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GEL FILTRATION OF PROTECTED PEPTIDES ON SEPHADEX G-50 IN HEXAMETHYLPHOSPHORAMIDE CONTAINING 5% WATER

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SUMMARY

Gel permeation chromatography on G-50 and G-75 Sephadex gels, using 5% water in hexamethylphosphoramide (phosphoric trisdimethylamide) has been applied to the purification of large protected peptides which are outside the molecular weight range of the Sephadex LH-20–dimethylformamide system.

INTRODUCTION

One of the advantages in synthesis of polypeptides by the fragment condensation approach is that the intermediates are capable of purification and characterisation. However, when the synthetic strategy involves maximal protection of side-chain functional groups, the normal processes of purification often fail due to the hydrophobic and insoluble nature of the peptide fragments. Ostensibly, large protected peptides which have been synthesised by fragment coupling would be amenable to purification by gel permeation chromatography, since the product could be designed to differ greatly from the starting materials in molecular weight.

The G-series of Sephadex gels is effective over a wide range of molecular weights (MW), but they are normally used with aqueous buffers in which fully protected peptides are usually insoluble. Sephadex LH-20 is a hydroxypropylated derivative of G-25 (ref. 1) and has proved to be useful in gel filtration of protected peptides using dimethylformamide (DMF) and other organic solvents[2,3]. From a number of examples it can be seen (Fig. 1) that the exclusion limit is between MW 2500 and 3000 for this system. In our experience arginine-containing peptides in which the guanidine function is protonated cannot generally be purified by this method. The MW range has been extended by hydroxypropylating G-50 to produce LH-60. This gel is not yet commercially available, but LH-60–DMF has been used by Zeiger and Anfinsen to purify a synthetic sequence of Staphylococcal nuclease after LH-20 had proved to be unsatisfactory[4].

We, therefore, decided to study the swelling characteristics of the available G series of gels in solvent mixtures, which had been found to be compatible with large protected peptides during the course of synthetic work. Hexamethylphosphoramide (phosphoric trisdimethylamide) (HMPA) containing 5% water was found to be suitable

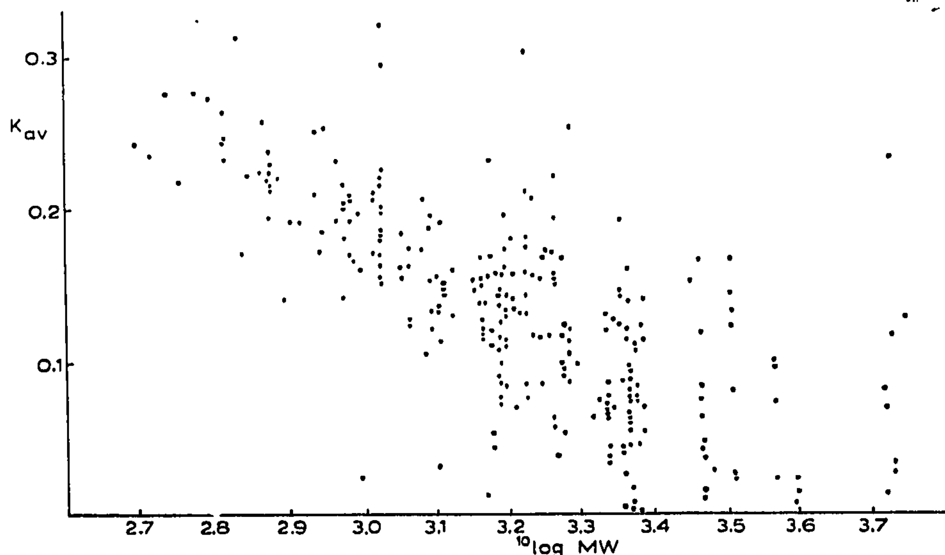

Fig. 1. Gel filtration of protected peptides on LH-20 Sephadex using DMF as solvent.

for gel filtration of synthetic peptides which were either insoluble in DMF or too large for LH-20 purification. In water, G-50 and G-75 Sephadex gels have effective MW limits of 30,000 and 70,000, respectively[5]. Even if the effect of HMPA as the mobile phase were to reduce this range to 30% of these values, these gels together with LH-20 would still cover the selection of peptides and enzymes currently considered to be targets for synthesis.

EXPERIMENTAL

Materials

Blue dextran 2000, Sephadex G-series and LH-20 were purchased from Pharmacia, London (Great Britain). The glass columns (2.5 and 5.0 cm I.D., resp.) were fitted with a porosity 3 sinter. DMF and HMPA were dried and distilled at 0.1 mm Hg.

