*Journal of Chromatography*, 445 (1988) 29–36
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 480

# EFFECT OF STERIC EXCLUSION ON THE SEPARATION OF PROTEINS BY HYDROPHILIC SIZE-EXCLUSION CHROMATOGRAPHY

QING CHENG MENG* and YIU-FAI CHEN
*Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294 (U.S.A.)*
LAWRENCE J. DELUCAS
*Institute of Dental Research, Macromolecular Crystallography and Department of Optometry, University of Alabama at Birmingham, Birmingham, AL 35294 (U.S.A.)*
and
SUZANNE OPARIL
*Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294 (U.S.A.)*
(Received March 15th, 1988)

## SUMMARY

On the basis of studying the retention and band broadening of proteins on the TSK SW column, diffusion coefficients ($D_s$) of solute in stationary phase were obtained which elucidate the hydrodynamic process of chromatographic resolution of proteins by hydrophilic size-exclusion chromatography (SEC). After calculating the correlation between $D_s$ and the molecular weight of the solute, the molecular dimensions of proteins in the process of chromatographic separation can be predicted. Deviations in diffusion coefficient of a protein from the calculated value reflect differences of measured molecular dimensions from molecular volumes predicted from the calibration curve of the SEC column. This study illustrates a convenient method for estimating the purity of proteins by SEC. Deviations from $2\lambda\ d_p$ (where $d_p$ is the particle diameter) in the intercept of the theoretical plate height ($H$) *versus* flow-rate ($U$) curve from the band broadening equation $H = C_sU + 2\lambda\ d_p + f(\alpha_M)_T$ {where $C_sU$ represents mass transfer resistance caused by solute diffusion in the stationary phase and $f(\alpha_M)_T$ an added term for polydisperse solutes as proposed by Knox and McLennan [*Chromatographia*, 10 (1977) 75]} reflect impurities in the proteins.

## INTRODUCTION

Size-exclusion chromatography (SEC) of water-soluble polymers is a rapidly growing area, especially with the introduction of high-performance liquid chromatography (HPLC). HPLC provides high resolution, high speed and high sensitivity, important advantages over conventional analytical methods, such as SEC, for the separation and purification of water-soluble polymers, including proteins. Conventional size-exclusion columns, which are made of a cross-linked dextran (*e.g.*, Sephadex), polyacrylamide (*e.g.*, Bio-Gel P), or agarose (*e.g.*, Sepharose or Bio-Gel A),

0021-9673/88/$03.50 

do not have the mechanical rigidity to withstand the pressure required to achieve rapid separation under high pressure or sufficiently small particle size to give good efficiency in separation. With the development of the newer microparticulate packings, many of these problems have been overcome. Sulfonated polystyrene (*e.g.*, Ion-Pak), hydroxylated polyester (*e.g.*, OHpak), hydrophilic monolayer bonded to porous silica (TSK Type SW), and hydroxylated polyether (TSK Type PW) are examples of packings available for aqueous exclusion work. Recent developments in commercially available microparticulate packings and columns for steric-exclusion chromatography of water-soluble polymers have been reviewed[1,2]. Since this is a very proprietary area, many manufacturers do not reveal the exact composition of their packing phase. Because of the tremendous growth of high-performance hydrophilic SEC in life science research, further study of separation mechanisms of proteins by SEC is important in order to understand the dynamic behavior of proteins during the chromatography process and to provide a basis for optimizing chromatographic resolution in the application of SEC. Recently, Ghrist *et al.*[3] studied the retention behavior of proteins in HPLC and developed a general model that predicts band widths in HPLC.

At present, TSK Type SW and TSK Type PW systems are commonly used in protein separation and purification. In the current study, we examined the retention behavior and band broadening of proteins of different molecular weights on a TSK type SW column containing microspheres having a particle size ($d_p$) of 10 $\mu$m. Experimental plate-height data were determined , and diffusion coeffients ($D_s$) of solute into stationary phase were calculated. The experimental results were discussed in terms of the model of Ghrist *et al.*[3]. From these data and our previous experiments[4,5], a method was developed for correlating molecular weight (MW) and hydrodynamic volume of standard proteins in the process of chromatographic resolution. With the aid of the experimental dependence of theoretical plate height ($H$) on flow-rate ($U$) and the band broadening equation[6] in the HPLC, the purity of standard proteins and protein mixtures was evaluated from the intercept of the $H$–$U$ curve. The intercept of the $H$–$U$ curve is a constant for pure proteins, and the extent of the deviation from the intercept of the $H$–$U$ curve reflects the level of impurity of a protein sample.

