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Effect of column length and elution mechanism on the separation of proteins by reversed-phase high-performance liquid chromatography

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ABSTRACT

Different theories have been proposed for the elution of proteins in reversed-phase high-performance liquid chromatography. To establish the correct elution mechanism, the effects of column length and the concentration of the organic solvent on column efficiency and the elution of high- and low-molecular-weight compounds were examined. It was concluded that protein elution principally involves the same retention process as for low-molecular-weight compounds, although the influence of partition is small under steep gradient conditions. In accordance with this, wide-pore packings in a short column (35 mm) gave excellent separations of proteins and were usable with a wide range of gradients.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) has become an important technique for protein analysis owing to its high resolution [1–9]. A gradient elution technique is commonly used for protein analysis. However, the behaviour of proteins is different from that of low-molecular-weight compounds, and new mechanisms, such as the so-called "on–off" or "critical behaviour" mechanism, which differs from that of conventional partitioning, have been proposed [10–22]. According to these mechanisms, proteins are trapped at the top of the column initially and then, after the concentration of organic solvent reaches a value sufficient for protein desorption from the packing material, the proteins are eluted through the column without further interaction with the packing. Recently, Nimura *et al.* [23] reported a high-speed protein analysis based on this adsorption–desorption mechanism. If these proposed concepts are valid, the structure of the packing material, such as pore size and particle size, and column length should have no influence on protein elution. Nevertheless, many studies concerning the influence of pore size and particle size on protein analysis have appeared [24–30]. Further, Snyder and co-workers [31–39] reported that protein elution can be explained by the same mechanism as for of small molecules. Therefore, the influence of the structure of the packing material and the protein elution mechanism still remain to be established. In order to clarify the mechanism of protein elution and to identify the best packings for protein analysis, we have studied the elution of proteins in RP-HPLC with variations in the column length and the concentration of organic solvent.

Our previous studies demonstrated that small amounts of metallic impurities in packing materials have a greater influence than pore size, alkyl chain length and residual silanols on protein elution [40]. In this study, capsule-type packings, the surface of

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which is covered with silicone polymer, were used to avoid the influence of metallic impurities in silica gels.

EXPERIMENTAL

Reagents and materials

Capsule-type packings having C_8-alkyl groups were prepared by using high-purity silica gels [41]. The pore size was 300 Å and particle diameter 5 μm. The materials were packed into 0.46 cm I.D. stainless-steel tubes of length 10, 35, 100 and 250 mm.

Bovine serum albumin (BSA), ovalbumin, cytochrome *c*, lysozyme, myoglobin and ribonuclease A were purchased from Sigma (St. Louis, MO, USA), peptides, alkylphenones (C_4–C_6) and trifluoroacetic acid (TFA) from Wako (Osaka, Japan) and HPLC-grade acetonitrile from Nacalai Tesque (Kyoto, Japan).Water was purified using a Milli-R/Q system (Millipore, Bedford, MA, USA).

Apparatus

A Shimadzu LC-6A system equipped with a high-pressure gradient mixer was used.Elution gradients were prepared from solvents A and B (A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile) and the gradient programmes are illustrated in the figures. The column temperature was maintained at 40°C, the flow-rate was 1.5 ml/min and the eluate was monitored at 214 nm.

Each protein was dissolved in water at a concentration of 1 mg/ml and each solution was mixed as an equal volume. An aliquot of the mixture (20 μl) was injected into the HPLC system.

RESULTS AND DISCUSSION

Gradient elution

Fig. 1 shows the effect of column length on the elution profile. The retentions of low-molecular-weight compounds, alkylphenones and peptides, decreased as the column was made shorter. The retention capacity of the 35-mm column was only 10–30% of that of the 250-mm column. On the other hand, the decrease in protein retentions was smaller than that with low-molecular-weight substances. Even the 10-mm column maintained more than 60% of the retention capacity as compared with the 250-mm column.

