Journal of Chromatography, 147 (1978) 424–428

CHROM. 10,345

Note

Improved method of gel filtration of protected peptides using Sephadex LH-60

I. J. GALPIN, A. G. JACKSON, G. W. KENNER, P. NOBLE and R. RAMAGE
The Robert Robinson Laboratories, The University of Liverpool, Liverpool L69 3BX (Great Britain)
(Received June 22nd, 1977)

Recently[1–3] we have investigated the application of gel filtration for the purification of large fully protected peptides. From our earlier work it emerged that Enzacryl K2 may be used to purify protected peptides of molecular weight up to 10,000 using N,N-dimethylformamide (DMF) or N-methylpyrrolidone (NMP) as eluents. Further use of Enzacryl K2 has shown this gel to have variable resolving power, particularly with sparingly soluble peptides.

Although Sephadex LH-20 has proved satisfactory as a gel filtration matrix for relatively low-molecular-weight peptides, the resolution is variably reduced in the molecular weight (MW) band 2000–3000. Recently a higher homologue of Sephadex LH-20 has become commercially available. This matrix, Sephadex LH-60, is formed by hydroxypropylation of Sephadex G-50 and has both hydrophilic and lipophilic properties. Since the beads are known to swell in a wide variety of organic solvents[4], we tested some of these solvents and others as eluents for the purification of protected peptides.

An early application of Sephadex LH-60–DMF on trial batches of Sephadex LH-60[5] showed the suitability of this gel filtration system for purification of a large synthetic fragment of staphylococcal nuclease; however, it was to be anticipated that DMF would not be useful for sparingly soluble protected peptides.

EXPERIMENTAL

Sephadex LH-60, supplied by Pharmacia, London, Great Britain, was swollen in the appropriate dry, distilled solvent at 50° for 3 h under degassed conditions. The gel was packed into the column under gravity flow and then allowed to equilibrate for several hours under flow conditions. Two total bed volumes of eluent were collected before checking the homogeneity of the column with modified dextran blue 2000 (ref. 1). About 1.2 total bed volumes of solvent were collected during each run. Elution was monitored by UV adsorption (280 nm) and optical rotation (546 nm) as described previously[1]. The values of V_0, V_e, V_t and K_{av}, were determined in the usual manner.

RESULTS AND DISCUSSION

From Table I it can be seen that many of the solvents used in peptide synthesis are suitable for use with Sephadex LH-60. In our studies DMF and NMP have proved

TABLE I

SWELLING CHARACTERISTICS OF SEPHADEX LH-60

Solvent	*Bed volume (cm^3/g)*
N,N-Dimethylformamide (DMF)	13
N,N-Dimethylacetamide (DMA)	14
N-Methylpyrrolidone (NMP)	11
Hexamethylphosphoramide (HMPA)	16
2,2,2-Trifluoroethanol (TFE)	20
2 *M* urea in DMF	13

TABLE II

COLUMN PARAMETERS

Eluent	*Diameter (cm)*	V_t *(cm^3)*	V_0 *(cm^3)*	*Optimum flow-rate (cm^3/h)*
DMF	2.5	469	117	20–25
DMF	5.2	2025	501	75–90
NMP	2.5	415	100	20–25
TFE	1.0	54	16	4–6
2 *M* urea in DMF	2.5	451	104	16–20

to be most generally applicable and can be removed *in vacuo* at 30°. 2,2,2-Trifluoroethanol is easily removed, provides excellent solubility properties and good chromatographic results but is expensive for large-scale work and may cause deprotection of very acid-labile protecting groups.

TABLE III

PROTECTED PEPTIDES PURIFIED BY GEL FILTRATION ON SEPHADEX LH-60 WITH DMF AS ELUENT

Acm = acetamidomethyl; Adoc = adamantyloxycarbonyl; Bpoc = β-Biphenylisopropoxycarbonyl; Bu^t = *tert.*-butyl; OBu^t = *tert.*-butoxy; OPh = phenoxy

