Separation of protected peptides on methylated Sephadex

In a previous paper we have described the preparation and use of methylated Sephadex in the separation of some lipids[1]. Because of the difficulties sometimes encountered in the purification of reaction products in peptide synthesis it appeared of interest to study the behaviour of protected peptides on columns of methylated Sephadex. Some results of this study have been reported[2].

Experimental

Columns of methylated Sephadex G-25 or G-50 were prepared as described previously[1]. Usually 25 g of methylated Sephadex G-25 or 12.5 g of methylated Sephadex G-50 were used for each column. The column diameter was usually kept at 2 cm whereas the column height varied with the solvent used[1].

Protected peptides were generously supplied by Dr. W. RITTEL, Ciba A.G., Basel, Switzerland. The compounds (usually 0.2–0.8 mg) were applied to the top of the column in 0.5–1.0 ml of the same solvent as that used for the preparation and elution of the column. When the sample had entered the gel, solvent was added and the elution performed with a flow rate of 0.5–1.0 ml/min. Fractions of 2–3 ml were collected. The solvents were evaporated and the protected peptide located by a ninhydrin reaction[3] after hydrolysis[4] or by thin-layer chromatography using the chlorine/*o*-tolidine reaction[5]. In all analyses about 0.01 μC of cholesterol-4-^{14}C was added as an internal standard to the sample. Radioactivity was determined by counting an aliquot of each fraction in a gas-flow counter, and the elution volumes of the protected peptides were calculated relative to that of the labeled cholesterol.

Results and discussion

As discussed in a previous paper[1], separations of lipid soluble compounds on methylated Sephadex are determined by several factors. Most important of these appear to be: (a) partition between a stationary gel–solvent phase and the mobile phase; and (b) gel filtration (molecular sieving). Furthermore, the methylated Sephadex contains negatively charged, fixed ions (about 10 μequiv./g in methylated Sephadex G-25, as determined by titration), which may influence the elution rate of compounds with basic and acidic groups (this effect is also seen with regular Sephadex in water[6]). Table I shows the relative elution volumes of some of the protected peptides on methylated Sephadex G-25 and G-50 using chloroform–methanol, 1:1 (v/v), and ethanol–water, 96.5:3.5 (v/v), as the solvents. The results indicate that the effects mentioned may all be of importance in determining the elution volumes of the protected peptides. With the solvents tested gel filtration seems to be most pronounced in chloroform–methanol, 1:1. A plot of relative elution volume in this solvent *versus* log molecular weight is shown in Fig. 1. Two peptides with a free carboxyl group are eluted earlier than peptides of similar molecular weight with protected carboxyl groups. Peptides containing histidine or histidine and nitroarginine are eluted later than peptides of similar molecular weight not having these residues. Occasionally difficulties with tailing of these compounds have been encountered which might indicate adsorption.

With ethanol–water, 96.5:3.5, gel filtration seems to be of minor importance in determining the elution volume. Thus the small peptides a–c (Table I) have elution

TABLE I

ELUTION VOLUMES RELATIVE TO THAT OF CHOLESTEROL OF PROTECTED PEPTIDES ON METHYLATED SEPHADEX G-25 AND G-50 IN TWO SOLVENT SYSTEMS

