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Note

Factors affecting the relationship between the plate height and the linear mobile phase velocity in gel filtration chromatography of proteins

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Gel filtration chromatography (GFC) has been widely used for the separation and purification of proteins. However, the number of components resolvable in GFC is strongly dependent on the column efficiency[1]. Therefore, GFC with smaller than conventional gel particles, known as medium- or high-performance GFC (MPGFC, HPGFC), has recently been employed to increase the resolution.

We have reported the heights equivalent to a theoretical plate (HETPs) for proteins on soft dextran gels[2] and in MPGFC[3]. In the latter study the effect of the column dimensions, sample volume and sample concentration on HETP were also investigated, and the effect of the column dimensions was found to be negligible for columns between 30.0 cm × 1.0 cm and 90 cm × 9.0 cm (ref. 3).

In this study, HETP values for proteins in MPGFC and HPGFC were measured as a function of the linear mobile phase velocity, u. The effects of the particle diameter, d_p, the gel type, the type of proteins and the temperature were examined on the basis of plots of the reduced HETP, $h(=\mathrm{HETP}/d_p)$ *vs.* the reduced flow-rate, $v(=ud_p/D_m)$[4,5], where D_m is the molecular diffusion coefficient.

EXPERIMENTAL

The apparatus and the method were essentially the same as in our previous study[2,3].

The MPGFC gels from Toyo Soda (Japan) employed in this study included Toyopearl (TSK gel) HW40F ($d_p = 44$ μm), HW55SF ($d_p = 35$ μm), HW55F ($d_p = 44$ μm) and HW55C ($d_p = 75$ μm).

In the case of a packed HPGFC column (TSK gel G3000SW, $d_p = 11$ μm, 30 cm × 0.75 cm), the sample was introduced by means of a Rheodyne 7120 injection valve connected to a Model 100 pump (Altex, U.S.A.).

Myoglobin (Mb) (Cat. No. M0630) from Sigma (St. Louis, MO, U.S.A.) and ovalbumin (OA) (five times crystallized) from Seikagakukogyo (Japan) were employed without further purification. The same purified fraction of bovine serum albumin (BSA) as that in our previous study[6] was used. All other reagents were analytical grade.

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RESULTS AND DISCUSSION

Factors affecting HETP

As shown in Fig. 1, the HETP values for Mb, OA, BSA and vitamin B_{12} (B_{12}) on an HW55F gel column were found to be a linear function of u. As the molecular weight increased, the slope of the line increased while the HETP value extrapolated to $u=0$ from the experimental results did not change appreciably. HETP values for proteins on HW40F were almost parallel to the u-axis and the HETP value extrapolated to $u=0$ was almost the same as that on the HW55F column. Similar experimental results were found previously[2,3,8–10].

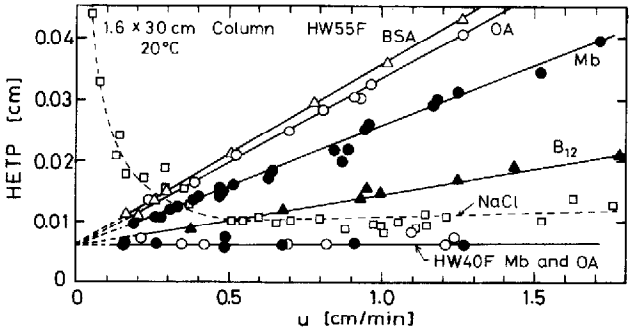

Fig. 1. Plots of HETP *vs.* u with MPGFC columns. The distribution coefficient, K, was measured from the peak elution volume, V_e, according to the relationship $K = (V_e - V_o)/(V_t - V_o)$ (ref. 7) where V_o = void volume and V_t = total column volume. $K=0.30$ for BSA, 0.34 for OA, 0.41 for Mb, 0.74 for sodium chloride and 0.9 for B_{12} on HW55F; $K=0$ for Mb and OA on HW40F. Sample volume: 0.5 ml. Sample concentrations: 0.3–0.5% for Mb, OA and BSA; 0.5 M for sodium chloride and 0.1% for B_{12}. Vitamin B_{12} was weakly adsorbed to the HW55F gel, although the exact mechanism of the adsorption is unknown. This is the reason why K for B_{12} is higher than that for sodium chloride. Note that the values of HETP at $u=0$ extrapolated from the experimental results are very similar except that for sodium chloride.