No.	*Compound*	*MW*	*log MW*	K_{av}
1	Bpoc-Cys(Acm)-Ala-Lys(Adoc)-Lys(Adoc)-Ile-Val-Ser(Bu^t)-Asp((OBu^t)-Gly-Asn-Gly-OH	1869	3.272	0.55
2	Bpoc-Phe-Asn-Thr(Bu^t)-Gln-Ala-Thr(Bu^t)-Asn-Orn(Adoc)-Asn-Thr(Bu^t)-Glu(OBu^t)-Gly-OPh	2027	3.307	0.52
3	Bpoc-Ser(Bu^t)-Thr(Bu^t)-Asp(OBu^t)-Tyr(Bu^t)-Gly-Leu-Leu-Gln-Ile-Asn-Ser(Bu^t)-Orn(Adoc)-Trp-Trp-Cys(Acm)-Ala-Asp(OBu^t)-Gly-OH	2866	3.457	0.56
4	Bpoc-Ser(Bu^t)-Thr(Bu^t)-Asp(OBu^t)-Tyr(Bu^t)-Gly-Leu-Leu-Gln-Ile-Asn-Ser(Bu^t)-Orn(Adoc)-Trp-Trp-Cys(Acm)-Ala-Asp(OBu^t)-Gly-OPh	2942	3.468	0.11
5	Bpoc-Ser(Bu^t)-Thr(Bu^t)-Asp(OBu^t)-Tyr(Bu^t)-Gly-Leu-Leu-Gln-Ile-Asn-Ser(Bu^t)-Orn(Adoc)-Trp-Trp-Cys(Acm)-Ala-Asp(OBu^t)-Gly-Orn(Adoc)-Thr(Bu^t)-Pro-Gly-Ser(Bu^t)-Ala-Asn-Gly-OPh	3930	3.594	0.37
6	Bpoc-Nle-Asn-Ala-Trp-Val-Ala-Trp-Orn(Adoc)-Asn-Arg(Adoc)$_2$-Cys(Acm)-Lys(Adoc)-Gly-Ser(Bu^t)-Asp(OBu^t)-Val-Ser(Bu^t)-Ala-Trp-Val-Orn(Adoc)-Gly-Cys(Acm)-Gly-Leu-OBu^t	4290	3.622	0.37
7	Bpoc-Cys(Acm)-Ala-Lys(Adoc)-Lys(Adoc)-Ile-Val-Ser(Bu^t)-Asp(OBu^t)-Gly-Asn-Gly-Nle-Asn-Ala-Trp-Val-Ala-Trp-Orn(Adoc)-Asn-Arg(Adoc)$_2$-Cys(Acm)-Lys(Adoc)-Gly-Ser(Bu^t)-Asp(OBu^t)-Val-Ser(Bu^t)-Ala-Trp-Val-Orn(Adoc)-Gly-Cys(Acm)-Gly-Leu-OBu^t	5902	3.772	0.44

Table II gives the column parameters and shows V_0 to be approximately $\frac{1}{3}$ of V_t. It was found that the gel performed well at relatively high flow-rates with good resolution.

The strategy of peptide synthesis by fragment coupling relies on effective purification of fully protected peptide fragments. Thus we compared Sephadex LH-60 with Enzacryl K2 which had been used previously for this purpose but was found in later applications to give only moderate resolution. Tables III and IV show the protected peptides employed in this study using DMF and NMP, respectively, as eluents. It was found that crude fragment 6 on application at Sephadex LH-60–DHF gave two sharp well resolved peaks. Reapplication of the first peak showed it to be homogeneous (Fig. 1). Amino acid analysis, deprotection followed by paper electrophoresis at pH 2.1, confirmed the purity of fragment 6 isolated after Sephadex LH-60–DMF gel filtration.

