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Note

Optimization of size-exclusion separation of proteins on a Superose column

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The chromatographic partition coefficient for size-exclusion chromatography (SEC) is obtained experimentally as

$$K_{SEC} = \frac{V_e - V_0}{V_t - V_0} \quad (1)$$

where V_e is the measured peak elution volume, V_0 is the column void volume or exclusion volume and V_t is the total column volume, *i.e.*, the sum of V_0 and the pore volume, V_p. Several treatments have appeared in which K_{SEC} is related to the dimensions of the stationary pores and the macromolecular solutes[1–3]. However, the migration of proteins on SEC columns is in fact typically influenced by electrostatic and hydrophobic factors, as well as by steric effects[4–6].

All aqueous SEC packings bear a discernible level of ionogenic groups which commonly produce a negative surface charge[8]; this stationary charge may interact with regions of similar or opposite charge on the proteins, leading to repulsion or retention. Such coulomb interactions are evidently most prominent in low ionic strength eluent, in the absence of screening by small ions. However, it is not at all clear that these interactions can be totally suppressed by the addition of salt, for two reasons. First, the reduction of protein solvation at high ionic strength may facilitate binding via enhanced hydrophobic interactions. Second, if the protein's ionic groups actually ion pair with those on the packing, as is indeed suggested by the common reference to "ion-exchange" mechanisms[9], then the interaction between the stationary phase and the protein may be so intimate that the ionic strength of the medium might not screen these interactions through the usual Debye–Hückel square root dependence. Put differently, the proximity of the stationary phase and the protein ionophores in the binding mode might preclude the intervention of the bulk electrolyte. Such considerations may be involved in the finding that protein retention volumes may display minima or maxima with increasing ionic strength for a number of SEC packings[4,10].

The role of the ionic strength (I) must primarily involve the effective distance of electrostatic forces, *i.e.*, the Debye length (see, *e.g.*, ref. 11), while the influence of pH

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on retention depends on the pK values of solute and packing ionogens. Repulsive forces should dominate at high pH, at which condition both packing and solute are negatively charged; the situation is more complex in neutral or acidic media, in which some regions of the protein bear a charge opposite in sign to that of the stationary phase. The net electrostatic force between the protein and the packing will then be quite sensitive to orientation and rotational freedom of the protein in the near proximity of the stationary phase.

The co-existence of several separation mechanisms is of course frequently exploited to maximize peak resolution[4]. In practise, this may involve the manipulation of solvent gradients with SEC packings that contain both hydrophobic and ionic substituents. Such mixed-mode chromatography however, vitiates the interpretation of retention volumes in terms of molecular dimensions. Furthermore, it is difficult to analyze the mangnitude of non-steric effects, unless conditions for "ideal" behavior can be identified as a point of reference. The determination of solvent conditions that correspond to "pure SEC" is therefore of practical and fundamental significance. In this report, we describe an empirical, but efficient procedure to identify such "ideal" conditions.

EXPERIMENTAL

Proteins obtained from Sigma (99+% purity) were thyroglobulin (bovine) (mol.wt. 669 000 daltons, pI = 4.6); apoferritin (horse spleen) (467 000 daltons, pI = *ca.* 5.0); catalase (bovine liver) (232 000 daltons, pI = 5.6), bovine serum albumin (66 000 daltons, pI = 4.8), ovalbumin (44 000 daltons, pI = 4.6), myoglobin (17 000 daltons, pI = 7.3) and cytochrome *c* (12 400 daltons, pI = 10.6). All proteins gave a single symmetrical chromatographic peak. Corresponding Stokes radii, obtained from a variety of references, were 85, 61, 52, 36, 28, 20 and 16 Å, respectively. Blue dextran was obtained from Pharmacia.

A prepacked Superose 6 column (30 cm × 1.00 cm I.D., Pharmacia) with a molecular weight range of 5000–500 000 (globular proteins) was eluted with sodium chloride–sodium phosphate buffer (9:1), at varying pH and ionic strengths, at 23 ± 1°C using a Milton Roy minipump. A Rheodyne procolumn filter (0.2 μm) was placed in-line to protect the column from particulate matter. The column exclusion volume and total permeable volume, as measured with blue dextran and deuterium oxide, were 14.58 and 22.42 ml, respectively. At a typical flow-rate of 0.35 ml min^{-1} the plate count, as measured by injection of 5% deuterium oxide, was 9000 m^{-1}. Samples, containing typically 3.5 mg ml^{-1} protein, were injected via a Rheodyne 7010 (200 μl) loop injector, after filtration through disposable nylon 0.45-μm syringe filter tips (National Scientific Co.). Detection was by refractive index (Waters Assoc., R401) or UV absorbance at 254 nm (Altex, Model 153).