Fig. 2 shows the influence of column length on band width. The values for low-molecular-weight compounds became smaller as the column length became longer, from 10 to 250 mm, as shown in Fig. 2A. These results indicate that the column efficiency for small compounds is improved by increasing the column length. In contrast, the band width of proteins was almost constant for lengths >35 mm, as shown in Fig. 2B. These results indicate that for proteins, a longer column does not give a high separation efficiency. Hence there is a clear difference

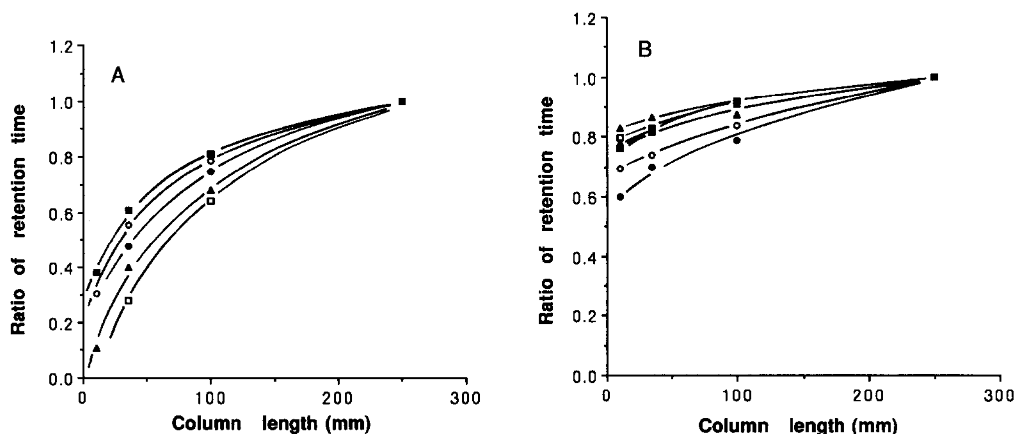

Fig. 1 Effect of column length on retention time of solutes of various molecular size. (A) Low-molecular-weight compounds: ● = C_4-phenone; ○ = C_5-phenone; ■ = C_6-phenone; □ = Lys-bradykinin; ▲ = Met-Lys-bradykinin. (B) Proteins: ● = ribonuclease A; ○ = cytochrome *c*; ■ = lysozyme; □ = BSA; ▲ = myoglobin; △ = ovalbumin. Conditions: linear gradient from 15 to 60% eluent B in 15 min.

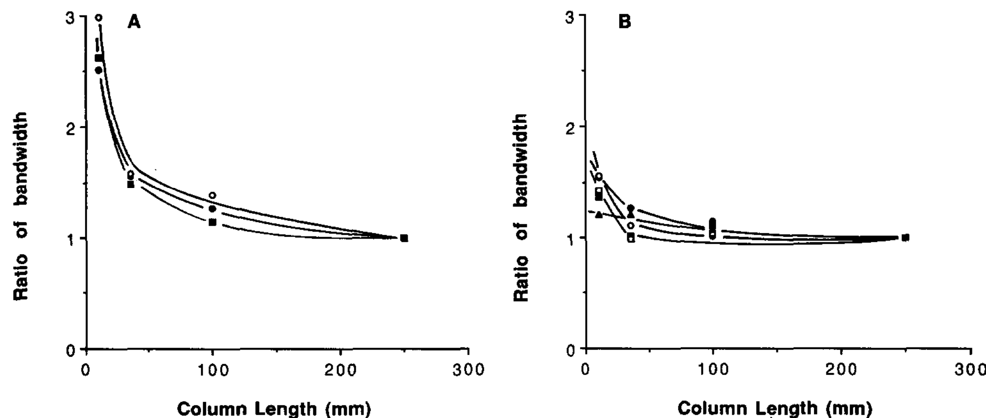

Fig. 2. Effect of column length on peak band width. (A) Low-molecular-weight compounds: ● = C_4-phenone; ○ = C_5-phenone; ■ = C_6-phenone. (B) Proteins: ● = ribonuclease A; ○ = lysozyme; ■ = BSA; △ = myoglobin; ▲ = ovalbumin. Conditions as in Fig. 1.

in elution behaviour between proteins and low-molecular-weight compounds.