TABLE IV

PROTECTED PEPTIDES PURIFIED BY GEL FILTRATION ON SEPHADEX LH-60 WITH NMP AS ELUENT

No.	*Compound*	*MW*	*log MW*	$K_{av.}$
8	Adoc-Lys(Adoc)-Val-Phe-Gly-Orn(Adoc)-Cys(Acm)-Glu(OBu^t)-Leu-Ala-Ala-Ala-Nle-Lys(Adoc)-Ala-Leu-Gly-OPh	2521	3.402	0.49
3	Bpoc-Ser(Bu^t)-Thr(Bu^t)-Asp(OBu^t)-Tyr(Bu^t)-Gly-Leu-Leu-Gln-Ile-Asn-Ser(Bu^t)-Orn(Adoc)-Trp-Trp-Cys(Acm)-Ala-Asp(OBu^t)-Gly-OH	2866	3.457	0.56
9	Bpoc-Leu-Ala-Gly-Tyr(Bu^t)-Orn(Adoc)-Gly-Tyr(Bu^t)-Ser(Bu^t)-Leu-Gly-Asn-Trp-Nva-Cys(Acm)-Ala-Ala-Lys(Adoc)-Phe-Glu(OBu^t)-Ser(Bu^t)-Gly-OPh	3229	3.509	0.43
10	(Adoc)Lys(Adoc)-Val-Phe-Gly-Orn(Adoc)-Cys(Acm)-Glu(OBu^t)-Leu-Ala-Ala-Ala-Nle-Lys(Adoc)-Ala-Leu-Gly-Leu-Ala-Gly-Tyr(Bu^t)-Orn(Adoc)-Gly-Tyr(Bu^t)-Ser(Bu^t)-Leu-Gly-Asn-Trp-Nva-Cys(Acm)-Ala-Ala-Lys(Adoc)-Phe-Glu(OBu^t)-Ser(Bu^t)-Gly-OPh	5418	3.734	0.30

Although the majority of protected peptides studied chromatographed satisfactorily on Sephadex LH-60, in some cases there was evidence for aggregation, which is well known for free proteins but not normally observed for protected peptides. No indication of such aggregation was observed using Sephadex LH-20 to purify smaller protected peptides. In several cases gel filtration on Sephadex LH-60 showed a peak at, or close to, the void volume. In the example shown in Fig. 2, a crude reaction mixture obtained by coupling of fragment 8 (after cleavage of OPh) with fragment 9 (after cleavage of Bpoc) afforded fragment 10 from peak (b). However, reapplication of this material gave fragment 10 together with a small second peak (a) close to the void volume. Deprotection of the material from peaks (a) and (b) followed by electrophoresis at pH 2.1 and amino acid analysis showed both (a) and (b) to contain the same compound. It would appear that Sephadex LH-60 is capable of promoting aggregation unlike Enzacryl K2. One explanation for this behaviour may be that Sephadex LH-60 is highly hydrophobic with few sites for hydrogen bonding and can not compete effectively with solute–solute interactions. In the case of Enzacryl K2 the amide functions within the gel could interact with the peptide solute by hydrogen bonding.

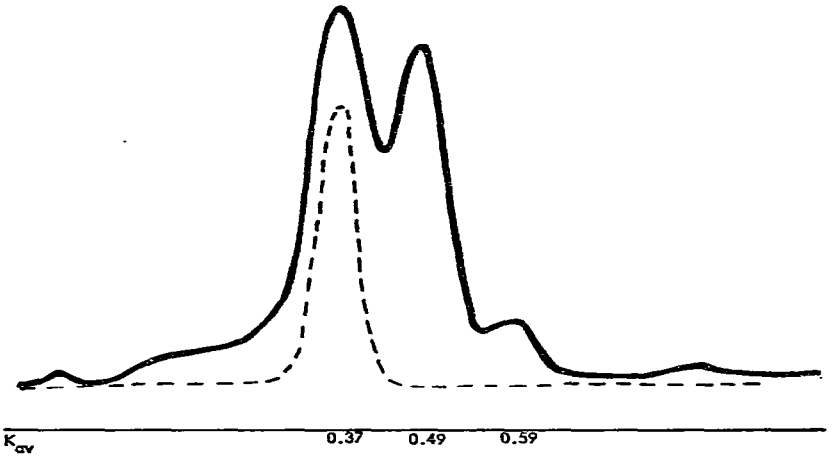

Fig. 1. Gel filtration of fragment 6 on Sephadex LH-60–DMF. ———, First elution; – – –, reapplication. Monitored by UV (280 nm).

In order to circumvent this problem it was decided to use 2 *M* urea in DMF as eluent in order to disrupt the hydrogen bonds in the solute–solute interactions. This idea found support when fragment 7 was applied to Sephadex LH-60. With this eluent a single symmetrical peak was observed ($K_{av_7} = 0.44$) in contrast to the experience using DMF alone when a peak was found at the void volume followed by other less clearly defined peaks. 2,2,2-Trifluoroethanol is a solvent which is known to disrupt aggregation; thus it was decided to use this eluent for gel filtration of fragment 7 on Sephadex LH-60. As predicted a single peak was found with little indication of aggregation.