Procedures

The swelling characteristics were determined by placing a known quantity of the appropriate Sephadex in a measuring cylinder and dispersing the beads in the solvent. After 24-h equilibration with occasional shaking the volume of the gel was measured.

For chromatographic use, the Sephadex gels were swollen in the appropriate solvent mixture at 50° for 16 h, then degassed. The G-50 columns, 2.5 and 5.0 cm I.D., were packed with flow-rates of 7.5 ml/h and 20 ml/h, respectively. The 2.5-I.D. G-10 column in DMF was eluted at a flow-rate of 15–30 ml/h. Elution was monitored by ultraviolet (UV) absorption at 280 nm using an LKB Uvicord.

The void volume, V_0, was determined using the completely excluded solute Blue dextran 2000, modified by the method of Gut and Cimrova[2]. The dark blue

material obtained from reaction of Blue dextran 2000 with $ClCOOC_2H_5$ was soluble in DMF and HMPA.

The total bed volume, V_t, of the gel was determined by calibrating the column with measured volumes of water prior to packing. The elution volume, V_e, of partially excluded solutes was characterised as the volume of eluent collected up to the point of maximal UV absorbance. The distribution coefficient, K_{av}, was given by the expression[5]

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

The constants for the columns used are given in Table I.

TABLE I

COLUMN PARAMETERS

Column	*I.D. (cm)*	*V_0 (ml)*	*V_t (ml)*
G-50, HMPA-5% water	2.5	27.0	103.5
G-50, HMPA-5% water	5.0	122.0	475.0
G-10, DMF	2.5	126.0	367.0

RESULTS AND DISCUSSION

The total bed volumes of G-50 Sephadex when swollen in DMF–water and HMPA–water are shown in Fig. 2. The swelling of the gel was found to be proportional to the water content over the range 2.5–15% water. A similar result was found by Bush and Jones[6] for G-25 in dioxane–water systems over the range 0–60% water. It was also found that G-75, G-100 and G-200 gels behaved in a similar manner when treated with a HMPA–water mixture. In all cases the swelling was greater in the HMPA–water than in the DMF–water systems. This may be due to

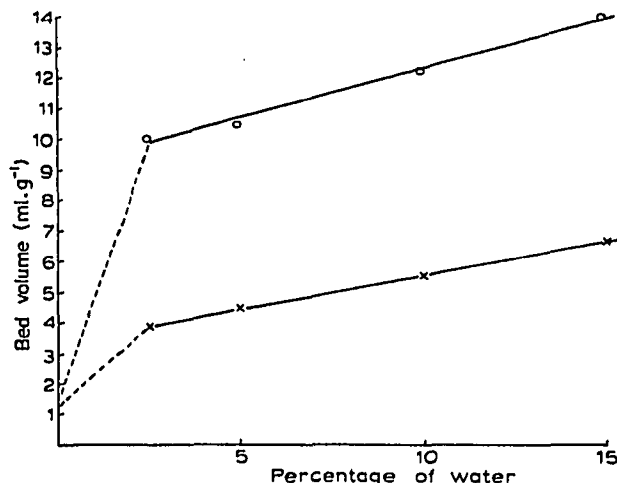

Fig. 2. Swelling characteristics of G-50 Sephadex in HMPA–water (○—○) and DMF–water (×—×).

the high solvating power of HMPA, which is well fitted for H-bonding through the hydroxyl groups of the Sephadex gel.

After preliminary swelling experiments, it was decided that G-50 would be most suitable for the MW range of protected peptides available. In order to obtain maximum solubility of the compounds the water content was kept at 5%. Lower proportions (2.5%) of water gave poorer separations. Removal of HMPA after gel permeation chromatography poses a problem in that, unlike DMF, HMPA is not removed by evaporation *in vacuo* at 30°. The protected peptide can be isolated after precipitation with brine, but this is not always satisfactory. Alternatively the solute can be separated from HMPA by gel filtration using a column packed with G-10 Sephadex and eluted with DMF. Large peptides would be expected to be eluted in DMF close to V_0 whereas HMPA would be eluted at V_t. It was found that many protected peptides, even those which were not very soluble in DMF, could be isolated by this method.