The effect of the initial concentration of the organic solvent on elution behaviour is shown in Fig. 3. The retention times of small compounds decreased in proportion to the increase in initial organic solvent concentration. Proteins showed similar behaviour. However, when the concentration of organic solvent exceeded a critical value, which was different for each protein, the elution rate increased rapidly. This phenomenon indicates that proteins are no longer retained when the organic solvent concentration exceeds this critical value. This is a second difference between proteins and low-molecular-weight compounds. These phenomena suggested us that protein elution involves a different mechanism to that from the theory for low-molecular-weight compounds.

Isocratic elution

The effect of column length on capacity factor underisocratic conditions is shown in Fig. 4. Under isocratic conditions, high-molecular-weight compounds showed similar elution profiles to low-molecular-weight compounds, *i.e.*, delayed retentions and higher theoretical plate numbers were obtained with longer columns.

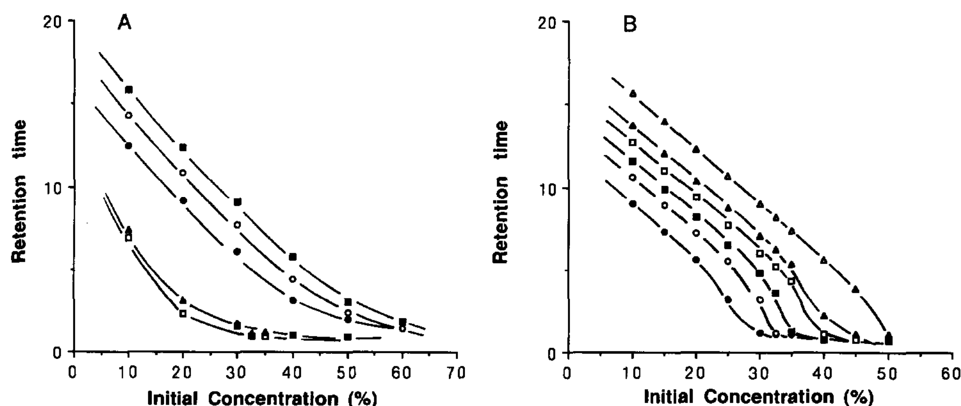

Fig. 3. Effect of initial concentration of organic solvent on retention time for (A) low-molecular-weight compounds and (B) proteins. Symbols as in Fig. 1. Conditions: linear gradient from the indicated initial concentration to 60% eluent B at 3%/min; column length, 250 mm.

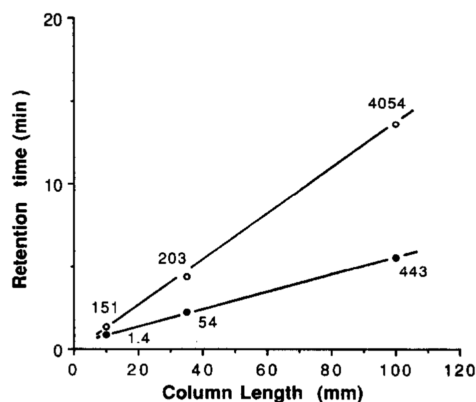

Fig. 4 Effect of column length on retention time and column plate number. The ratio of retention time is represented on the basis of the retention time of a 250-mm column. ● = Lysozyme; ○ = C_6-phenone. Numbers indicate the column plate number. Conditions; isocratic, 31% eluent B.

Fig. 5 shows the variation of the capacity factor with change in the concentration of the organic solvent. The capacity factors of low-molecular-weight solutes decreased linearly with increase in organic solvent concentration. In isocratic elution, proteins gave similar results to small compounds, although the changes in capacity factors were far greater for the proteins. From this result, it is concluded that protein elution in RP-HPLC is not based on the putative "on–off" or "critical behaviour" mechanism, but on the same repeated partition mode as operates for low-molecular-weight substances. However, the influence of organic solvent concentration on the capacity factors of proteins is very large. This steep change in capacity factor is the reason why proteins show curious behaviour in gradient elution.

