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Note

Molecular sieve effects in ion-exchange chromatographic separations of oligopeptides

HARTMUT FRANK and HANSPAUL HAGENMAIER
Chemisches Institut der Universität Tübingen, D-74 Tübingen (G.F.R.)
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In the last few years, we have carried out many ion-exchange chromatographic analyses of oligopeptides[1,2], synthesized by the Merrifield procedure[3]. In the course of these analyses, we noticed that, in general, the elution volumes of oligopeptides become smaller with increasing length of the peptide chain. In this paper, we describe how the elution behavior of oligopeptides on a cation exchanger with a 7% cross-linked polystyrene matrix can be correlated with their molecular weights.

EXPERIMENTAL

A 20-μmole amount of synthetic oligopeptide was dissolved in 5 ml 0.1 *M* pyridine acetate buffer, pH 2.8, containing 5 μmole of cysteic acid. A 0.5-ml volume of this solution was chromatographed on a modified automated amino acid analyzer (Beckman Unichrom)[1] under the following conditions: cation-exchange resin, Beckman M72; column dimensions, 60 × 0.9 cm; jacket temperature, 50°; buffer flow-rate, 84 ml/h; elution volume of cysteic acid (V_0), 13.5 ml; eluent, linear gradient of 0.1 *M* pyridine in 28% (v/v) acetic acid, pH 2.8, and 0.1 *M* pyridine in 12% (v/v) acetic acid, pH 3.3. Peaks were detected after reaction of the eluate with ninhydrin reagent[4].

Each oligopeptide was chromatographed at least four times. For each peak, the number of theoretical plates, N, was calculated according to the Glueckauf equation[5]:

$$N = 8 \cdot \frac{(V_e - V_0)^2}{B^2} \text{ or } \sqrt{N} = 2.84 \cdot \frac{V_e - V_0}{B}$$

where V_e = elution volume of the oligopeptide, V_0 = elution volume of cysteic acid and B = peak width at 0.368 of the maximum height.

RESULTS AND DISCUSSION

Table I shows the elution volumes, peak widths and number of theoretical plates for all of the oligopeptides and amino acids chromatographed.

On examining the elution diagrams of peptide mixtures[1,2], we noticed that (i) starting with tripeptides, the elution volumes become smaller with increasing chain

TABLE I

PROPERTIES OF OLIGOPEPTIDES AND AMINO ACIDS

M = molecular weight; V_e = elution volume; V_0 = elution volume of cysteic acid; B = peak width at 0.368 of maximum height; N = number of theoretical plates.

No.	*Compound*	*Log M*	$V_e - V_0$	*B*	*N*	$\sqrt{N}$
1	Pro	2.061	136	4.45	7 485	86.4
2	Ala	1.950	336	7.76	14 980	122.0
3	Gly	1.876	353	7.22	19 170	138.1
4	Val	2.069	307	7.50	13 420	116.0
5	Ala–Pro	2.270	424	15.09	6 315	79.5
6	Gly–Pro	2.236	382	11.97	8 320	91.3
7	Gly–Ala–Pro	2.386	210	8.00	5 550	74.5
8	Gly–Val–Gly	2.364	451	16.40	6 070	77.8
9	Val–Gly–Ala–Pro	2.535	130	7.94	2 150	46.4
10	Gly–Val–Ala–Pro	2.535	122	6.65	2 700	52.0
11	Gly–Gly–Ala–Pro	2.478	136	6.71	3 290	57.3
12	Gly–Val–Gly–Ala–Pro	2.602	92	7.01	1 375	37.1
13	Ile–(12)	2.710	53	5.87	650	25.5
14	Ala–Ile–(12)	2.766	29	4.00	420	20.5
15	Thr–Ala–Ile–(12)	2.836	17	4.35	122	11.1
16	Gln–Ala–Ile–(12)	2.852	18	4.65	120	10.9
17	Gln–Thr–Ala–Ile–(12)	2.910	6	5.40	10	3.2
18	Gln–Thr–Ala–Ile–(12)	2.895	10	4.81	35	5.9
19	Gln–Thr–Thr–Ala–Ile–(12)	2.961	3	4.90	3	1.7

length, and (ii) two compounds with similar elution volumes can be roughly classified on the basis of peak width: the substance with a greater peak width is always the one with a higher molecular weight. The latter observation is especially noticeable when the peaks obtained for di- and tripeptides are compared with those obtained for amino acids. Amino acid 1 (Table I) has an elution volume similar to those of oligopeptides 9, 10 and 11, while amino acids 2, 3 and 4 have elution volumes similar to those of oligopeptides 5, 6 and 8. The peak widths for the amino acids are, however, only about half those for peptides. A similar observation was made when we examined published ion-exchange chromatograms of partial hydrolyzates of proteins[6–10]. In most instances, we found that of two peptides with similar elution volumes, the one with the greater peak width was the one with the higher molecular weight.

