CHROM. 5708

Partition chromatography of glucagon and secretin on Sephadex

Of the methods employed for the purification of polypeptides, partition chromatography on Sephadex or other supports has the potential to be among the most convenient, rapid, and efficient. The method was introduced for use with polypeptides by YAMASHIRO[1] and by SCHMIDT-KASTNER[2]. YAMASHIRO *et al.*[3], provided an excellent example of the power of the technique by showing that it could be applied to the separation of the diastereomers oxytocin and [1-hemi-D-cystine]-oxytocin. For this separation, the method was shown to be as effective as counter-current distribution.

We have adapted YAMASHIRO's procedures to routine application in the separation and purification of a number of natural and synthetic polypeptides, and we wish to report here the development of solvent systems suitable for the partition chromatography of glucagon and secretin, two moderate-sized polypeptide hormones[4]. A brief discussion of the factors that affect the R_F values of these polypeptides is also presented.

Experimental

Solvents. *n*-Butanol was distilled through a 30-cm Vigreux column over 2-amino-2-methyl-1,3-propanediol (3–6 g per 5.2 l of A.R. *n*-butanol). From 5.2 l, a forerun of 500 ml was discarded and the next 4 l were collected for use. A.R. acetic acid and A.R. benzene were purified by distillation through a 30-cm Vigreux column. A.R. absolute ethanol was used without further purification. Deionized water was employed throughout.

Preparation of solvent systems. The appropriate amount of each component solvent was deaerated at the water aspirator for 10–15 min. The solvents were then combined in a separatory funnel and thoroughly shaken. A pH meter was used and the pH of the lower phase was adjusted to the desired level by the addition of small amounts of acetic acid or ammonium hydroxide to the combined system. After equilibrating for several hours, the upper and lower phases were ready for use.

Sephadex. Sephadex G-25 (Pharmacia, Piscataway, N.J.) was sieved and the 100–200-mesh fraction was prepared as described by YAMASHIRO *et al.*[3].

Hormones. Crystalline glucagon was obtained from Schwarz-Mann (Van Nuys, Calif.). The secretin was a generous gift from Professor VIKTOR MUTT, Karolinska Institutet, Stockholm, Sweden.

Columns. For the analytical runs, a 1 × 49 cm glass column (column I), with a teflon needle valve at the column exit for flow-rate control, was used. This column was filled with Sephadex to a height of approximately 37 cm. For larger scale work, a 2.2 × 70 cm column (column II) filled with Sephadex to a height of approximately 65 cm was used. The columns were packed and prepared for use as described by YAMASHIRO *et al.*[3].

Chromatographic method. Each chromatographic run was carried out in a cycle of five stages at a temperature of 22–24°. These consisted of stage A, equilibration of the column to the lower (stationary) phase by passage of about 1.5 bed volumes through the column; stage B, equilibration to the upper (mobile) phase and measure-

ment of the hold-up volume, V_H (*vide infra*) by passage of about 0.25 bed volume of the upper phase through the column; stage C, chromatography of the sample by elution with 2 to 3 bed volumes of the upper phase; stage D, discharge of the two-phase system and material not eluted in stage C by passage of two bed volumes of the appropriate solvent system (*vide infra*); stage E, regeneration of the column for re-use by equilibration with 1.5 bed volumes of 0.2 *N* acetic acid. Stage E is usually not essential if the column is to be re-used for partition chromatography, but is necessary if the column is to be used for gel filtration.

The general solvent used for stage D is a pyridine–0.2 *N* acetic acid (X : Y) mixture, where the ratio X : Y (determined by the nature of the solvent system used in stages A, B, and C) should not be greater than 4 : 1. The empirical rule to establish the ratio X : Y is that no more than four volumes of the washing solvent should be required to obtain miscibility with one volume of the upper phase of the solvent system to be discharged. The less expensive washing solvent acetic acid–water (X : Y) can be employed when the solvent system contains little or no pyridine (a pyridine to water ratio of less than 1 to 50 in the solvent system)[5].

The hold-up volume, V_H, for each solvent system during stage B was calculated by subtracting the volume of bed shrinkage during equilibration to the upper phase plus the dead volume at the column exit from the measured volume of displaced lower phase (eqn. 1). This is the volume of the mobile phase in the column, and its value was usually about one-fourth of the bed volume.

$$V_H = \text{volume of displaced lower phase} - (\text{volume of bed shrinkage} + \text{dead volume}) \quad (1)$$

For example, the V_H for column I (*ca.* 26-ml bed volume) was approximately 6.3 ml; for column II (*ca.* 240-ml bed volume) the V_H was approximately 60 ml. These values varied slightly with solvent composition.

