COLUMN CHROMATOGRAPHY OF *BACTERIUM CADAVERIS* EXTRACTS USING CARBOXYMETHYLCELLULOSE

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During the past few years the chromatographic separation of proteins on columns of cellulose ion exchangers using gradient elution techniques has won wide approval. However, the lack of standard experimental conditions and of chromatographic characteristics of the separated substances does not often permit comparison of the data of different authors. This paper gives the results of some experiments on the chromatography of proteins on carboxymethylcellulose columns and deals with the possibility of using the elution constant as a new chromatographic characteristic for the various substances.

EXPERIMENTAL AND DISCUSSION

Five protein fractions possessing asparaginase activity were obtained by chromatographing an aqueous extract of *B. cadaveris* on a carboxymethylcellulose (CMC) column. Fraction I consists of a protein that is not adsorbed on CMC from 0.01 *M* pyridine solution. Fractions II, III and IV were extracted from the CMC column using the gradient elution technique as described by PARR[1] at concentrations of pyridine of 0.023, 0.027 and 0.032 *M*, respectively. Fraction V was eluted by 0.1 *M* pyridine or 0.1 *M* Na_2HPO_4 (Fig. 1). On rechromatography of fraction I under the conditions described, two subfractions were obtained. One of these corresponds to fraction I, the other to fraction V of the initial chromatogram. On rechromatography of fractions V and IV, we obtained in a similar way two and three subfractions respectively. In the case of fraction V, the subfractions correspond to fractions V and IV, in that of fraction IV, to fractions III, IV and V of the initial chromatogram.

Fraction IV contains about 1/10,000 of the total protein applied to the column. The specific activity of asparaginase in this fraction is increased 250-fold as compared with that of the initial *B. cadaveris* acetone powder.

The change in the eluent concentration on elution by the method of PARR can be expressed by the equation

$$C = C_2 - (C_2 - C_1)\left(1 - \frac{v}{V}\right)^{A_2/A_1} \qquad (1)$$

where C is the eluent concentration at the moment when a volume v of the eluate has

passed the column; C_1 and C_2 are the concentrations of the solution in the mixing and reservoir chambers; A_1 and A_2 are the cross-sectional areas of the mixing and

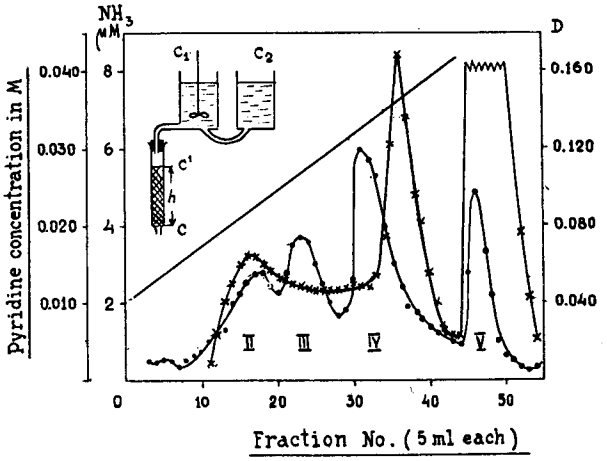

Fig. 1. Column chromatography of *B. cadaveris* extract using CMC. Column size 2.0 × 6.5 cm; q = 2.5 ml; washed with 0.010 M pyridine. 25 ml of aqueous extract containing 180 mg protein applied on the column after dialysis against 0.010 M pyridine. Before starting elution by the concentration gradient procedure, the column was washed with 240 ml of 0.010 M pyridine. Elution with aqueous solutions of pyridine: C_1 = 0.010 M, C_2 = 0.085 M, V = 500 ml, $G = 3.7 \cdot 10^{-4}$. Asparaginase activity was determined by the method of MARDASHEV AND LESTROVAYA[2], protein as described by LOWRY[3]. (●—●—●) μmoles NH_3; (×—×—×) optical density (D); (————) pyridine concentration.

reservoir chambers; V is their total volume. If $A_1 = A_2$, the equation becomes linear, which is the most favourable case for most separations.

