SCALING UP OF MEDIUM-PERFORMANCE GEL FILTRATION CHROMATOGRAPHY OF PROTEINS

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Since gel filtration chromatography with particles having a relatively small diameter and high mechanical stability (medium-performance gel filtration chromatography, MPGFC) is useful not only in scaling up the separation of proteins but also in reducing the separation time, HETP of proteins and pressure drop were measured for MPGFC (particle diameter, 44 µm) columns of various column dimensions. The smallest column was 1.0 cm in diameter and 30 cm in length (24 cm³), while the largest was 9 × 90 cm (5726 cm³). The dependences of HETP and of pressure drop per unit length on column dimensions were found to be negligible for these columns. The sample volume dependence of HETP was predicted well on the basis of the HETP at small sample volumes, when the protein concentration is low. However, when the protein concentration was increased to above 3%, HETP increased sharply. This increase of HETP was considered to be due to the relative difference between the viscosity of the sample and that of the elution buffer. From the above results, we expect that the scaling up of MPGFC of proteins can be easily carried out on the basis of the data obtained with a small column.

Introduction

Gel filtration chromatography (GFC) has been widely used for the separation and purification of proteins. Usually, it is operated at low pressure and low flow rates, since dextran or agarose gels for protein separation are very soft and compressible. In addition, scaling-up with such compressible gels requires some special devices for supporting gel beds, as reviewed by Janson and Hedman. These devices, however, might decrease the column efficiency considerably.

On the other hand, several rigid or semi-rigid gels for protein separation, which are resistant to pressures up to several atmospheres, are now commercially available. These gels may not only increase speed of separation but also make scaling-up easy. Furthermore, the particle diameter of such gels is usually much smaller than that of conventional soft gels. With such small gels, column efficiency is also expected to be much higher than that of conventional GFC. Therefore, we adopt the term "medium-performance gel filtration chromatography, (MPGFC)" for this type of GFC. It is quite important to examine whether a large MPGFC column can be designed on the basis of the data obtained with a small one.

In this study, the dependence of HETP (height equivalent to a theoretical plate) and of pressure drop per unit column length on the column dimensions are examined with Toyopearl gels, which are mechanically stable and have a relatively small particle diameter (20-60 µm). Special attention is paid to the method of sample introduction and of packing gels. Since a large amount of proteins is applied to the column in preparative chromatography, the effects of sample volume and concentration on HETP were also investigated.

1. Experimental

1.1 Materials

Myoglobin (abbreviated Mb) from equine skeletal muscle (Sigma, Cat. No. M0630) and five-times crystallized ovalbumin (abbreviated OA, Seikagaku Kogyo) were employed without further purification. Elution curves of Mb and OA purified once by GFC had almost the same width as those of the original material although a small amount of leading and/or tailing decreased. Therefore, the contribution of heterogeneity of these proteins to zone spreading was considered to be negligible. Blue Dextran 2000 and Dextran T-10 were obtained from Pharmacia Fine Chemicals. The buffer used was 14 mM Tris-HCl (pH 7.9) containing 0.3 M NaCl. The gel employed in this study was Toyopearl HW55F (Toyo Soda). Its average particle diameter \( d_p \) calculated by the equation proposed by Nakanishi et al. is 44 µm.
1.2 Apparatus for column experiment

The chromatographic column tubes employed were purchased from Amicon. Since the material of the column tubes was transparent, we were able to observe the movement of the sample zones during elution by using colored substances such as Mb and vitamin B12. The concentrations of Mb and vitamin B12 at the outlet of the column were measured with a UV-detector (UVIDEC 100-II, JASCO) and recorded continuously. A peristaltic pump (Model SJ-1211H, Atto) was employed. To obtain high volumetric flow rates for larger-diameter columns, two pumps were used in parallel. The experiments were carried out in a constant-temperature room maintained at 20 ± 0.5°C. Since the packing procedure and the method of sample introduction are very important factors affecting elution behavior, they will be described in RESULTS.