The compounds chromatographed on G-50 Sephadex are given in Table II and the graph of K_{av} against $^{10}\log(MW)$ is shown in Fig. 3. It was found that the large peptides having an MW greater than 5000 were eluted near the void volume (V_0/V_t = 0.27 for G-50 in HMPA–5% water). In order to extend the method, G-75 Sephadex

TABLE II

PROTECTED PEPTIDES PURIFIED BY GEL FILTRATION ON SEPHADEX G-50 IN HMPA–5% WATER

No.	*Compound*	*MW*	K_{av}	V_e/V_t
1	Bpoc-Cys(Acm)-Asn-Ile-Pro-Cys(Acm)-Ala-Ala-Leu-Nva-Ser(Bu^t)-Gly-OPh	1560	0.43	0.56
2	Bpoc-Phe-Asn-Thr(Bu^t)-Gln-Ala-Thr(Bu^t)-Asn-Orn(Adoc)-Asn-Thr(Bu^t)-Glu(OBu^t)-Gly-OH	1951	0.44	0.58
3	Bpoc-Leu-Leu-Gln-Ile-Asn-Ser(Bu^t)-Orn(Adoc)-Trp-Trp-Cys(Acm)-Ala-Asp(OBu^t)-Gly-OPh	2197	0.44	0.58
4	Bpoc-Cys(Acm)-Asn-Ile-Pro-Cys(Acm)-Ala-Ala-Leu-Nva-Ser(Bu^t)-Gly-Asp(OBu^t)-Ile-Thr(Bu^t)-Ala-Ser(Bu^t)-Val-Gly-OPh	2373	0.43	0.58
5	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	0.45	0.60
6	Bpoc.-Leu-Ala-Gly-Tyr(Bu^t)-Arg(H$^+$TosO$^-$)-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	3265	0.31	0.49
7	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	0.29	0.48
8	Z-Lys(Z)-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)-Arg(H$^+$TosO$^-$)-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	5358	0.14	0.37
9	Bpoc-Phe-Asn-Thr(Bu^t)-Gln-Ala-Thr(Bu^t)-Asn-Orn(Adoc)-Asn-Thr(Bu^t)-Glu(OBu^t)-Gly-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	5625	0.095	0.32

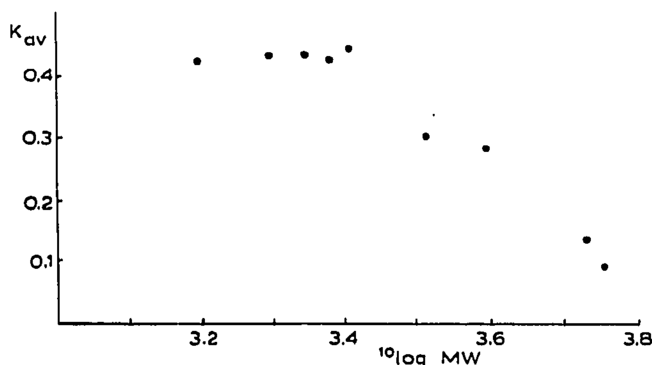

Fig. 3. Gel filtration of protected peptides as listed in Table II on G-50 Sephadex using HMPA containing 5% water as solvent.

was used with HMPA–5% water resulting in approx. 12 ml/g swelling. Compound 9 (Table II) was found to have a V_e/V_t ratio of 0.46 compared with a ratio of 0.32 for the G-50 system.

The method of G-50 gel permeation chromatography has proved useful not only for purification of protected peptides, but also for isolation of pure materials directly from fragment coupling reactions carried out in HMPA solution. The isolated materials were all shown to be homogeneous by amino acid analysis, thin-layer chromatography and, in some cases, electrophoresis of the deprotected peptides. It was found that the K_{av} value obtained from the G-50 Sephadex column was a further useful parameter for characterisation of a particular protected peptide since it was often possible to assess the expected V_e of the coupled product from a knowledge of its MW.

We hope to extend this method further into the MW range of 5,000–20,000 during the course of an approach to synthesize lysozyme analogues.

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REFERENCES

1 *Sephadex LH-20*, Technical Booklet, Pharmacia, Uppsala, 1972.
2 V. Gut and M. Cimrova, *Coll. Czech. Chem. Comm.*, 34 (1969) 1620.
3 *Amino-acids, Peptides and Proteins*, Specialist Periodical Reports, Vol. 3, The Chemical Society, London, 1971, pp. 274 and 275.
4 A. R. Zeiger and C. B. Anfinsen, *J. Amer. Chem. Soc.*, 95 (1973) 880.
5 *Sephadex-gel filtration in theory and practice*, Technical Booklet, Pharmacia, Uppsala, 1969.
6 B. Bush and T. E. L. Jones, *J. Chromatogr.*, 49 (1970) 448.