Glucagon or secretin (1–3 mg for column I) was applied as a solution in *ca.* 1 ml of the upper phase to which three drops of 1 *N* HCl was added in order to insure complete solubility, and two 1-ml washes were added. Elution with the upper phase was continued and approximately 80 ml was eluted through column I (400 ml for column II) in each run. The flow rate was about 4–7 ml/h for column I and about 25 ml/h for column II. Fractions of 0.45–0.85 ml were collected from column I and 3.5–4.0 ml from column II by use of an automated fraction collector. The R_F values were determined from eqn. 5 (see *Discussion*) after measurement of the appropriate elution volumes. Equivalent R_F values were obtained on both column I and column II.

Analysis. Upon completion of each chromatographic run, FOLIN-LOWRY color values[6] were determined on 0.10–0.20 ml fractions from every other tube. The absorbance values at 625 mμ were determined. Some typical plots of absorbance *vs.* tube number are shown in Figs. 1 and 2. The R_F value was determined from V_E, the elution volume at the peak maximum, and from V_H, the hold-up volume, by use of eqn. 5 (see *Discussion*). To recover the secretin or glucagon appropriate fractions were pooled, twice the volume of deionized water was added, and the mixture was evaporated to 10–20 ml on a rotary evaporator at 20–30° *in vacuo*. The final solution was then lyophilized.

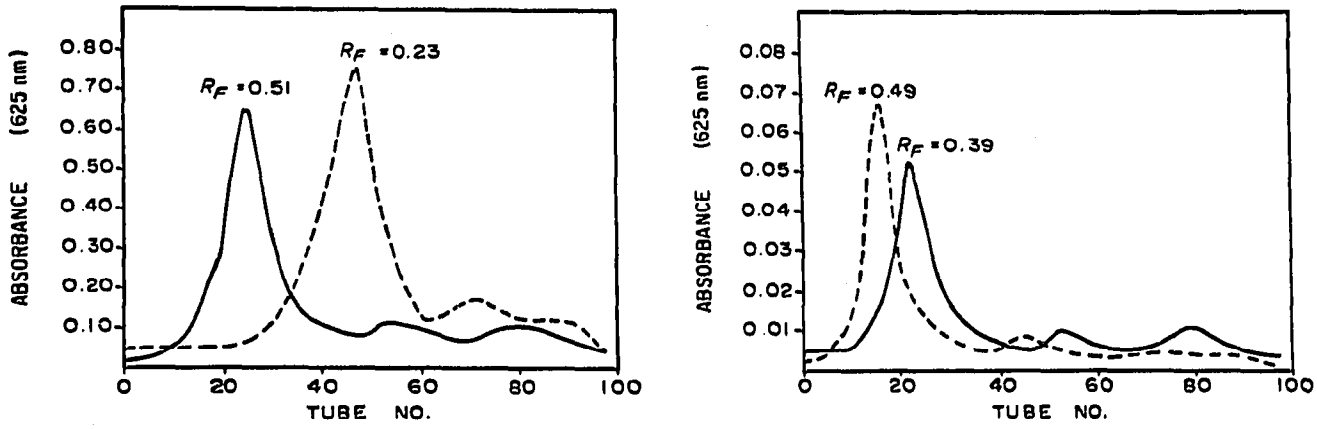

Fig. 1. Partition chromatography of glucagon on column I. ———, Solvent system 1a; – – – –, solvent system 4.

Fig. 2. Partition chromatography of secretin on column I. ———, Solvent system 3d; – – – –, solvent system 3c.

Discussion

According to DIXON's treatment[7], R_F can be defined as follows:

$$R_F = \frac{1}{1 + (V_S/V_H)(1/K)} \tag{2}$$

where V_S is the volume of the stationary phase, V_H is the hold-up volume (see *Experimental*), and K is the distribution coefficient. Rearrangement of eqn. 2 gives

$$K = \frac{V_S}{V_H} \cdot \frac{R_F}{1 - R_F} \tag{3}$$

Experiments indicate that on Sephadex G-25 the ratio V_S/V_H is in the range 1.5–2.0 (ref. 3). For the purpose of testing suitable solvent systems in this type of partition chromatography, K can be estimated as follows:

$$K = \frac{\text{concentration of solute in upper phase}}{\text{concentration of solute in lower phase}} \tag{4}$$

For our purposes, the R_F was calculated according to

$$R_F = \frac{V_H}{V_E} \tag{5}$$

where V_E is the elution volume of the peak (volume of mobile phase required to move the substance the length of the column). It has been suggested[7] that although the R_F should be kept low, no great advantage is gained by further diminution below 0.3, since beyond this value there is little improvement in the resolving power. Arbitrary practical limits of the R_F in the range 0.6–0.13 therefore correspond to distribution coefficients (K) of approximately 2.6–0.4.