*Protected peptide**	*Approx. mol. wt.*	*Relative elution volume*			
		G-25		*G-50*	
		Chloroform–methanol (1:1)	*Ethanol–water (96.5:3.5)*	*Chloroform–methanol (1:1)*	*Ethanol–water (96.5:3.5)*
(a) Z·Gly-Gly·OMe	280	1.06	1.55	1.00	1.22
(b) Z·Gly-Gly-Gly·OMe	337	0.97	1.51	0.98	—
(c) Z·Gly-Gly-Gly-Gly·OMe	394	0.93	1.53	1.00	—
(d) Z·Val-Lys(BOC)·OH	479	0.68	0.72	0.78	—
(e) Z·Lys(BOC)-Val-Tyr-Pro·O*t*Bu	795	0.69	0.98	0.86	0.96
(f) H·Lys(BOC)-Val-Tyr-Pro·O*t*Bu	661	0.79	1.00	1.00	1.00
(g) Z·Val-Lys(BOC)-Val-Tyr-Pro·O*t*Bu	894	0.66	0.93	0.83	0.94
(h) H·Val-Lys(BOC)-Val-Tyr-Pro·O*t*Bu	760	0.71	0.97	—	0.94
(i) H·Tyr(*t*Bu)-Ser(*t*Bu)·O*t*Bu	436	0.80	1.04	1.00	1.00
(j) Z·Val-Tyr(*t*Bu)-Ser(*t*Bu)·O*t*Bu	669	0.74	0.90	0.89	0.97
(k) Z·Leu-Val-Tyr(*t*Bu)-Ser(*t*Bu)·O*t*Bu	782	0.72	—	—	—
(l) Z·Leu-Leu-Val-Tyr(*t*Bu)-Ser(*t*Bu)·O*t*Bu	761	0.71	0.91	1.00	0.95
(m) Z·His-Leu-Leu-Val-Tyr(*t*Bu)-Ser(*t*Bu)·O*t*Bu	1032	0.71	0.84	0.86	0.92
(n) H·His-Leu-Leu-Val-Tyr(*t*Bu)-Ser(*t*Bu)·O*t*Bu	898	0.72	0.78	0.83	0.89
(o) Z·Val-Tyr-Val-His-Pro-Phe·OMe	908	0.76	1.18	1.07	1.06
(p) Z·Arg(NO_2)-Val-Tyr-Val-His-Pro-Phe·OMe	1109	0.77	—	0.84	—
(q) H·Arg(NO_2)-Val-Tyr-Val-His-Pro-Phe·OMe	975	0.85	—	0.88	—
(r) Z·Asp(OBzl)-Arg(NO_2)-Val-Tyr-Val-His-Pro-Phe·OMe	1314	0.74	0.72	0.78	—
(s) Z·Asp(O*t*Bu)-Glu(NH_2)-Leu-Ala-Glu(O*t*Bu)-Ala-Phe-Pro-Leu-Glu(O*t*Bu)-Phe·O*t*Bu	1636	0.60	—	—	—
(t) Z·Glu(O*t*Bu)-Asp(O*t*Bu)-Glu(NH_2)-Leu-Ala-Glu(O*t*Bu)-Ala-Phe-Pro-Leu-Glu(O*t*Bu)-Phe·O*t*Bu	1821	0.50	—	—	—

* Abbreviations: Z = benzyloxycarbonyl; BOC = *tert.*-butyloxycarbonyl; OMe = methyl ester; OBzl = benzyl ester; O*t*Bu = *tert.*-butyl ester; *t*Bu = *tert.*-butyl ether.

