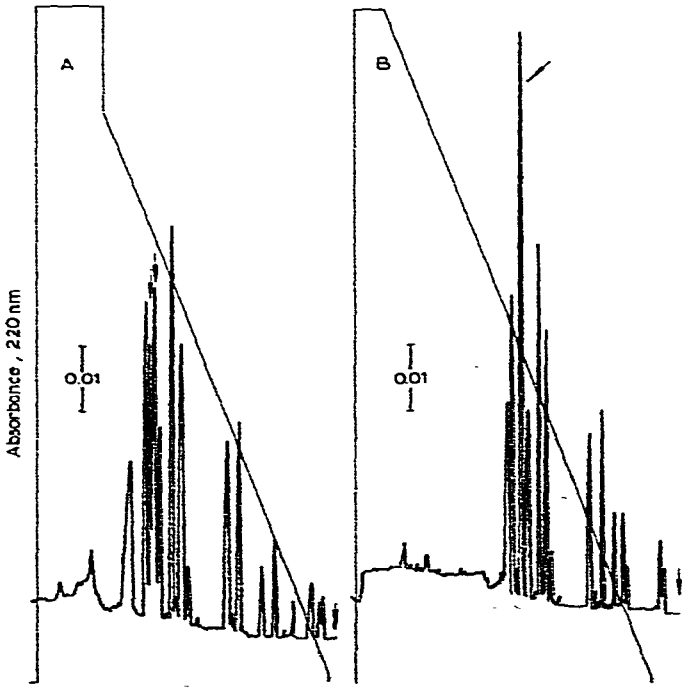

Fig. 2. Comparison of the resolution for peptides achieved at pH 2.1 by 100- (A) and 300-Å (B) supports. The peptide mixtures (2.5 nmol) were chromatographed as above using buffer system I.

earlier, *i.e.* at lower buffer *B* concentrations (*cf.* Figs. 2B and 3A). Such pH changes do not effect all peptides equally, *e.g.* resulting simply in an elution at lower acetonitrile concentrations while maintaining the same elution order. Some peptides were found to be greatly effected while others not at all. Table I gives the sequences of the peptides and their chromatographic as well as charge characteristics at both pH 2.2 and 7.0. At the lower pH the peptides do elute as a function of their increasing apolarity when the newer values from Meek and Rossetti[37] for amino acid hydrophobicities are used to calculate peptide hydrophobicity. This would be expected since the buffer systems used in this study and that of Meek and Rossetti[37] are essentially the same. However, when the amino acid hydrophobicity values determined by us in a different buffer system[36] (*e.g.* pyridine–formate–acetate using 1-propanol as the organic eluant) are used the estimation of peptide elution order is less satisfactory. Using the values from a previous investigation by Meek[38], calculated peptide hydrophobicities for pH 7.4 digress considerably from the observed elution order seen in Fig. 3A. Conceivably this might be due to one (or more) of the amino acid hydrophobicity values being incorrectly assessed. The differences in peptide chromatographic behaviour are sufficient enough, however, to utilize simply a change in the pH as a further isolation step or as a check for purity. Utilizing these columns at pH 7.0 to 7.5 for several weeks has not resulted in any noticeable chromatographic changes in either resolution or yield. Similarly, switching from one buffer system to another with a different pH is conveniently performed without effecting column performance.