These results can be interpreted on the basis of the equation[2,10–12]

$$\text{HETP} = 2(D_L/u) + R(1-R)ud_p^2/(30D_s) = A + Cu \qquad (1)$$

where D_L is the axial dispersion coefficient, D_s the gel phase (intraparticle) diffusion coefficient and R is defined as the equilibrium fraction of solute in the mobile phase[4,12].

The first term on the right-hand side of eqn. 1 expresses the contribution from axial dispersion, which can be assumed to be constant under the usual conditions in GFC of proteins[2,10–13]. The A value is therefore obtained from the HETP value extrapolated to $u=0$. The second term is for the gel phase (intraparticle) diffusion and the C value can be obtained from the slope of a plot of HETP *vs.* u. Since the distribution coefficient, K, of proteins was 0 for the HW40F column, eqn. 1 simplifies to $\text{HETP} = 2D_L/u = A = \text{constant}$.

The HETP for sodium chloride on the HW55F column showed a minimum at

around $u = 0.5$ cm/min and below this value it increased sharply. This is due to the contribution from the molecular diffusion coefficient, D_m, to D_L. If we split D_L into $\lambda d_p u$ and $\gamma_m D_m$ where λ is the packing characterization factor and γ_m the tortuosity factor in interparticle space as shown by Van Deemter *et al.*[14], eqn. 1 becomes[11,12]:

$$\text{HETP} = 2\lambda d_p + 2\gamma_m D_m/u + R(1-R)ud_p^2/(30D_s) = A + B/u + Cu \quad (2)$$

This equation is quite similar to the Van Deemter equation[14].

When $A = 2\lambda d_p = 66$ μm (the HETP value extrapolated to $u = 0$ in Fig. 1), $D_m = 1.5 \cdot 10^{-5}$ cm^2/s (value for sodium chloride[15]) and $\gamma_m = 0.6$[4,14] are inserted to eqn. 2, the second term becomes more than half of the total HETP at $u = 0.1$ cm/min.

On the other hand, in the case of Mb, D_m is $1.1 \cdot 10^{-6}$ cm^2/s (ref. 16), which is 14 times lower than that of sodium chloride. With this D_m, $2\lambda d_p = 66$ μm and $\gamma_m = 0.6$[4,14], the second term does not contribute to the total HETP even at $u = 0.1$ cm/min. Therefore, there will be a minimum in the HETP *vs.* u relationship only at extremely low flow-rates[17]. This minimum will be discussed later in terms of the reduced velocity.

GFC is often performed at subambient temperature for the separation of unstable substances such as proteins and at high temperature for highly viscous samples such as sugars. As in Fig. 2, the lower the temperature the higher is the HETP. It is interesting that the HETP value extrapolated to $u = 0$ (the A value) is affected little by temperature whereas the slope changes markedly. Since the K value ($= 0.41$) did not vary with temperature, this change in the slope is due to the variation of D_s.

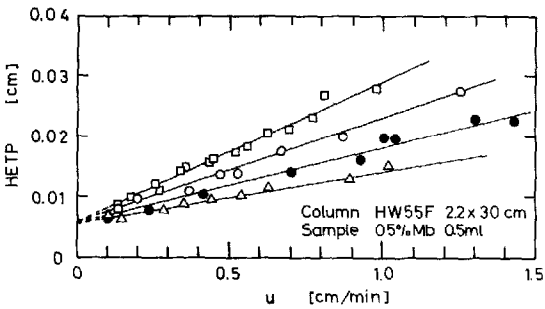

Fig. 2. Effect of temperature on HETP. Note that the values of HETP at $u = 0$ extrapolated from the experimental results for different temperatures are very similar, although the slopes are different. □ = 10°C; ○ = 20°C; ● = 30°C; △ = 40°C.

Reduced HETP

As already suggested by several researchers[4,5], a plot of the reduced HETP, h, *vs.* the reduced velocity, v, is useful for examining the influence of various factors such as d_p and temperature, since the HETP values over a wide range of experimental conditions can be compared in the same diagram. Egn. 1 is rewritten as [12]

$$h = (2/Pe) + R(1-R)v(30\gamma_{sm}) \quad (3)$$

where Pe is the Peclet number (ud_p/D_L) and $\gamma_{sm} = D_s/D_m$.