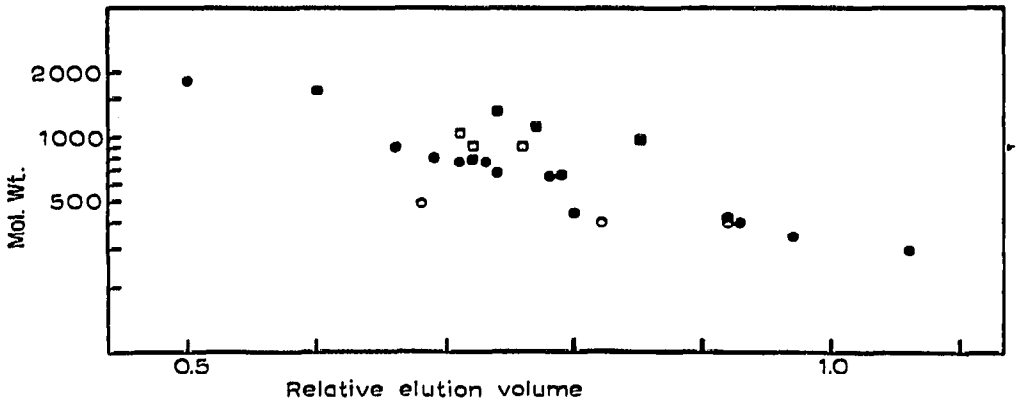

Fig. 1. Plot of relative elution volume *versus* molecular weight. (●) Protected peptides not having histidyl and nitroarginyl residues or a free carboxyl group; (○) peptides with a free carboxyl group; (□) peptides with a histidyl residue; (■) peptides with a histidyl and a nitroarginyl residue. Column: 20 mm. Solvent: chloroform–methanol (1:1); flow rate: 1.0 ml/min.

volumes which are larger than the total column volume (see ref. 1). Partial exclusion from the inner phase seems to occur with peptides above mol. wt. 1000.

With methylated Sephadex G-50 the elution volumes of the peptides studied are closer to that of the reference cholesterol than with methylated Sephadex G-25. This may be due to the higher porosity of this gel giving less separation by gel filtration of the low molecular weight compounds studied.

The results indicate that molecular weight, polarity and charged groups influence the behaviour of protected peptides on columns of methylated Sephadex. In spite of the difficulties involved in predicting the elution volume of a protected peptide it appears, however, that chromatography on methylated Sephadex may be of potential value for the separation and purification of these compounds. Examples of chromatograms are shown in Fig. 2. The method is non-destructive, and since high flow rates can be obtained the separations can be carried out rapidly. Suitable solvent systems may be chosen to increase the difference in polarity between the gel–solvent phase and the mobile phase thereby increasing differences in partition coefficients. It is also possible to use methylated Sephadex of higher or lower polarity by controlling the degree of methylation (the present material had a mean of about 2.3 methoxyl groups per glucose unit[1]).

When the separation factors are small it has been found advantageous to use recycling chromatography[7] permitting the separation of compounds with a separation factor of 1.02–1.03.

Chromatography on methylated Sephadex has been used on a gram scale for removal of *p*-nitrophenol from reaction mixtures in peptide syntheses with the *p*-nitrophenyl ester method[9]. Analysis of microgram amounts can be carried out in capillary columns. The separation shown in Fig. 3 was obtained with a column of methylated Sephadex G-25, superfine, packed in chloroform–methanol–heptane, 1:1:2, in a teflon tubing 1750 × 1.5 mm. The sample was injected in 10 μl of solvent[8]. Material in the effluent was detected with a modified flame ionization detector[10] kindly lent to us by Dr. E. HAAHTI.