1.3 Data acquisition

A void fraction $\varepsilon$ was determined from the peak retention time $t_R$ of a Blue Dextran 2000 pulse. Similarly, the distribution coefficient $K$ was determined from the measurements of $t_R$ according to Eq. (1).\(^\text{1.2,13}\)

$$t_R = t_{R0} + (t_0/2) = (Z/u)(1 + HK) + (t_0/2) \quad (1)$$

where $t_{R0}$ is $t_R$ at infinitely small sample volumes and $t_0$ is the sample injection time ($=\text{sample volume}/v$).

When the elution curve is Gaussian, its variance $\sigma^2$ is calculated with the width of the curve measured at $C = \exp(-1) \times C_{max} = 0.3679 \times C_{max}$ (see Fig. 1 for the meanings of these symbols) as

$$\sigma^2 = W^2/8 \quad (2)$$

Height equivalent to a theoretical plate, $HETP$, was then calculated from the observed elution curves on the basis of Eq. (3).\(^\text{1.2,13}\)

$$HETP = Z(\sigma/t_R)^2 = Z(W/t_R)^2/8 \quad (3)$$

The total pressure drop for packed columns $\Delta P_t$ and the pressure drop in connection tubes and column end fittings $\Delta P_c$ were measured by a mercury manometer at 20°C, and $\Delta P$ was calculated as $\Delta P = \Delta P_t - \Delta P_c$.

2. Results

2.1 Method of sample introduction

As already mentioned by several investigators,\(^\text{3,8,12}\) in order to examine precisely the dependence of $HETP$ on the column dimensions, an artificial increase of $HETP$ due to poor sample introduction should be avoided. Therefore, we employed the following method, although it is rather tedious and requires empirical skill. The top of the gel bed packed in the column was first made smooth and horizontal, and a filter paper (Whatman No. 2, England) having almost the same cross-sectional area as that of the column was put onto the top of the gel bed.

For the sample introduction, the elution buffer above the gel bed was first withdrawn. Then, a sample was poured onto the gel bed by use of a pipette, so carefully that the sample solution was equally distributed over the entire cross section of the gel bed. After the sample was introduced, the flow was started and the time was recorded. As soon as the sample disappeared, the elution buffer was poured carefully and the top end fitting was connected. We observed the movement of zones by using colored substances such as myoglobin and vitamin B12. When the observed zone was not horizontal but tilted, the top of the gel bed was re-made until the horizontal zone could be obtained. This method of sample introduction was highly reproducible, although its operation is rather cumbersome. It should be noted that even with this method the shape of the zone deviated from the horizontal gradually with increasing usages of the column, and $HETP$ was increased. This increase of $HETP$ was probably ascribable to the fouling of the gel bed which causes uneven flow there, since $HETP$ was recovered by re-packing the top part of the gel bed (usually 0.5–1 cm).

2.2 Packing method

Two packing methods were attempted in this study. The first method is that reported by Kato et al.,\(^\text{9}\) in which all the slurry was added in one operation. This type of packing method is most commonly employed for GFC columns, but is not suited for larger columns since it requires a slurry reservoir or an extension column tube. The second method is as follows. A slurry (40–50%) was first introduced into a column until it filled two-thirds of the column height. The column was left until 2 to 3 cm of gel bed appeared at the bottom of the column. Then, a pump was started which pumped an effluent from the bottom of the column at a flow rate of 1.2 cm\(^3\)/cm\(^2\) min. Occasionally, a supernatant of the slurry was removed and additional slurry was applied. After a desired height of gel bed was reached, the column was equilibrated for 2 to 3 hours with relatively high flow rates (1.4–1.5 cm\(^3\)/cm\(^2\) min).
The first and second methods gave almost the same HETPs. Furthermore, although the void fraction of a 1.6 x 30 cm column decreased from 0.38 to 0.33 with increasing flow rate during the packing from 1.2 cm³/cm² min to 3.0 cm³/cm² min in the second method, no appreciable difference was found in HETP values. Since the second method does not require any extension tube or reservoir, it was chosen as the packing method for this study.

2.3 Pressure drop

When soft compressible gels are employed, the flow rate increases linearly with pressure below a certain value (usually, much less than 0.1 MPa). Above this value, the pressure drop increases with flow rate rapidly due to gel bed compression. In addition, the maximum flow rate usually decreases with increasing column dimensions. This compression of gel bed makes scaling-up and increase of speed of separation difficult.