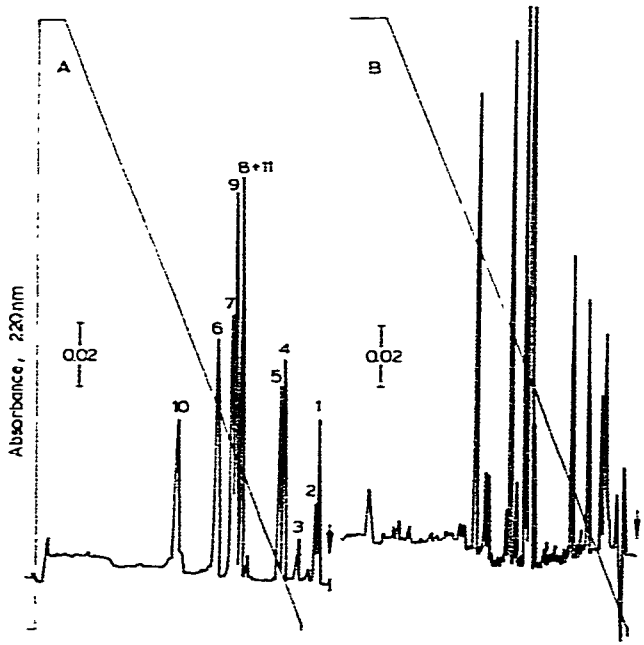

Fig. 3. Comparison of the resolution for peptides achieved at neutral pH using 100-(A) and 300-Å (B) supports. The buffer systems were for (A) as in Figs. 1 and 2 except that the pH of the buffer was titrated to 7.0 and for (B) a 50 m*M* Tris–HCl, pH 7.5, 60% (v/v) acetonitrile system (see Experimental). The peptide amounts injected were for 5.0 nmol for both (A) and (B). The peaks in (A) are numbered so that comparisons can be made with Fig. 1B; see Table I.

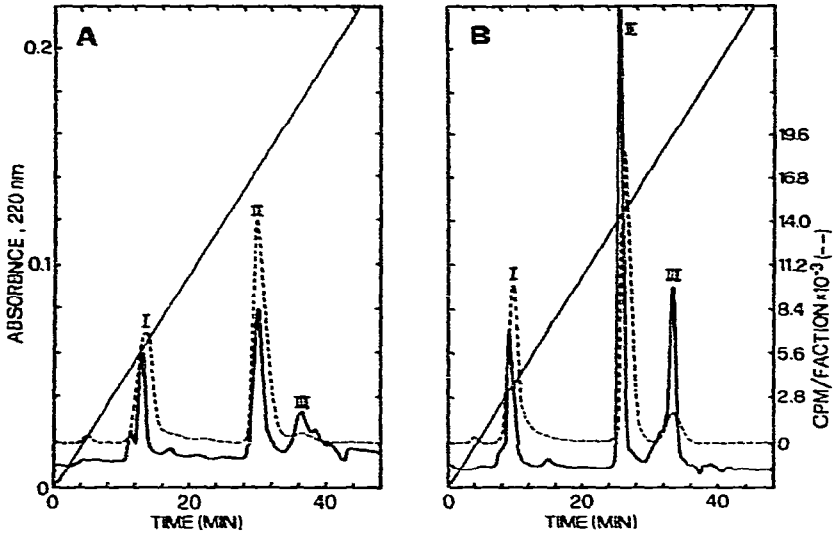

Fig. 4. HPLC of [^{14}C]methylated proteins on 100-(A) and 300-Å (B) packings. The samples injected were: I = bovine pancreatic trypsin inhibitor (57 μg, $1.6 \cdot 10^5$ cpm); II = myoglobin (48 μg, $2.2 \cdot 10^5$ cpm); III = ovalbumin (76 μg, $5.3 \cdot 10^4$ cpm). Elution was carried out with a 2-propanol gradient at low pH (buffer system II, see Experimental) and 1.4-ml fractions were collected. Solid lines represent absorbance at 220 nm and dashed the ^{14}C-label incorporated into the proteins by reductive methylation (see Experimental).

The chromatography of a protein mixture on such supports indicates that resolution as well as yield are a function of both the packing pore size and protein molecular weight. On the 300-Å column (Fig. 4A) peak broadening is significantly

TABLE II

COMPARISON OF PROTEIN REVERSED-PHASE CHROMATOGRAPHY ON 100- AND 300-Å (10 μm) PACKINGS

Elution carried out using buffer system II: A buffer, 10 m*M* $NaClO_4$ in 0.1 % H_3PO_4; B buffer, as A except 60 % (v/v) in 2-propanol.