## EXPERIMENTAL

HPLC experiments were performed with a Beckman 165 system [consisting of a Model 165 variable-wavelength detector (operated at 280 nm), a Model 112 solvent delivery module, a Model BD 41 recorder (Kipp and Zonen), and a Model 340 injector (Rheodyne)]. A Bio-Sil TSK-250 HPLC gel filtration 300 × 7.5 mmm I.D. column (Bioanalytical Systems) was employed. Standard protein samples (gel filtration standard) were obtained from Bio-Rad. These included: thyroglobulin (bovine), MW 670 000; $\gamma$-globulin (bovine), MW 158 000; Ovalbumin (chicken), MW 44 000; myoglobin (horse), MW 17 000; vitamin $B_{12}$, MW 1350. The mobile phase consisted of 0.041 $M$ disodium hydrogensulphate, 0.019 $M$ sodium dihydrogenphosphate and 0.15 $M$ sodium sulphate, adjusted to pH 6.8. All measurements were conducted at ambient temperature (25°C).

On the basis of a plot of plate height equivalent to $H$ *versus* $U$, diffusion coefficients ($D_s$) of solute in the stationary phase were determined.

## RESULTS AND DISCUSSION

In HPLC, band broadening effects can be expressed as follows:

$$H = B/U + C_sU + (\tfrac{1}{2}\ \lambda d_p + D_m/d_p^2u)^{-1} \quad (1)$$

The term $B/U$ represents molecular diffusion in the longitudinal direction in the mobile phase. The $C_sU$ term represents mass transfer resistance caused by solute diffusion in the stationary phase. The last term $(\frac{1}{2}\ \lambda d_p + D_m/d_p^2u)$ (where $D_m$ is the solute diffusion coefficient and $u$ is the flow-rate of the mobile phase) is the coupling of Eddy diffusion and mobile phase terms for solute dispersion in the mobile phase. The diffusion coefficient of protein solutes in water is *ca.* $10^{-7}$ cm$^2$/s, and usually the flow-rate used is of the order of mm/s and the particle size $d_p$ is *ca.* 10 $\mu$m, so the contribution of the last term is mainly due to $2\lambda d_p$. Since $D_m$ is *ca.* $10^5$ times smaller in liquids than in gases, this longitudinal term does not make an important contribution to band broadening in liquid chromatography. Thus, the column efficiency of the non-permeable protein thyroglobulin (MW 670 000) is constant throughout the flow-rate range shown in Fig. 1. This phenomenon is consistent with the results of Dawkins and Yeadon[7] on hydrophobic gel permeation chromatography (GPC) using silica microspheres. Thus, eqn. 1 can be simplified as follows:

$$H = C_sU + 2\lambda d_p \quad (2)$$

In general, $C_s$ is a function of the diffusion coefficient ($D_s$) of the solute in the stationary phase, the relative migration rate of the solute in the mobile phase, and particle size and pore structure parameters. $C_s$ can be computed from the equation of Damkins and Yeadon[7]

$$C_s = R(1 - R)d_p^2/30D_s \quad (3)$$

where $R$ is the retention ratio, defined here for each solute by $V_0/V_R$, and $V_0$ is the interstitial (or void) volume of the column. Thus, $D_s$ values for proteins of different

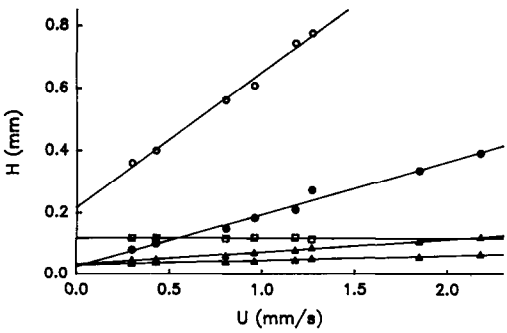


Fig. 1. $H$–$U$ dependence of proteins on TSK-250 gel. Eluent: 0.041 $M$ $Na_2HPO_4$, 0.019 $M$ $NaH_2PO_4$ and 0.15 $M$ $Na_2SO_4$ (pH 6.8). Key to symbols: ○ = $\gamma$-globulin; ● = ovalbumin; △ = myoglobin; ▲ = vitamin $V_{12}$; □ = thyroglobulin.