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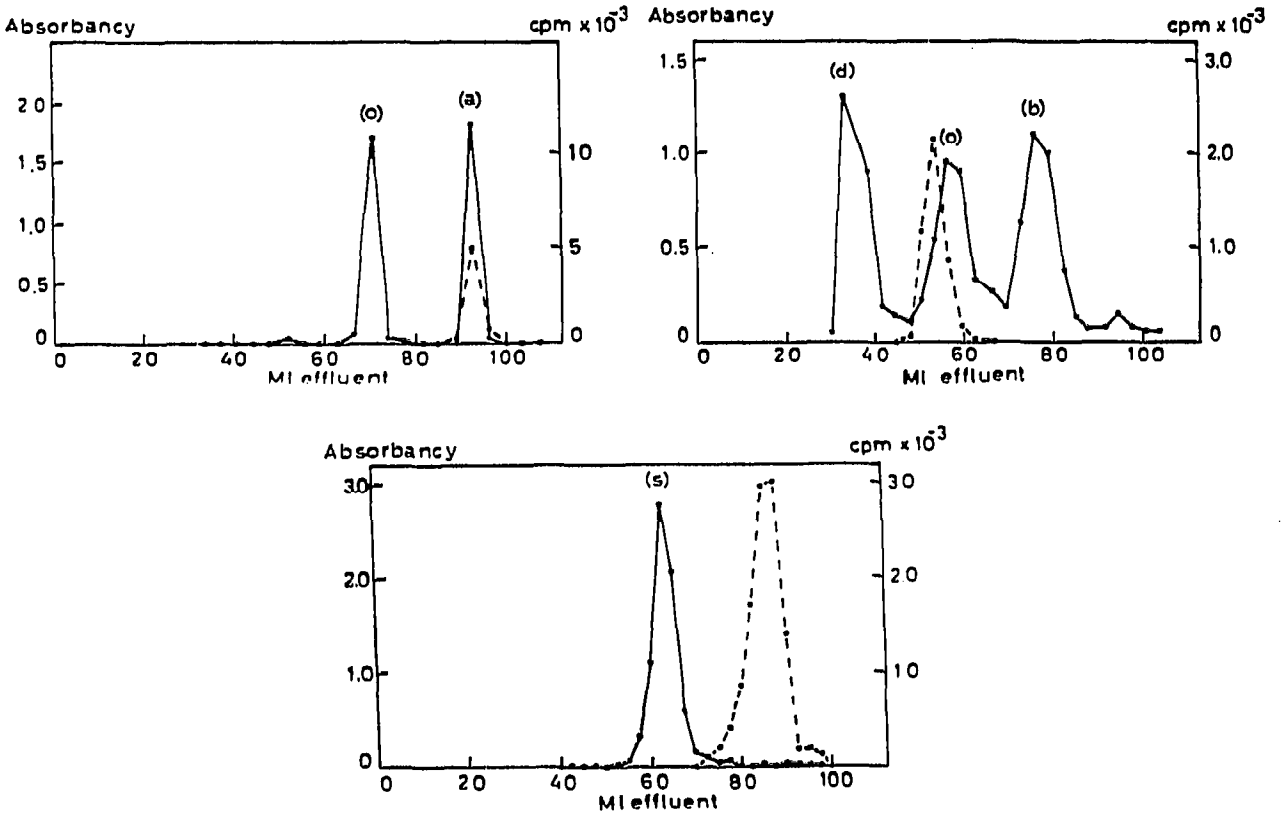

Fig. 2. Upper left curve: Separation of Z·Val-Tyr-Val-His-Pro-Phe·OMe (0.46 mg; o), Z·Gly-Gly·OMe (0.44 mg; a), and cholesterol-4-^{14}C (trace amount: broken line). Column: 25 g methylated Sephadex G-25, 1580 × 8 mm. Solvent: chloroform–methanol (1:1); flow rate: 0.37 ml/min. Fractions of 3.7 ml collected. Upper right curve: Separation of Z·Val-Lys(BOC)·OH (0.27 mg; d), Z·Val-Tyr-Val-His-Pro-Phe·OMe (0.26 mg; o), Z·Gly-Gly-Gly·OMe (0.26 mg; b), and cholesterol-4-^{14}C (trace amount: broken line). Column: 20 g methylated Sephadex G-25, 230 × 20 mm. Solvent: ethanol–water (96.5:3.5); flow rate: 0.8 ml/min. Fractions of 3.0 ml collected. Lower curve: Separation of Z·Asp(O*t*Bu)-Glu(NH_2)-Leu-Ala-Glu(O*t*Bu)-Ala-Phe-Pro-Leu-Glu(O*t*Bu)-Phe·O*t*Bu (0.49 mg; s), and cholesterol-4-^{14}C (trace amount: broken line). Column: 12.5 g methylated Sephadex G-50, 390 × 20 mm. Solvent: chloroform–methanol (4:1); flow rate 0.5 ml/min. Fractions of 2.5 ml collected.