Protein	*Molecular weight (ref.)*	*A, LiChrosorb RP-18, 100 Å*		*B, Aquapore RP 300, 300 Å*		*Differences (column A minus column B)*	
		B (%)	*Yield (%)*	*B (%)*	*Yield (%)*	*B (%)*	*Yield (%)*
Bovine pancreatic trypsin inhibitor	6500 (39)	29.0	81.1	20.5	59.8	+ 8.5	+21.3
Cytochrome *c*	11,700 (39)	58.5	48.0	56.7	45.6	+ 1.8	+ 2.4
Parvalbumin	11,700 (33)	74.0	102.9	60.5	78.2	+13.5	+24.7
Lysozyme	14,300 (39)	62.5	67.1	63.4	44.1	− 0.9	+23.0
Myoglobin	17,200 (39)	64.0	89.0	59.0	94.9	+ 5.0	− 5.9
Creatine kinase	42,000 (40)	73.5	33.7	69.0	67.2	+ 4.5	−33.5
Ovalbumin	43,000 (41)	82.0	36.5	75.5	57.3	+ 5.5	−20.8
Serum albumin, chicken	65,000 (42)	62.5	76.5	56.0	101.1	+ 6.5	−24.6
Serum albumin, human	69,000 (43)	59.5	48.3	50.6	98.5	+ 8.9	−50.2

decreased in comparison to a 100-Å packing (Fig. 4B) and the yields of the larger proteins, here ovalbumin, are improved. As shown in Table II the protein elution times also decrease by 5–10% owing to the more hydrophilic character of the support. The differences in per cent yields thus appear to be a function of both protein molecular weight and pore size, *i.e.* under *ca.* 15,000 the yields are higher with the 100-Å material; conversely, the 300-Å support is superior for higher-molecular-weight proteins.

Overall yields remain constant or slightly increase when more protein is progressively chromatographed (Fig. 5). That portion not recovered in the effluent appears to remain irreversibly bound to the column since neither radioactivity nor absorbance can be detected when changing to either higher concentrations of 2-propanol or other buffer solutions (*e.g.* 0.1% trifluoroacetic acid). This observation is supported by the results from the isolation of various proteins from both muscle and brain extracts by reversed-phase HPLC using above-mentioned chromatographic conditions[44,45]. In such cases the eluted proteins were collected, desalted and radioactively labelled by reductive methylation. They exhibited only single spots by either protein staining or autoradiography following two-dimensional sodium dodecyl sulphate isoelectric focusing, thereby indirectly supporting the idea that once protein is lost (presumably by either precipitation or hydrophobic association with the packing) it is slowly, if at all, eluted during subsequent separations.

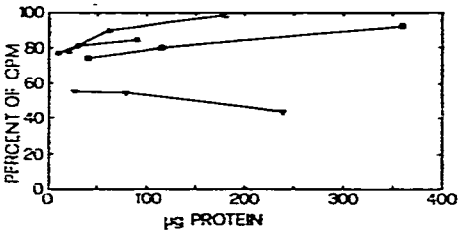

Fig. 5. Influence of protein quantity on per cent [^{14}C] recovery. The samples injected on the 300-Å C_{18} column were: ▲, myoglobin; ●, bovine pancreatic trypsin inhibitor; ■, carbonic anhydrase; ▼, ovalbumin. Elution was carried out with the 2-propanol gradient (buffer system II).

Table III summarizes the results from the chromatography of a number of proteins, molecular weights ranging between 6500 and 69,000, on the RP 300 support with buffer systems I and II. With the exception of myoglobin, all the proteins chromatographed were eluted in higher yields when 2-propanol, rather than acetonitrile, was used as the organic eluent. The protein elution points were decreased by approximately 25% owing to the higher solvent strength while maintaining comparable peak heights and widths (results not shown). Other proteins which have been chromatographed with similar yields are α- and β-chains of haemoglobin, calmodulin, insulin-like growth factors, S-100 protein and troponin c[44,45].

In summary, the C_{18} reverse-phase support of 300-Å pore size offers a number of improvements: (a) proteins of molecular weights exceeding *ca.* 15,000 can be chromatographed with higher yields; (b) the resolution on such supports for proteins is better; (c) peptide mapping can be performed under conditions which give results approximating those of the smaller-pore-size packings; and (d) smaller molecules

TABLE III

ELUTION POSITIONS AND YIELDS FROM PROTEIN REVERSED-PHASE CHROMATOGRAPHY ON AQUAPORE RP-300 (300 Å, 10 μm) PACKINGS UTILIZING ACETONITRILE AND 2-PROPANOL GRADIENTS

nd = Not determined.