TABLE I

DIFFUSION COEFFICIENTS OF PROTEINS ON TSK-250 GEL

| *Protein* | *MW* | $D_s$ ($\times 10^{-8}$ $cm^2/s$) |
|---|---|---|
| γ-Globulin | 158 000 | 48.00 |
| Ovalbumin | 44 000 | 20.05 |
| Myoglobin | 17 000 | 4.92 |
| Vitamin $B_{12}$ | 1350 | 1.67 |

molecular weights can be obtained from eqns. 2 and 3 and the H–U curves in Fig. 1, as listed in Table I. In SEC, the interpretation of chromatograms requires a valid correlation between elution volume ($V_R$) and a stable molecular dimension. Deviations from this correlation occur when molar volume or size in solution does not correlate with molecular weight. The $D_s$ value exactly describes the relative migration rate of solute in the stationary phase and then reveals the correlation between the molecular weight and the true molecular dimensions of protein in the process of chromatographic resolution. From our previous studies[4,5] and the data of Dawkins and Yeadon[7] the MW *vs.* $D_s$ plot is linear for either the standard polystyrene in the hydrophobic GPC or for standard dextrans in hydrophilic GPC. These results provide information about ascertaining the correlation between MW and the hydrodynamic volume of solute in SEC. In our current study, the diffusion coefficients of proteins shown in Table I were obtained from the slopes of *H–U* curves and eqn. 3. The flow-rate was defined here by $L/V_0$, where $L$ is the length of the SEC column, and the retention volume of thyroglobulin (MW = 670 000) is equal to the void volume of the column.

Walters[8] showed that bandwidths (values of $N$) in SEC agree (approximately) with experimental values when ($D_p/D_m$) is calculated by:

$$D_p/D_m = 1 - 2.10r_{sp} + 2.09r_{sp}^3 - 0.95r_{sp}^5 \quad (4)$$

where $D_p$ is the solute diffusion coefficient in the stagnant mobile phase within the packing material, $D_m$ is the solution diffusion coefficient in the bulk mobile phase, and $r_{sp}$ is the ratio of solute Stokes' diameter to the pore diameter of the column packing. Knox and McLennan[9] also found a decrease in $D_p/D_m$ for large polystyrenes separated by SEC, as a result of the hindered diffusion of large molecules in small pores. Eqn. 4 has been used to estimate the restricted diffusion of proteins in other HPLC systems[10]. The calculated data of $D_p/D_m$ and $r_{sp}$ in our experiments are

TABLE II

$D_p/D_m$ AND $r_{sp}$ OF PROTEINS ON TSK-250 GEL

| *Protein* | $D_p/D_m$ | $r_{sp}$ |
|---|---|---|
| γ-Globulin | 0.265 | 0.416 |
| Ovalbumin | 0.563 | 0.218 |
| Myoglobin | 0.692 | 0.150 |

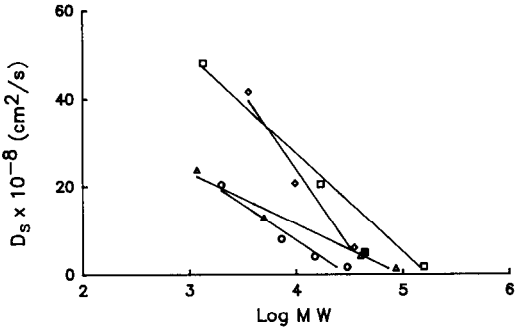


Fig. 2. Dependence of experimental diffusion coefficient ($D_s$) on molecular weight (MW). Key to symbols: □ = proteins eluted from TSK-250 gel, ■ = albumin; △ = dextrans eluted from EGPM (polar polymeric gel of ethyleneglycol dimethacrylate–glycidylmethacrylate) gel; ◇ = polystyrene eluted from silica gel; ○ = polystyrene eluted from ethylstyrene–divinylbenzene copolymer gel.

summarized in Table II. The values of $D_p/D_m$ increase with decreasing molecular weight of proteins, consistent with the results of Knox and McLennan as described below.

Fig. 1 shows that the slopes increase with the molecular weights of the solute molecule, the larger the mass transfer resistance of the solute in the stationary phase will be. In the SEC of proteins, the size-exclusion mechanism has been considered[11], so the correlation between the MW and the $D_s$ should be reasonably straightforward. In our experiment, only in the case of ovalbumin was there a lack of correlation between MW and $D_s$. The $D_s$ of ovalbumin was much smaller than predicted by eqn. 3, as indicated in Fig. 2. If the molecular dimension of ovalbumin were consistent with the correlation line of the MW and $D_s$, the molecular radius of ovalbumin would be 25.2 Å, significantly smaller than the value of 27.3 Å calculated by Ackers[12] from data obtained from analytical gel chromatography. The larger molecular dimension caused the large mass transfer resistance, which, of course, led to the smaller $D_s$. Therefore, the $D_s$ values obtained by this method indicate that the true molecular dimensions of some proteins are not consistent with the calibration curve of the SEC column during protein separation by SEC.