$$C = C_1 + \frac{v}{V}(C_2 - C_1) \tag{2}$$

However, the separations depend on the actual concentration gradient along the length of the column, which varies not only with C_1, C_2 and V, but also with the cross-section of the column. Therefore, eqn. (2) fails to characterize completely the conditions of separation in gradient elution. To evaluate the concentration gradient along the length of the column we suggest using the value of the change in the concentration of the solution per unit length of the column. If the distance from the base to the top of the column is plotted against the concentration of the solution at various levels, the straight line obtained will represent the change in concentration along the length of the column. The slope of the line determined by angle α expresses the gradient value G

$$G = \operatorname{tg}\alpha = \frac{\Delta C}{h} \tag{3}$$

If a volume of solution v, has passed through the column at a certain moment of time, the concentration at the outflow of the column becomes C; the concentration

at a certain distance h from the bottom of the column will at that moment be C'; then

$$G = \frac{\Delta C}{h} = \frac{C' - C}{h} \tag{4}$$

It follows from eqn. (2) that

$$C' = C_1 + \frac{v + v_{bH}}{V}(C_2 - C_1) \tag{5}$$

where v_{bH} is the volume of exchangeable liquid in the pores of a column of length h. Substituting the values of C and C' from eqn. (2) and (5) into eqn. (4), we find:

$$G = \frac{v_{bH}}{h} \cdot \frac{C_2 - C_1}{V} \tag{6}$$

The factor v_{bH}/h is the volume of liquid in the pores of a 1 cm long column, *i.e.*, the "specific pore volume" of the column. Denoting it as q, we get

$$G = q\frac{C_2 - C_1}{V} \tag{7}$$

The value of G represents the actual change in concentration of the eluent along the length of the column. Since q depends on the column radius, the steepness of the gradient change in columns with different radii will be different, other conditions determined by eqn. (2) being constant. Identical conditions of separation are defined by eqns. (8) or (9):

$$G = G' \tag{8}$$

$$q\frac{C_2 - C_1}{V} = q'\frac{C'_2 - C_1}{V'} \tag{9}$$

For $q = 2.5$ [column i.d. (inner diameter) 2 cm], $C_1 = 0.010\ M$, $C_2 = 0.085\ M$ and $V = 500$ ml, the value of G will be:

$$2.5\frac{0.085 - 0.010}{500} = 3.7 \cdot 10^{-4}$$

In order to have the same gradient operating in a column with $q = 0.6$ ml (column i.d. 1 cm) and the C_1 and V values the same as in the previous experiment, it is necessary to change C_2:

$$C_2 = \frac{VG}{q} + C_1 = \frac{500 \times 3.7 \times 10^{-4}}{0.6} + 0.010 = 0.318$$

If it is required to keep C_1 and C_2 constant, the V value must be changed according to the relationship:

$$\frac{q}{V} = \frac{q'}{V'}$$

which is derived from eqn. (9) if $C_2 = C_2'$.

With such a characteristic of the gradient, the relative distances between the peaks on the chromatogram of a certain mixture will remain the same, independent of the dimensions of the column used.

It can be shown that:

$$\frac{v}{q} = \frac{v'}{q'} = \text{const.} = K_e \qquad (10)$$

where v and v' are the volumes of solutions that have passed through columns with the specific pore volumes q and q' up to the moment when the concentration C in both effluents becomes equal, under the condition that $G = G'$. Therefore, the ratio of the volume of effluent collected from the beginning of the elution to the moment of appearance of the maximal concentration of the given substance, to the specific pore volume of the column—the elution constant K_e—may be considered as an accurate chromatographic constant of a substance and may be used for purposes of identification. From certain theoretical considerations and experimental data[4,5] it follows that in gradient elution the rate of migration of the zone of a substance tends to attain a maximum equal to the rate of flow of the solvent itself. This means that K_e is independent of the column length, provided the length is not less than that necessary for attaining maximum velocity (and simultaneously best separation) for the concentration gradient selected.

The K_e values for the three asparaginase peaks at $G = 3.7 \cdot 10^{-4}$ obtained in columns of various sizes are given in Table I.

TABLE I

Column size cm	q	K_e		
		II	*III*	*IV*
2.0 × 6.5*	2.5	36.0	46.0	60.0
1.2 × 10	0.9	37.7	46.4	63.3
1.0 × 16	0.6	38.2	48.3	66.6

* The first figure represents the inner diameter, the second the length.

SUMMARY

1. A method is suggested for the evaluation of the changes in concentration of an eluent along the length of a column in gradient elution chromatography.

2. A new chromatographic constant—the elution constant K_e—is proposed for substances being chromatographed. K_e is defined as the ratio of the effluent volume, collected up to the moment of appearance of the maximal concentration of the substance being chromatographed, to the specific pore volume of the column.

3. The elution constants have been determined for three protein fractions of a *B. cadaveris* extract possessing asparaginase activity.

4. By means of column chromatography using carboxymethylcellulose, it was possible to attain a 250-fold purification of asparaginase.

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