Protein	*Molecular weight*	*A, buffer system I (acetonitrile)*		*B, buffer system II (2-propanol)*		*Differences (column A minus column B)*	
		B (%)	*Yield (%)*	*B (%)*	*Yield (%)*	*B (%)*	*Yield (%)*
Bovine pancreatic trypsin inhibitor	6500 (39)	42.5	36.1	20.5	59.8	22.0	−23.7
Cytochrome *c*	11,700 (39)	nd	nd	56.7	45.6	–	–
Parvalbumin	11,700 (39)	85.6	65.0	60.5	78.2	25.1	−13.2
Lysozyme	14,300 (39)	nd	nd	63.4	44.1	–	–
Myoglobin	17,200 (39)	88.6	98.3	59.0	94.9	29.6	+ 3.4
Carbonic anhydrase	31,000 (46)	nd	nd	68.5	81.3	–	–
Maleic dehydrogenase	35,000 (47)	nd	nd	?	0	–	–
Alcohol dehydrogenase	36,700 (46)	nd	nd	78.4	18.9	–	–
Creatine kinase	42,000 (40)	88.7	60.3	64.0	67.2	24.7	− 6.9
Ovalbumin	43,000 (41)	>100	36.9	75.5	57.3	?	−20.4
3-Phosphoglycerate kinase	47,000 (48)	nd	nd	78.4	37.9	–	–
Serum albumin, chicken	65,000 (42)	93.7	97.7	56.0	101.1	37.7	− 3.4
Serum albumin, human	69,000 (43)	85.0	68.0	50.6	98.5	34.4	−30.5

such as Dns-amino acids chromatograph extremely well on the larger-pore-size material[3].

REFERENCES

1 R. Schuster, *Anal. Chem.*, 52 (1980) 617–620.
2 M. K. Radjai and R. T. Hatch, *J. Chromatogr.*, 196 (1980) 319–322.
3 G. J. Hughes, K. H. Winterhalter, E. Boller and K. J. Wilson, *J. Chromatogr.*, 235 (1982) 417–426.
4 C. de Jong, G. J. Hughes, E. van Wieringen and K. J. Wilson, *J. Chromatogr.*, in press.
5 B. R. Larsen and F. G. West, *J. Chromatogr. Sci.*, 19 (1981) 259–265.
6 N. D. Johnson, M. W. Hunkapiller and L. E. Hood, *Anal. Biochem.*, 100 (1979) 335–338.
7 K. J. Wilson, K. Rodger and G. J. Hughes, *FEBS Lett.*, 108 (1979) 87–91.
8 M. Rubinstein, S. Stein, L. D. Gerber and S. Udenfriend, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 3052–3055.
9 S. Kimura, R. V. Lewis, A. S. Stern, J. Rossier, S. Stein and S. Udenfriend, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 1681–1685.
10 R. C. C. Chang, W.-Y. Huang, T. W. Redding, A. Arimura, D. H. Coy and A. V. Schally, *Biochim. Biophys. Acta*, 625 (1980) 266–273.
11 K. J. Wilson and G. J. Hughes, *Chimia*, 9 (1981) 327–333.
12 G. J. Hughes, K. H. Winterhalter and K. J. Wilson, *FEBS Lett.*, 108 (1979) 81–86.
13 M. Rubinstein, S. Chen-Kiang, S. Stein and S. Udenfriend, *Anal. Biochem.*, 95 (1979) 117–121.
14 P. Böhlen and G. Kleeman, *J. Chromatogr.*, 205 (1981) 65–75.