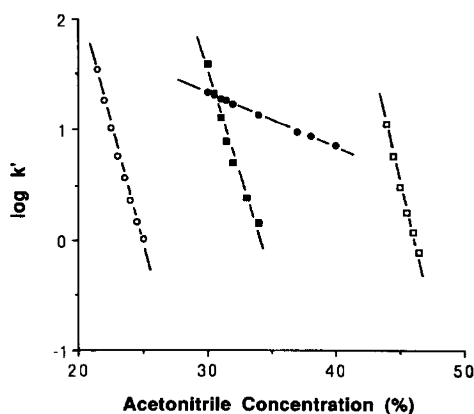

Fig. 5. Variation of capacity factor (k') with the concentration of acetonitrile. Column length, 250 mm. ● = C_6-phenone; ○ = ribonuclease A; ■ = lysozyme; □ = ovalbumin.

Protein elution mechanism

In gradient elution, the behaviour of proteins was apparently different from that of low-molecular-weight compounds, as if a peculiar "on–off" or "critical behaviour" elution mechanism were operating. These observations have been explained by a model proposed by Armstrong and co-workers [16,19], in which macromolecules are precipitated at the head of a column in the presence of a low-strength mobile phase and they never migrate until the mobile phase strength increases to some critical composition which induces dissolution and subsequent elution. However from our isocratic results, it was considered that protein elution involves essentially the same partition mode as for low-molecular-weight compounds. Evidently, as shown in Fig. 5, the plot of k' *versus* volume of organic solvent is far steeper than that for hexanophenone.

Snyder and co-workers [31,37,38] have shown that the separation of proteins by gradient elution can be described quantitatively by a model based on a small-molecule separation mechanism.The retention time in gradient elution can be predicted from data for corresponding isocratic systems. For isocratic systems there is a linear relationship between the k' value of a sample and the volume fraction of organic solvent (φ) in the mobile phase:

$$\log k' = \log K_w - S\varphi$$

where S is the slope of the plot of log k' *vs.* φ, and K_w is the k' value for water as mobile phase. In gradient elution, at any point in time during the separation, the composition of the mobile phase in contact with the solute band determines an isocratic value of k', and this in turn determines the instantaneous velocity of the band through the column. Consequently, a band elutes under a given set of gradient conditions with some average value of k' [38]. It was demonstrated that the large values of S is the reason for the curious behaviour in gradient elution.

Because proteins take a large value of K_w, despite the large value of S for proteins, k' will not change much with increase in organic solvent with a low-strength mobile phase. However, after the organic solvent has reached a sufficient concentration for elution, k' changes considerably owing to the large value of S, as shown in Fig. 5. This is the reason for the unchanging retention time in Fig. 1B and critical point in Fig. 3B.

The range of concentration of organic solvent within which proteins can have the appropriate value of k' for separation is very narrow, at most within a few per cent. This means that when the concentration of the organic solvent is less than the lower limit, the k' value of proteins become very large, and so proteins move through the column only very slowly, as reported by Bussolo and Gant [39]. In contrast, at an organic solvent concentration even slightly over the upper limit, the k' value of proteins becomes too small for retention on the stationary phase. Therefore, under gradient conditions, an injected protein moves very slowly in the column until the organic solvent concentration reaches the value at which the protein has the appropriate value of k' for elution. Beyond this point, the protein moves through the column, being repeatedly partitioned on the stationary phase, for a while until the organic solvent concentration reaches the upper limit. Above this upper limit, the protein has only small value of k', so it moves through the column very rapidly and the interaction with the stationary phase is very small.