Figure 2 shows the pressure drop per unit column length as a function of the linear mobile phase velocity, \( u \), for various column dimensions. The values of \( \varepsilon \) ranged between 0.33 and 0.38. The results of all the column dimensions employed in this study obey the Kozeny-Carman equation given by Eq. (4).

\[
AP/(ZH^2) = 180 \mu u/d^2 \tag{4}
\]

The straight line shown in Fig. 2 indicates that the gels employed in this study are neither compressed nor deformed in the column under the present experimental conditions.

2.4 Effect of column dimensions on HETP

Figure 3 shows HETP for Mb as a function of \( u \) for various column dimensions. The sample volume was chosen so that it was around 0.5-1.0% of the total column volume. The elution curves were almost symmetrical, as shown in Fig. 1, and the distribution coefficient \( K \) obtained from \( t_R \) was 0.41 regardless of flow rate or column dimensions. HETP for all the columns can be expressed by a single straight line within small experimental error. It is considered that there is a region near the wall of the column where the packing density is low, and consequently the flow is faster than that near the axis of the column. Therefore, if a sample is injected into the center of the column and cannot reach the region near the wall until it is eluted from the column, the resulting HETP will be lower than that with the sample injected into the whole cross-section of the gel bed. This is, what is called an "infinite diameter column" as reported by Knox and Parcher. We also applied the central injection method in order to investigate the contribution of the wall effect on HETP. A small amount of sample (0.02 cm³) was injected into the center of the top of the gel bed (1.0 x 30 cm). In this injection method, no movement of the zone could be observed until the zone was eluted from the column. The results, shown in Fig. 3 by filled circles, indicated no appreciable change of HETP, although a very small amount of tailing and/or leading of elution curves was eliminated. With increasing column diameter, the contribution of the wall effect to HETP is considered to decrease. Accordingly, HETP did not depend on the column dimensions appreciably.

2.5 Effect of sample volume and concentration

In the case of preparative scale separation, it is desirable to apply a large amount of sample without significant loss of separation efficiency. The effect of sample volumes on HETP is shown in Fig. 4. HETP remains constant up to a certain sample volume and above that it increases rapidly with sample volume. Similar experimental results have been reported by many investigators. Since the contribution of sample volume to the variance of the elution curve is equal to \( t_0^2/12 \), the variance at infinitely small sample volume, \( \sigma_0^2 \), is given by

\[
\sigma_0^2 = \sigma^2 - (t_0^2/12) \tag{5}
\]

Then, HETP as a function of sample injection time \( t_0 \) is given from Eqs. (1) and (5) as

\[
HETP = Z(\sigma_0^2 + t_0^2/12)/(t_{R0} + t_0/2)^2 \tag{6}
\]

A calculated line by Eq. (6) with the values of \( \sigma_0^2 \) and
In GFC, substances are separated according to their molecular shapes. Thus the distribution coefficient is constant and skewed elution curves due to a non-linear isotherm are not observed under the usual operating conditions. However, since the protein concentration is often high in preparative chromatography, it is important to know whether proteins of high concentrations behave ideally. HETP for ovalbumin of various concentrations was measured with a HW55F gel column. HETP was almost constant when the protein concentration was less than 3%, as shown in Fig. 5. However, the elution curve became asymmetrical with a further increase in concentration and finally had two peaks or one peak with a shoulder. Consequently, HETP increased rapidly, as is seen in the figure. As reported by many investigators,1,4,14 it is not the concentration but the viscosity of the sample, i.e., the hydrodynamic instability of the viscous zone, which causes this phenomenon. This is supported by the fact that similar experimental results were obtained in the following cases: (1) when 0.5% Mb containing 5% Dextran T-10 (viscosity at 20°C is 1.6x10^{-3} Pa·s) was employed as a sample and (2) when the buffer containing 5% Dextran T-10 and 0.5% Mb without Dextran were used as the elution buffer and the sample, respectively. We examined the shape of the zones by slicing frozen gel beds. Typical shapes of the observed zones for case (1) are shown in the inset of Fig. 5. The viscous sample zone spreads widely in the radial and longitudinal directions. This overload effect should be more pronounced when a short column of high efficiency is used. In fact, such an effect was also observed with a high-performance gel filtration chromatography column (TSK G3000SW). Therefore, it is recommended that preliminary experiments be carried out to examine whether this undesirable overload phenomena occurs when the sample viscosity is different from the solvent viscosity by a factor of 2 or more.