It can be seen from the R_F values of Table I that several solvent systems

J. Chromatog., 63 (1971) 423–428

TABLE I

PARTITION CHROMATOGRAPHY SYSTEMS AND R_F VALUES FOR GLUCAGON AND SECRETIN

System No.	*Solvent systems*	*pH*	*R_F value of glucagon*	*R_F value of secretin*
1a	*n*-butanol–ethanol–0.2 *N* ammonium hydroxide (4:1:5)	9.4	0.51	
1b	*n*-butanol–ethanol–0.2 *N* ammoniumhydroxide (4:1:5)	9.0	0.51	0.95
2a	*n*-butanol–ethanol–benzene–0.2 *N* ammoniumhydroxide (3:1:1:5)	9.4	0.05	
2b	*n*-butanol–ethanol–benzene–0.2 *N* ammoniumhydroxide (3:1:1:5)	9.0		0.64
3a	*n*-butanol–ethanol–benzene–0.2 *N* ammoniumhydroxide (12:3:2:17)	9.0	0.15	0.53
3b	*n*-butanol–ethanol–benzene–0.2 *N* ammoniumhydroxide (12:3:2:17)	8.0		0.84
3c	*n*-butanol–ethanol–benzene–0.2 *N* acetic acid (12:3:2:17)	5.0		0.49
3d	*n*-butanol–ethanol–benzene–0.2 *N* acetic acid (12:3:2:17)	4.6		0.39
3e	*n*-butanol–ethanol–benzene–0.2 *N* acetic acid (12:3:2:17)	2.9		0.05
4	*n*-butanol–ethanol–benzene–0.2 *N* ammoniumhydroxide (5:2:1:8)	9.0	0.23	
5	*n*-butanol–ethanol–benzene–0.2 *N* ammoniumhydroxide (4:1:1:6)	9.0		0.53

have been developed which fit this range of suitability for both glucagon and secretin. Since an R_F value of about 0.3 is considered optimal, the systems of choice would be system 4 for glucagon and system 3d for secretin. Figs. 1 and 2 show representative plots of the chromatograms for a few of the systems examined.

Although the two hormones have remarkably similar amino acid sequences (about half of the amino acids occupy analogous positions and the charge distribution is quite similar)[4,8], they behave quite differently in every solvent system. For glucagon, it was possible to adjust the R_F to a suitable value by altering the polarity of the system (which was done by changing the proportion of benzene). A possible qualitative explanation of this effect on the partition coefficient is that the solubility of glucagon in the mobile phase relative to the stationary phase is altered sufficiently by this polarity change alone, and that this effect dominates other possible effects such as pH and absorption. The result is that glucagon has a different residence time in the gel, and the changes in elution volume and therefore in R_F can be predicted simply by changes in polarity.

This simple explanation, however, is not satisfactory to explain the behavior of secretin which is rapidly eluted (high R_F values) even with a relatively non-polar solvent system such as 3b (Table I). For secretin, it was found necessary to decrease the pH of the system to a slightly acidic value in order to obtain a reasonable R_F. As seen in Table I (systems 3a–e) secretin showed great sensitivity toward pH changes in the same solvent system, and it was necessary to employ a system whose lower phase pH was between 4.5 and 4.7 in order to obtain an R_F-value near 0.3.

Apparently there are several subtle solubility and partitioning effects which interact simultaneously to determine the elution characteristics for this polypeptide. The interdependence of the charged nature of the polypeptide at various pH values[9], the adsorption properties of the polypeptide on the Sephadex gel, and actual partitioning effects between the two phases apparently all affect the observed R_F value.

From these studies it appears that partition chromatography on Sephadex offers significant promise for continued and expanded use in the separation and purification of polypeptides and other natural products[10,11]. The method provides a convenient, rapid and practical adjunct, and in some cases an alternative to other purification procedures such as gel filtration, ion-exchange chromatography and counter-current distribution.

We wish to thank Dr. DONALD YAMASHIRO for his critical comments. We sincerely thank Drs. VIKTOR MUTT and ERIK JORPES for the generous gift of secretin. The technical assistance of Mr. ROBERT GRUBB and Mr. JOEL ROSS is gratefully acknowledged. This work was supported in part by the U.S. Public Health Service, Grant No. NIH-71-2125.

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Received September 13th, 1971