15 G. J. Hughes, C. De Jong, R. W. Fischer, K. H. Winterhalter and K. J. Wilson, *Biochem. J.*, 199 (1981) 61–67.
16 M. van der Rest, H. P. J. Bennett, S. Solomon and F. H. Glorieux, *Biochem. J.*, 191 (1980) 253–256.
17 W. C. Mahoney and M. A. Hermodson, *J. Biol. Chem.*, 255 (1980) 11199–11203.
18 A. Honegger, G. J. Hughes and K. J. Wilson, *Biochem. J.*, 199 (1981) 53–59.
19 P. Böhlen, F. Castillo, N. Ling and R. Guillemin, *Int. J. Peptide Protein Res.*, 16 (1980) 306–310.
20 J. D. Pearson, W. C. Mahoney, M. A. Hermodson and F. E. Regnier, *J. Chromatogr.*, 207 (1981) 325–332.
21 A. N. Kotake and Y. Funae, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 6473–6475.
22 A. Fallon, R. V. Lewis and K. D. Gibson, *Anal. Biochem.*, 110 (1981) 318–322.
23 H.-J. Friesen, S. Stein, M. Evinger, P. C. Familletti, J. Maschera, J. Meienhoffer, J. Shively and S. Pestka, *Arch. Biochem. Biophys.*, 206 (1981) 432–450.
24 S. H. Chang, K. M. Gooding and F. E. Regnier, *J. Chromatogr.*, 125 (1976) 103–114.
25 M. Rubinstein, *Anal. Biochem.*, 98 (1979) 1–7.
26 R. V. Lewis, A. Fallon, S. Stein, K. D. Gibson and S. Udenfriend, *Anal. Biochem.*, 104 (1980) 153–159.
27 T. D. Schlaback, J. A. Fulton, P. M. Mockridge and E. C. Toren, Jr., *Anal. Chem.*, 52 (1980) 729–733.
28 R. A. Bradshaw, W. H. Garner and F. R. N. Gurd, *J. Biol. Chem.*, 244 (1969) 2149–2158.
29 E. E. Strehler, H. M. Eppenberger and C. W. Heizmann, *FEBS Lett.*, 78 (1977) 127–133.
30 N. Gitzelmann-Cumarasamy, R. Gitzelmann, K. J. Wilson and C. C. Kuenzle, *Proc. Nat. Acad. Sci. U.S.*, 76 (1979) 2960–2963.
31 C. H. W. Hirs, *Methods Enzymol.*, 11 (1967) 199–203.
32 N. Jentoft and D. G. Dearborn, *J. Biol. Chem.*, 254 (1979) 4359–4365.
33 M. W. Berchtold, C. W. Heizmann and K. J. Wilson, submitted for publication.
34 K. J. Wilson, A. Honegger and G. J. Hughes, *Biochem. J.*, 199 (1981) 43–51.
35 R. E. Offord, *Nature (London)*, 211 (1966) 591–593.
36 K. J. Wilson, A. Honegger, R. P. Stötzel and G. J. Hughes, *Biochem. J.*, 199 (1981) 31–41.
37 J. L. Meek and Z. L. Rossetti, *J. Chromatogr.*, 211 (1981) 15–28.
38 J. L. Meek, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 1632–1636.
39 M. O. Dayhoff, *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Washington, DC, 1972.
40 D. M. Dawson, H. M. Eppinenberger and N. O. Kaplan, *J. Biol. Chem.*, 242 (1967) 210–217.
41 F. J. Castellino and R. Barker, *Biochemistry*, 7 (1968) 2207–2217.
42 C. W. Heiznann, G. Müller, E. Jenny, K. J. Wilson, F. Landon and A. Olomucki, *Proc. Nat. Acad. Sci. U.S.*, 78 (1981) 74–77.
43 C. Tanford, K. Kawahara and S. Lapanje, *J. Amer. Chem. Soc.*, 89 (1967) 729–736.
44 M. W. Berchtold, C. W. Heizmann and K. J. Wilson, in preparation.
45 K. J. Wilson, M. W. Berchtold, P. Zumstein, S. Klauser and G. J. Hughes, in M. Elzinga (Editor), *Methods in Protein Sequence Analysis*, Humana Press, Clifton, NJ, 1982, in press.
46 M. O. Dayhoff, *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Washington, DC, 1978.
47 J. I. Harris and R. N. Perham, *J. Mol. Biol.*, 13 (1965) 876–884.
48 W. K. G. Krietsch and T. Bücher, *Eur. J. Biochem.*, 17 (1970) 568–580.