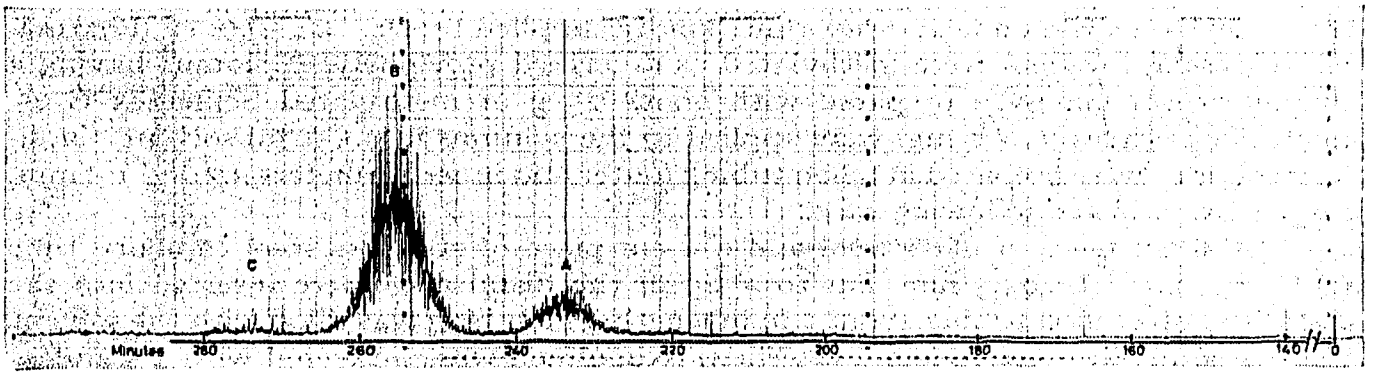

Fig. 3. Separation of Z·Val-Lys(BOC)-Val-Tyr-Pro·O*t*Bu (about 10 μg; A) and Z·Lys(BOC)-Val-Tyr-Pro·O*t*Bu (about 30 μg; B). The peak eluted at C is an impurity. Column: methylated Sephadex G-25, superfine, 1750 × 1.5 mm. Solvent: chloroform–methanol–heptane (1:1:2), flow rate: 0.57 ml/h. The effluent was monitored with a flame ionization detector[10].

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Separation of vitamins K_2 on capillary columns of methylated Sephadex

In the course of studies of the potential uses of methylated Sephadex for the separation of lipid soluble compounds[1] it was considered of interest to study the separations that could be obtained with a series of isoprenologue compounds. A series of vitamins K_2 were chosen since the effluent could be easily monitored by measurement of the U.V. absorption.

Experimental

Vitamins $K_{2(10)}$–$K_{2(40)}$ were generously supplied by Drs. O. WISS AND U. GLOOR, Hoffmann-la Roche, Basel, Switzerland.

Sephadex G-25 fine and superfine (kindly supplied by Dr. B. GELOTTE, Pharmacia, Uppsala, Sweden) were methylated as described previously[1]. Columns having a diameter of 2 cm were prepared with about 25 g of methylated Sephadex G-25, fine[1]. The samples (0.2–1 mg) were applied to the columns in 0.5–1 ml solvent. Capillary columns were prepared in teflon tubing (outer and inner diameters 2.3 and 1.5 mm, respectively) in the following way:

A small piece of glass wool and a 2 cm piece of stainless steel capillary tube (O.D. $^1/_{16}$ in., I.D. 0.25 mm, cut to a tip in the distal end) were inserted into the distal end of a teflon tubing about 2 m in length. The tubing was filled with the solvent to be used for the chromatography. The proximal end was connected with a stainless steel tubing (O.D. $^1/_{16}$ in., I.D. 0.6 mm, length 5 cm) silver soldered to a stainless steel cylindrical reservoir (O.D. 30 mm, length 100 mm) which contained a slurry of methylated Sephadex G-25, superfine, in the same solvent. The upper end of the