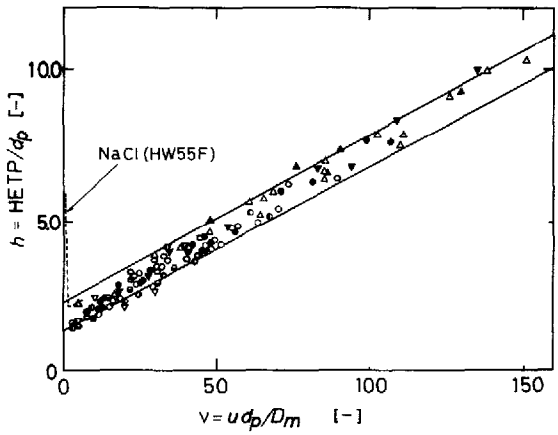

Fig. 3. Plots of reduced HETP *vs.* reduced velocity. The experimental points are cited from Figs. 1 and 2 except those for Mb on HW55SF and HW55C columns (30 cm × 1.6 cm). The data for sodium chloride were calculated from the broken curve in Fig. 1. The D_m values were from refs. 15, 16 and 18 and its temperature dependence was estimated according to the relationship $D_m\eta/T$ = constant[18], where η is the solvent viscosity. The two straight lines indicate the region of the experimental points and are also shown in Fig. 4. ○ = Sample Mb, HW55F gel (d_p = 44 μm), 10°C; ● = Mb, HW55F, 20°C; ◐ = Mb, HW55F, 30°C; ◒ = Mb, HW55F, 40°C; △ = Mb, HW55C gel (d_p = 75 μm), 20°C; ▽ = MB, HW55SF gel (d_p = 35 μm), 20°C; ▲ = sample OA, HW55F, 20°C; ▼ = sample BSA, HW55F, 20°C.

Fig. 3 shows plots of h *vs.* v for the present results with MPGFC columns. All the results are gathered in a very narrow range encompossed by the two lines. This implies the following. (1) Since the plots of HETP *vs.* u for different particle diameters are reduced to a single h *vs.* v relationship, the first and the second terms in eqn. 3 are independent of the particle diameter. (2) The D_L/u ($=A/2=\lambda d_p$) value is hardly dependent on temperature, the type of proteins or d_p, and is approximated to 0.8–

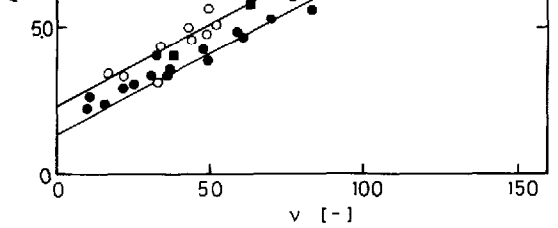

Fig. 4. Plots of h *vs.* v for low-pressure GFC and HPGFC. The two straight lines are the same as those in Fig. 3 and are given for comparison. Experimental conditions for G3000SW HPGFC: sample, 0.4% Mb, 20 μl; 0.1% OA, 100 μl; 20°C. The data for Mb on a Sephadex G-150 column are from ref. 2. ○ = sample Mb, G150 gel (d_p = 201 μm), 13 cm × 1.5 cm column; ● = Mb, G3000SW gel (d_p = 11 μm), 30 cm × 0.75 cm column; ■ = sample OA, G3000 SW, 30 cm × 0.75 cm column.

$1.5 \cdot d_p$, *i.e.*, $Pe = 0.7$–1.3. This Pe value is similar to that reported by Cluff and Hawkes[13] for the range of v employed in this study, but slightly higher than that calculated from the data reported by Katz *et al.*[17]. (3) The h *vs.* v plots for different temperatures are very similar as shown in Fig. 3. According to eqn. 3, this implies that the temperature dependence of D_s is similar to that of D_m (4). When the HETP *vs.* u plots for different proteins shown in Fig. 1 are expressed in the reduced variables h and v, they are similar. This is because v increases with molecular weight at certain u and d_p due to the decrease in D_m with molecular weight[18]. (5) h shows its minimum at v less than 5. This indicates that the HETP for proteins will not show a minimum with the flow-rates normally used and it can be regarded as a linear function of u due to the low D_m of proteins (less than $1 \cdot 10^{-6}$ cm^2/s at 20°C[18]).

In Fig. 4 the above results are compared with those obtained by conventional low-pressure GFC and HPGFC. It is interesting that the results also fall in a very narrow range. It should be noted that the flow-rate in GFC is usually low with larger particle diameters and high with smaller ones. Therefore, v ranges between 10 and 150 under the usual operating conditions.

The present study has shown that the HETP for proteins in GFC can be described by eqn. 1 and that a plot of the reduced HETP *vs.* the reduced velocity is useful for assessment of the column efficiency.

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