RESULTS AND DISCUSSION

Geometric considerations suggest that, for solutes and pores with well defined symmetrical geometries

$$K_{SEC} = (1 - \alpha)^{\lambda} \qquad (2)$$

where $\alpha = R/r_p$ is the ratio of solute and pore sizes, and λ depends on pore geometry, *i.e.* $\lambda = 2$ for cylindrical pores and 3 for spherical pores[13]. Although direct methods fail to reveal a uniform pore geometry for any gel packing[14], data for neutral polysaccharides on porous glass packings are well fit by eqn. 2 using $\lambda = 2$ (ref. 15). Furthermore, these same results indicate that the effective cylindrical radius of the pores may be identified with the value from mercury porosimetry.

The evaluation of R is hardly straightforward: the macromolecular dimensional parameter that controls elution in SEC is the subject of debate. For solutes of near-spherical symmetry, retention may be predicted, with equal accuracy, by a number of variables, including the Stokes radius and the viscosity radius[16]. We have recently shown, however, that neither of these parameters uniformly determines K_{SEC} for asymmetric macromolecules of varying shapes[17]. For proteins, however, R may be identified with dimensions determined by hydrodynamic and other techniques. In this work, the Stokes radius, R_S, is employed.

If electrostatic and hydrophobic effects influence the retention of proteins, deviations from a geometric relation such as eqn. 2 are expected. For a series of globular proteins with different isoelectric points, such as those studied here, with pI ranging from 4.6 to 10.6, it is unlikely that these deviations could be uniform, because the charge states of the proteins studied vary widely at any chosen pH. Conversely, congruence of the data with eqn. 2 may be taken as indicating an absence of non-ideal interactions. The magnitude of the deviations from eqn. 2 may be quantitatively parameterized by the regression coefficient of plots of K_{SEC} *vs.* R_S; for this purpose it is not necessary to assume a specific form for $K_{SEC}(R_S)$, *i.e.*, a specific value for λ. Optimization of solvent pH and ionic strength may then be guided by maximization of the regression coefficient.

Since the dependence of K_{SEC} on I and pH is not known *a priori*, an empirical optimization procedure was used to identify the combination of I and pH corresponding to the largest value of the regression coefficient, presumably 1.00. A simplex method[18] was used to maximize the regression coefficient of $K_{SEC}(R_S)$ in the I, pH coordinate system, as shown in Fig. 1. Fig. 2 illustrates the dependence of K_{SEC} on R_S at conditions far from the optimum, while Fig. 3 shows the results obtained at the optimum conditions, $I = 0.38$, pH 5.5. As evident from the insert of Fig. 3, the data conform rather well to eqn. 2, with $\lambda = 2$. The result for thyroglobulin, not shown in the insert, deviates slightly from the curve, and we suggest that this largest protein might sample an average pore size different from that "seen" by the others.

Our results differ from those of Waldmann-Meyer[19] whose data for proteins on Agarose and Sephadex gels conformed to eqn. 3

$$K_{SEC} = k - R_S/R_x \cos\theta \tag{3}$$

corresponding to conical pore geometry. (Here R_x and θ are the geometric parameters of the pore.) This difference could arise in several ways, including an actual difference in pore geometry between the packings used in the two studies, or distortion of the data in ref. 19 through protein–packing interactions. In addition, discerning between eqns. 2 and 3 may require precision beyond that typically available.