For a separation on the basis of ion-exchange phenomena alone, one would expect that elution from a cation-exchange column would occur in the order of increasing pK_1 values. In general, the pK_1 values of peptides increase with increasing chain length up to a certain limit[11], provided that the peptides consist of bifunctional amino acids only. As the elution volume is proportional to the selectivity constant[12], one would therefore expect elution to occur in the order amino acids, dipeptides, tripeptides, etc. On the other hand for a constant number of theoretical plates, N, the peak width, B is dependent only upon the distribution coefficient, K:

$$B \approx \frac{K}{\sqrt{N}}$$

The ratio of the elution volume to the peak width should therefore remain constant

for all distribution coefficients. If this is not the case, as our chromatograms of oligopeptides show, we must assume that the number of available ion-exchange groups and therefore the number of theoretical plates become smaller with increasing molecular size. The result is equivalent to a molecular sieve effect.

In Fig. 1, elution volumes of amino acids and peptides are plotted *versus* the logarithms of molecular weights. With the exception of proline and oligopeptides 5, 6 and 8 (Table I), all points can be connected by a curve that is similar in shape to that obtained in gel filtration of polypeptides[13–16].

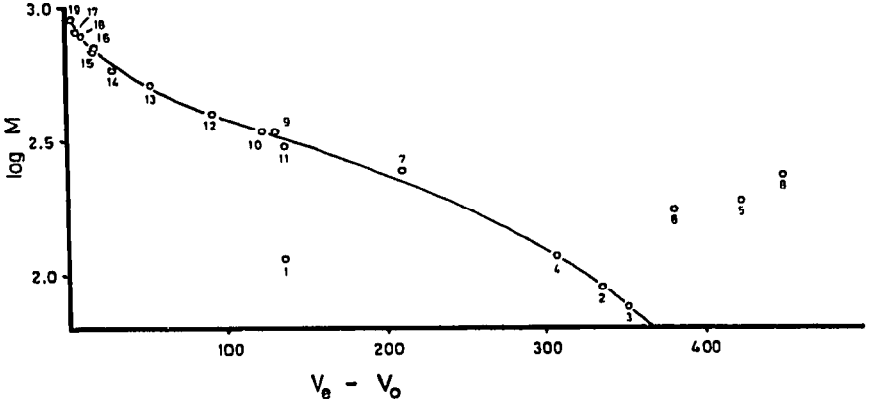

Fig. 1. Logarithms of molecular weights of the peptides and amino acids listed in Table I *versus* elution volume.

A linear relationship is obtained when the logarithms of the molecular weights are plotted against $\sqrt{N'}$. $\sqrt{N'}$ can be considered as a "reduced" elution volume, an elution volume from which that part has been eliminated which depends on ionic interactions:

$$\sqrt{N'} = \frac{2.84}{B} \cdot (V_e - V_0)$$

We find the empirical relationship

$$\log M = [2.93 - 0.0078\sqrt{N'}] \pm 0.05^*$$

where M is the molecular weight.

Proline behaves like a molecule of greater molecular size, and slight deviations are also found for the largest molecules studied. These are eluted slightly after cysteic acid, owing to the fact that a small number of ion-exchange groups at the surface of the exchanger particles are accessible even to the larger molecules. Not all the deviations are readily explicable. The model is crude and does not take into consideration other effects such as adsorption and hydrophobic interactions. Peptides with side-chain functional groups also fit poorly in the described elution scheme.

The described effect could, in principle, be used for molecular weight deter-

* Observed maximum deviation, with the exception of proline.

minations of oligopeptides, but it is a rather laborious task to obtain the necessary calibration curve. Studies of the type described are useful in determining the properties of ion-exchange resins with regard to peptide separation. Fig. 2 shows that the 7% cross-linked ion-exchange resin used gives excellent resolution for peptides up to a molecular weight of about 800. For the separation of mixtures that contain substances with higher molecular weights, ion-exchange resins with lower degrees of cross-linking have to be used, which, owing to greater volume changes with changes in ionic strength, are more inconvenient to handle.

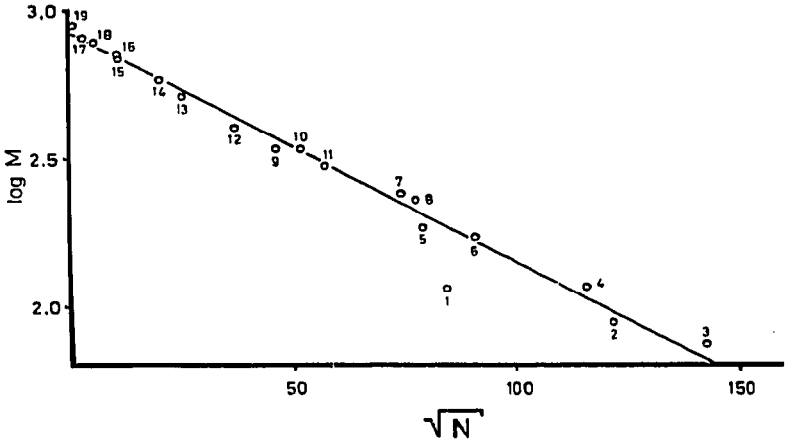

Fig. 2. Logarithms of molecular weights of the peptides and amino acids listed in Table I *versus* $\sqrt{N}$, where N is the number of theoretical plates calculated for each compound.

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