The simplified eqn. 2 described previously applies to a monodiosperse solute. For polydisperse solutes, it is necessary to add the term $f(\alpha_M)_T$, as proposed by Knox and McLennan[13]. Thus:

$$H = C_s U + 2\lambda d_p + f(\alpha_M)_T \quad (5)$$

As $f(\alpha_M)_T$ is flow-rate independent, sample deviation $\alpha_M$ from the intercept of the $H$–$U$ curve can be evaluated. Fig. 1 is a set of H–U plates of standard protein samples of different molecular weights. For ovalbumin, myoglobin and vitamin $B_{12}$, the value of the intercept of each curve was exactly equal to the value of $2\lambda d_p$. These data indicate that of the intercept of the $H$–$U$ curve is close to the value of $2\lambda d_p$, the protein is highly pure. In contrast, for $\gamma$-globulin, the value of the intercept was much larger than the constant $2\lambda d_p$, which is discussed below. Thus, the closer to $2\lambda d_p$ intercept of the $H$–$U$ curve is, the more pure the protein will be. These results illustrate

a convenient method to evaluate the purification of biomacromolecules, including proteins.

Knox and McLennan[9] have shown for the SEC separation of polystyrenes that bandwidths are predictable from the theory developed for small molecules (so-called Knox equation).

$$h = Av^{1/3} + B/v + Cv \tag{6}$$

Here $h$ is the reduced plate height, $v$ is the reduced mobile phase velocity, and $A$, $B$ and $C$ are constants that can be calculated for a given solute and set of conditions. The plate number $N$ for the separation of a given solute is given by the following standard relationships:

$$N = L/H \tag{7}$$

$$h = H/d_p \tag{8}$$

$$V = ud_p/D_m \tag{9}$$

$D_m$ can be calculated from the following eqn. 3:

$$D_m = 9 \cdot 10^{-6}(T/298)^7(2.5M^{-1/3} + 62/M)/\eta_{25} \tag{10}$$

Here $M$ is the molecular weight and $\eta_{25}$ is mobile phase viscosity in centiPoise at 25°C.

Values of $A$ are independent of separation conditions and vary only with how well the column has been packed. Typically, $0.5 \leqslant A \leqslant 1.0$ for well packed columns.

In SEC the parameter $B$ is given[14] as

$$B = 2\gamma \tag{11}$$

where $\gamma$ is an obstructive factor.

Ghrist *et al.*[3] give an expression for $C$[3]:

$$C = K_D\{1/[K_D + x/(1 - x)]\}^2 x/30\gamma(1 - x)\,(D_p/D_m)$$

$$C \cong K_D x/19.2\{K_D + [x/(1 - x)]\}(1 - x)(D_p/D_m) \tag{12}$$

They can relate $C$ to the SEC distribution constant $K$, defined as

$$K_D = (V_R - V_0)/V_0 \tag{13}$$

Here $V_R$ is the retention volume of the solute in an SEC system, $V_0$ is the volume of mobile phase in the column that is outside the pores. The quantity of $k''$ is given as[9].