We believe that Fig. 3 corresponds to pure size exclusion; therefore, under the conditions represented by the other vertices in Fig. 1, some protein–stationary phase

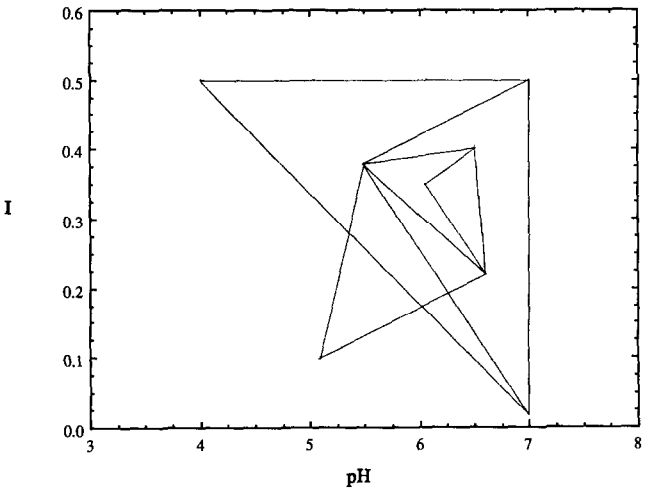

Fig. 1. Simplex optimization of the regression coefficient, r, of plots of K_{SEC} *vs.* R_S, for globular proteins in eluents of varying ionic strength, I, and pH, on Superose 6 column.

interactions take place. Such interactions are apparently difficult to suppress, even though the charge on Superose is presumably quite low in the pH range studied, and even though the hydrophobicity parameter[20] for this packing is low compared to other gels. It is also of interest to note that the optimal pH is below the isoelectric point of most of the proteins, so that the average protein net charge is opposite in sign to the charge on the packing. This effect, which would be expected to lead to enhanced retention through coulombic attraction, is obviously outweighed by the reduction of the packing charge at the lower pH. Kopaciewicz and Regnier[4] also noted the simultaneous effects of protein and packing charge for derivatized silica supports, and

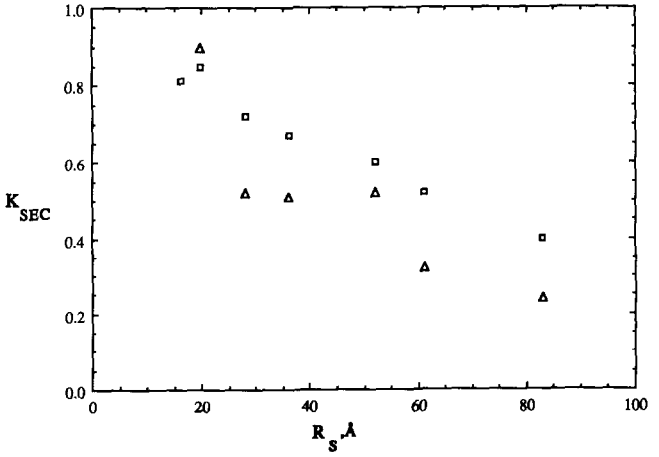

Fig. 2. K_{SEC} *vs.* R_S in mobile phase: (□) pH 6.04, I = 0.35 M; (△) pH 7.0, I = 0.01 M.

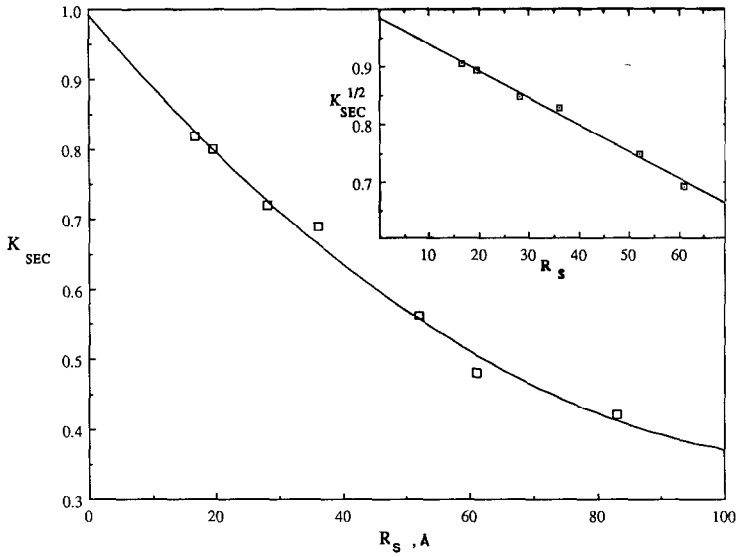

Fig. 3. K_{SEC} *vs.* R_S in optimized mobile phase, with pH 5.5, $I = 0.38$ *M*. Insert: data plotted according to eqn. 2 (see text for explanation).

Mori and Kato[21] similarly described the retention behavior of proteins on diol-bonded porous glass. Both of theses packings would be expected to have considerably larger surface charge densities than Superose.