Because the suitable range of organic solvent concentration for the partition mode is very narrow, as described above, when the gradient rate is high the participation of the partition mode becomes very small. As a result, the peak shape and elution profile of proteins are almost independent of the column length, as shown in Figs. 1 and 6. Differences in retention time essentially reflect the void volume of each column, and under this condition protein elution apparently looks like "on–off" or "critical behaviour" elution. When the gradient rate was low, a shorter column exhibited peak broadening, as shown in Fig. 7. This result is ex-

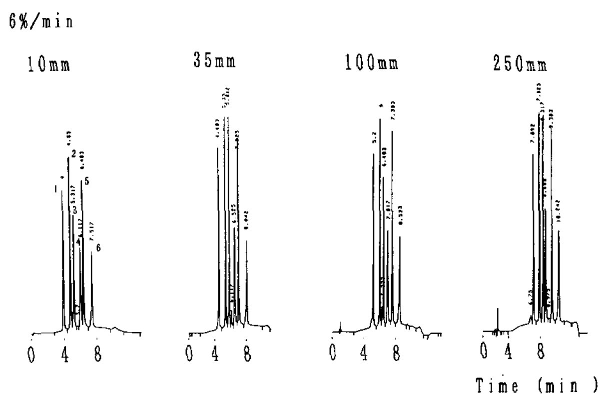

Fig. 6. Effect of column length (10–250 mm) on protein elution under fast gradient conditions. Conditions: linear gradient from 15 to 75% eluent B in 10 min. Peaks: 1 = ribonuclease A; 2 = cytochrome c; 3 = lysozyme; 4 = BSA; 5 = myoglobin; 6 = ovalbumin.

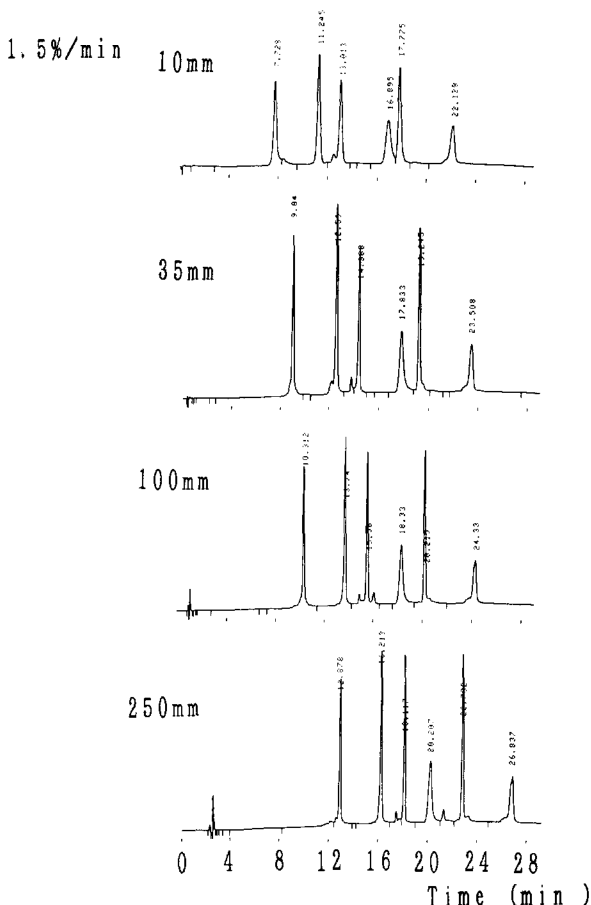

Fig. 7. Effect of column length (10–250 mm) on protein elution under slow gradient conditions. Conditions: linear gradient from 15 to 60% eluent B in 30 min. Peaks as in Fig. 6.

plained by the greater participation of the partition mode. Under such a slow gradient rate condition, the increase in organic solvent concentration is slower, so the time for which the partition mode dominates is extended. The peak widening obtained in a short column is considered to reflect the limited surface area where the protein can be repeatedly partitioned on the stationary phase. If the protein elution occurred in an "on–off" or "critical behaviour" elution mode, peak broadening should not be seen even in a short column. Hence the results also support the idea that protein elution involves a partition mode.

In conclusion, this study has indicated that protein elution occurs principally in the partition mode. Therefore, wide-pore packings, which have a sufficient surface area for protein partition, are expected to be more suitable for protein analysis than small-pore or non-porous packings, especially under low gradient rate conditions.

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