$$k'' = K_D(V_p/V_0) \tag{14}$$

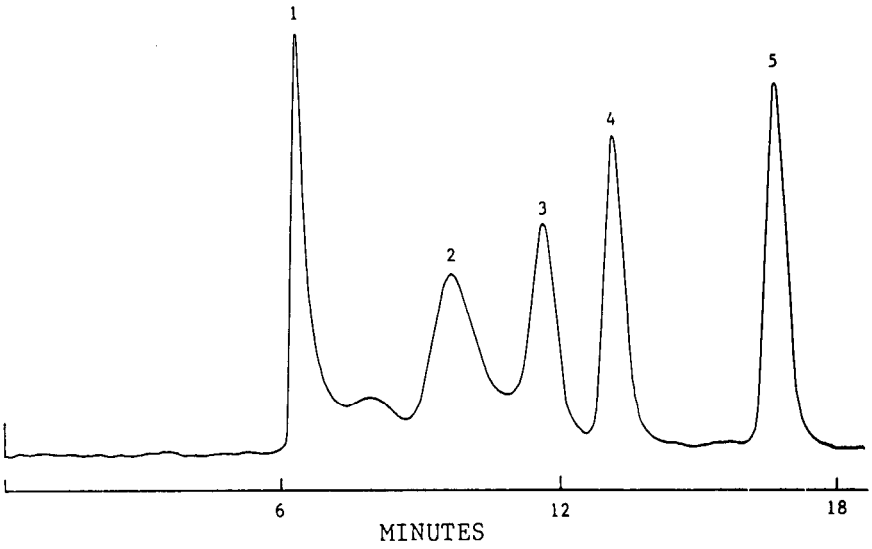


Fig. 3. Characteristic gel permeation chromatogram of TSKH-250 gel. Eluent same as in Fig. 1. Flow-rate, 0.6 ml/min; chart speed, 0.2 mm /s; temperature, 25°C. Peak 1 = thyroglobulin; peak 2 = γ-globulin; peak 3 = ovalbumin; peak 4 = myoglobin; peak 5 = vitamin $B_{12}$.

where $V_p$ is the volume of mobile phase inside the pores and $(V_p/V_0)$ is given in terms of $x$ as

$$x = V_0/(V_p + V_0) \tag{15}$$

From eqns. 6 and 12, the plate numbers of γ-globulin predicted by the model of Ghrist *et al.*[3] were $N = 4391$. The experimental values was $N = 540$, significantly lower than theoretical value.

Protein can be retained in SEC systems by processes other than exclusion. These processes, which include ion exchange and hydrogen bonding with silanols on the silica surface of the packing, should be more important for smaller solute molecules, which presumably have easier access to the silica surface. Secondary retention, when it occurs, often leads to excessive broadening of the solute band and $K_D$ values of more than 1. In the current study, the $K_D$ value of γ-globulin was 0.2. This information, coupled with the considerations described above, provides further proof that the breath of the γ-globulin peak was caused by impurity of solute rather than secondary retention in the elution profile (Fig. 3). From these data, it is evident that the polydispersity of a solute contributes to band broadening, but is insignificant in determining mass transfer resistance.

The plate number of a non-permeating solute depends on how well the column has been packed and also the interstitial (or void) volume of the column. Dawkins and Yeadon[15] found that for non-permeating polystyrenes, the values of $H$ for PS-47000 were always greater than values for PS-11000, which means that a high-molecular weight non-permeating solute probably causes high values of $H$. In our experiments, thyroglobulin, which has a high molecular weight, showed high values of

$H$, an observation which is in agreement with the results of Dawkins and Yeadon[15]. In addition, non-permeating proteins exhibited very little or no change in $H$ as $U$ was varied during the process of separation, which leads to a high intercept for the $H$–$U$ curve, as shown in Fig. 1. As the SEC separation mechanism operates mainly by size exclusion, the molecular dimension and diffusion coefficients of the solute molecules outside the range of the column used cannot be assessed by SEC.

ACKNOWLEDGEMENTS

The authors wish to thank Ms. Charlane Crouse for her help in the preparation of this manuscript. This work has been supported in part by the National Heart, Lung and Blood Institute Grants HL-22544, HL-35051 and HL-39041 and by the National Dairy Board and administered in cooperation with National Dairy Council.

REFERENCES

1 H. G. Barth, *J. Chromatogr. Sci.*, 18 (1980) 409.
2 E. E. Majors, *J. Chromatogr. Sci.*, 18 (1980) 488.
3 B. F. D. Ghrist, M. A. Stadalius and L. R. Snyder, *J. Chromatogr.*, 387 (1987) 1–19.
4 Meng Qing Cheng, Si Tian Sheng and Deng Liru, *Kexue Tongbao*, 12 (1984) 1622.
5 Meng Qing Cheng, Su Tian Sheng and Deng Liru, *Scientia Sinica XXVIII*, 9 (1985) 906.
6 J. J. Kirkland, *Modern Practice of Liquid Chromatography*, Wiley Interscience, New York, 1971, Ch. 1, p.29.
7 J. V. Dawkins and G. Yeadon, *J. Chromatogr.*, 206 (1981) 215.
8 R. R. Walters, *J. Chromatogr.*, 249 (1982) 19.
9 J. H. Knox and F. McLennan, *J. Chromatogr.*, 185 (1979) 289.
10 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 296 (1984) 61.
11 J. J. Kirkland, *Modern Practice of Liquid Chromatography*, Wiley Interscience, New York, 1971, Ch. 7, p. 237.
12 G. K. Ackers, *Adv. Protein Chem.*, 24 (1970) 392.
13 J. H. Knox and F. McLennan, *Chromatographia*, 10 (1977) 75.
14 R. W. Stout, J. J. DeStefano and L. R. Snyder, *J. Chromatogr.*, 282 (1983) 263.
15 J. V. Dawkins and G. Yeadon, *J. Chromatogr.*, 188 (1980) 333.