3. Discussion

One disadvantage of GFC is its low selectivity for substances. This is the reason why the column of GFC is much longer than that in other types of chromatography such as ion exchange chromatography and affinity chromatography. The column length of GFC is usually larger than 50 cm, sometimes exceeding 100 cm. In the case of soft gels, one difficulty in evaluating the dependence of HETP on the column dimensions lies in the fact that it becomes difficult to obtain gel beds having a similar structure with increase in column dimensions due to the compression of the gel bed. Janson and Hedman6) measured HETP of a flat, thin column (37 cm diameter and 15 cm length) and compared it with HETP obtained with a small one (5 cm diameter and 15 cm length). No significant difference was found between these two HETP values. Sada et al.19) also reported that the elution profiles of 1.65 cm and 18.4 cm diameter columns (column length, 8 cm) were very similar. However, such short columns must be stacked as Janson and Hedman6) mentioned, since the column for GFC should be long as noted above. On the other hand, our experimental results have shown that in the case of MPGFC, HETP obtained with small columns can be directly employed for the design calculation of a large column such as 9x90 cm. However, it is expected that a further increase of column diameter and/or column length from the 9x90 cm column might cause bed compression to some extent. Therefore, it is not advantageous to lengthen the column to increase the resolution when the diameter of the column is very large. It is advisable instead that the flow rate be reduced to increase the resolution in such a case.

In gradient elution, it is known that a zone sharpening effect, which can make tilted zones due to a poor sample introduction horizontally, exists.13,17,20) However, since the zone sharpening effect is not expected in isocratic elution chromatography...
such as GFC, the method of sample introduction is very important. Our method is, however, not suited for the continuous use of the column or for operation at high pressure. Further investigations of the method of sample introduction, including design of a column end piece which gives a horizontal zone and has high reproducibility are needed.

Conclusions

1. The HETP of proteins was almost the same for both 1.0 x 30 cm (24 cm³) and 9.0 x 90 cm (5726 cm³) columns. This is attributed to the high mechanical stability of the gels employed and the negligible contribution of the wall effect.

2. The sample volume dependence of HETP was predicted well on the basis of the measured HETP with a small sample volume when the protein concentration is low.

3. A sharp increase of HETP was observed when the protein concentration was above 3%. This increase of HETP was considered to be due to the relative difference between the protein and solvent viscosities.

4. From the above findings, we expect that the scaling up of medium-performance gel filtration chromatography can be easily carried out on the basis of the data obtained with a small column.

Nomenclature

\[ A = \text{cross-sectional area of column} \quad [\text{cm}^2] \]
\[ C = \text{height of elution curve} \quad [\text{arbitrary unit}] \]
\[ C_{\text{max}} = \text{maximum peak height of elution curve} \quad [\text{arbitrary unit}] \]
\[ d_e = \text{column diameter} \quad [\text{cm}] \]
\[ d_p = \text{particle diameter} \quad [\text{cm}] \]
\[ H = (1 - \varepsilon)/s \quad [-] \]
\[ \text{HETP} = \text{height equivalent to a theoretical plate} \quad [\text{cm}] \]
\[ K = \text{distribution coefficient} \quad [-] \]
\[ \Delta P = \text{pressure drop for a gel bed} \quad [\text{KPa}] \]
\[ \Delta P_c = \text{pressure drop in connection tubes and column end fittings} \quad [\text{KPa}] \]
\[ \Delta P_p = \text{total pressure drop for a packed column} \quad [\text{KPa}] \]
\[ t_0 = \text{sample injection time} \quad [\text{min}] \]
\[ t_R = \text{peak retention time} \quad [\text{min}] \]
\[ t_{R0} = \text{peak retention time at an infinitely small} \]

Literature Cited