Fig. 3 illustrates a reasonable linear relationship between log MW and K_{av_7} for the compounds cited in Tables III and IV. By extrapolation the exclusion limit of Sephadex LH-60 was found to be *ca.* 15,000 in DMF or NMP, indicating the great potential for purification of the matrix for the range of protected peptides presently being contemplated.

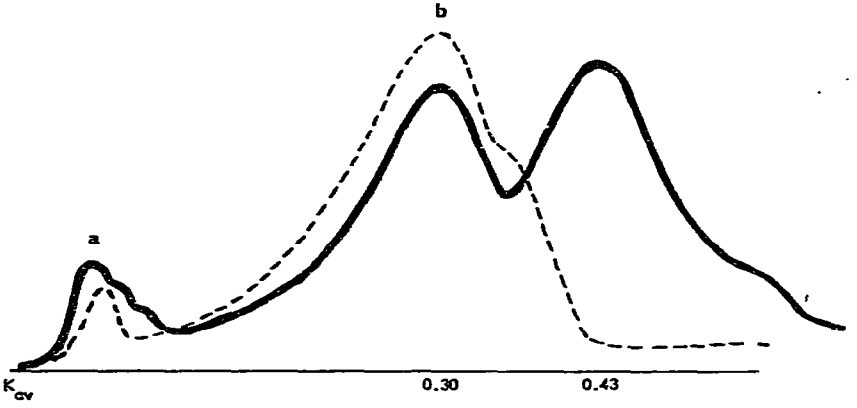

Fig. 2. Gel filtration of fragment 10 on Sephadex LH-60–NMP. ———, First elution; – – –, Reapplication. Monitored by UV (280 nm).

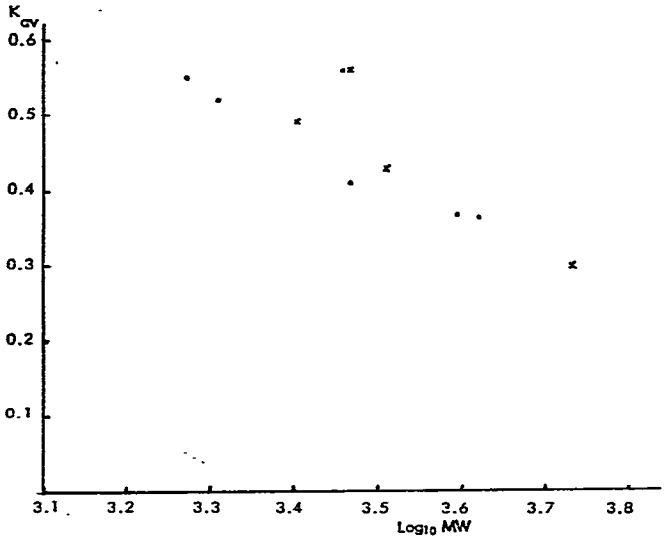

Fig. 3. Gel filtration on Sephadex LH-60. × = NMP; ● = DMF.

From our studies it appears that Sephadex LH-60 affords better resolution than Enzacryl K2 although suffering from aggregation of large peptide solutes. In addition to using Sephadex LH-60 for purification of precipitated products, we have also used the method for isolation of protected peptides from reaction mixtures directly applied to the columns in a variety of solvents. It is important that the viscosity of the applied solution should be similar to that of the eluent. If this is not controlled then violent contraction of the bed may occur and prevent further elution.

ACKNOWLEDGEMENTS

We thank the University of Liverpool and Science Research Council for financial support, and Pharmacia Fine Chemicals for providing a sample of Sephadex LH-60.

REFERENCES

1 I. J. Galpin, G. W. Kenner, S. R. Ohlsen and R. Ramage, *J. Chromatogr.*, 106 (1975) 125.
2 I. J. Galpin, B. K. Handa, G. W. Kenner, S. Moore and R. Ramage, *J. Chromatogr.*, 123 (1976) 237.
3 I. J. Galpin, B. K. Handa, G. W. Kenner, S. Moore and R. Ramage, *Macromolecules Symposium, Birmingham, 1976, Chromatography of synthetic and biological polymers*, Vol. I, 1977, p. 331.
4 *Sephadex LH-60*, Technical Booklet, Pharmacia, Uppsala, 1976.
5 A. R. Zeiger and C. B. Anfinsen, *J. Amer. Chem. Soc.*, 95 (1973) 880.