Three semi-empirical treatments have dealt with electrostatic interactions in SEC[22–24], all of them focussing on repulsive effects that lead to early elution. These three approaches all relate the reduction in V_e to the dimensions of an electrical double layer, but the first two[22,23] assign the effect to the potential around the solute, while the third[24] emphasizes the double layer near the packing. While all three descriptions are cleary incomplete, the present results show the importance of the charge on the packing.

CONCLUSIONS

An empirical optimization procedure leads to mobile phase conditions under which a number of globular proteins with differing isoelectric points, show a dependence of K_{SEC} on Stokes radius in close agreement with that predicted from a simple geometric model. It is suggested that these conditions correspond to "ideal" SEC, and deviations therefrom to electrostatic interactions with the packing. Studies are currently underway to establish whether such "ideal" conditions exist for SEC stationary phases that are more hydrophobic or more highly charged than Superose 6.

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REFERENCES

1 E. F. Cassasa, *Macromolecules*, 9 (1976) 182–185, and references cited therein.
2 J. C. Giddings, E. Kucera, C. P. Russell and M. N. Meyers, *J. Phys. Chem.*, 72 (1968) 4397–4408.
3 M. E. Himmel and P. G. Squire, in P. L. Dubin (Editor), *Aqueous Size-Exclusion Chromatography*, Elsevier, Amsterdam, 1988, Ch. 1.
4 W. Kopaciewicz and F. E. Regnier, in M. T. W. Hearn, F. E. Regnier and C. T. Wehr (Editors), *High-Performance Liquid Chromatography of Proteins and Peptides*, Academic Press, Orlando, FL, 1983, pp. 151–159.
5 H. D. Crone, *J. Chromatogr.*, 92 (1974) 127–135.
6 G. Siepke, H. Müllner and U. Grau, *Angew. Chem., Int. Ed. Engl.*, 25 (1986) 535–552.
7 P. Roumeliotis, K. K. Unger, J. Kinkel, G. Brunner, R. Wieser and G. Tschank, in F. Lottspeich, A. Henschen and K. P. Hupe (Editors), *HPLC in Protein and Peptide Chemistry*, Walter de Gruyter, Berlin, 1981, pp. 72–82.
8 P. L. Dubin, in P. L. Dubin (Editor), *Aqueous Size-Exclusion Chromatography*, Elsevier, Amsterdam, 1988, Ch. 3.
9 H. G. Barth, *J. Chromatogr. Sci.*, 18 (1980) 409–429.
10 E. Pfannkoch, K. C. Lu, F. E. Regnier and H. G. Barth, *J. Chromatogr. Sci.*, 18 (1980) 430.
11 W. J. Moore, *Basic Physical Chemistry*, Prentice-Hall, Englewood Cliffs, NJ, 1983, p. 373.
12 T. Andersson, M. Carlsson, L. Hagel, P.-Å. Pernemalm and J.-C. Janson, *J. Chromatogr.*, 326 (1985) 33–44.
13 E. F. Cassasa, *J. Phys. Chem.*, 75 (1971) 3929–3939.
14 L. Hagel, in P. L. Dubin (Editor), *Aqueous Size-Exclusion Chromatography*, Elsevier, Amsterdam, 1988, Ch. 5.
15 H. Waldmann-Meyer, *J. Chromatogr.*, 350 (1985) 1–13.
16 M. Potschka, *J. Chromatogr.*, 441 (1988) 239–260.
17 P. L. Dubin and J. M. Principi, *Macromolecules*, 22 (1989) 1891–1896.
18 K. W. Burton and G. Nickless, *Chemometrics and Intelligent Laboratory Systems*, 1 (1987) 135–149.
19 H. Waldman-Meyer, *J. Chromatogr.*, 410 (1987) 233–248.
20 P. L. Dubin and J. M. Principi, *Anal. Chem.*, 61 (1989) 780–781.
21 S. Mori and M. Kato, *J. Liq. Chromatogr.*, 10 (1987) 3113–3126.
22 M. G. Styring, C. J. Davison, C. Price and C. Booth, *J. Chem. Soc., Faraday Trans. 1*, 80 (1984) 3051–3058.
23 M. Potschka, *Anal. Biochem.*, 162 (1987) 47–64.
24 P. L. Dubin, C. M. Speck and J. I. Kaplan, *Anal. Chem.*, 69 (1988) 895–900.