Optimization of conditions for size-exclusion chromatography of proteins

D. HAGAROVÁ, M. HORVÁTHOVÁ, V. ŽÚBOR, and A. BREIER

Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, CS-833 34 Bratislava

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Chromatographic properties of bead celluloses (types Perlose MT 100 M, 200 M, 500 M and the low porous Perlose MT 5L) were investigated in conditions for size-exclusion chromatography. With mobile phase containing no detergent the retention time for elution of proteins was considerably long, the sample remained on the top of the column. Increasing content of sodium dodecyl sulfate (SDS) present in the mobile phase lowered the retention volume. At $w(\mathrm{SDS}) = 0.1\%$ the retention volumes could be established safely for all proteins tested. From values of retention volumes the distribution coefficients K_{av} could be calculated for all proteins applied. For Perloses MT 100 M, 200 M, and 500 M the relationship between K_{av} and the relative molecular mass of proteins could be described by the equation $K_{av} = a' + b' M_w^{1/3}$ An extrapolation of respective equations for $K_{av} = 0$ provided the following estimated values for upper exclusion limits: 155 000 ± 28 000, 263 000 ± 75 000, and 547 000 ± 75 000 for MT 100 M, 200 M, and 500 M, respectively. The exclusion limit for low porous cellulose MT 5L was less than 12 400. Results indicate that Perloses may be utilized in size-exclusion chromatography of proteins.

Preparation of macroporous bead cellulose [1, 2] enabled to avoid the known shortages of cellulose materials as carriers for immobilization of proteins applied in enzyme engineering. Concerning its chemical reactivity, bead cellulose is similar to other types of celluloses [3]; characteristic of the former is, however, a high porosity $P = 90$ vol. %, corresponding to 5—5.5 g H_2O/g of dry mass and to bed volume of 7.5—9 cm^3/g of dry mass [4]. Spheric shape of bead cellulose particles enables easy manipulation in both, batch and column applications. Moreover, at increased pressure bead celluloses exhibit relatively high mechanical stability over the current dextran gels [5]. These suitable physical properties and the chemical reactivity of Perloses enabled preparation of basic types of ion exchangers [6, 7] and adsorbents chelating heavy metals [8, 9]. It was the porous structure of bead cellulose which allowed its application in immobilization of enzymes catalyzing hydrolysis of macromolecular substances such as trypsin [10—13]. Determinate factor influencing the course of enzyme reactions was the size-excluding property of cellulose matrix [11—13]. Not less suitable proved to be the bead cellulose also in the development of hydrophobic

[14, 15], biospecific [16, 17], and chemoselective adsorbents [18, 19] for binding or chromatography of proteins. The above properties lead to high-capacity production of bead celluloses (Perloses MT). Since Perloses MT have not been applied yet in size-exclusion chromatography (SEC) of proteins the present paper deals with characterization and optimization of conditions for SEC of proteins on Perloses MT.

Experimental

Bead celluloses applied, Perloses MT 100 M, 200 M, 500 M, and the low porous Perlose MT 5L (Table 1), originated from the North Bohemian Chemical Industries, Lovosice (under the commercial name Perloza). Ferritin (M_w = 440 000), ferritin (heavy chain, 220 000), bovine serum albumin (BSA, 67 000), ovalbumin (45 000), and cytochrome C (12 400) (all from Serva, Heidelberg), thyreoglobulin (heavy chain, 330 000; Pharmacia, Uppsala), further chymotrypsin (25 000; Sigma, St. Louis) as well as human serum albumin and immunoglobulin G (heavy chain, 69 000 and 50 000, respectively; Imuna, Š. Michaľany) were applied as protein standards. Dead volume and total volume of the column were established by means of Blue Dextran (2 000 000; Pharmacia, Uppsala) and dinitrophenylalanine (225; Serva, Heidelberg). Relative molecular masses of single standards were obtained with the aid of gel chromatography.

All the other chemicals applied were of anal. grade (Lachema, Brno).

Table 1

List of Perloses, their characteristics and values of exclusion limits

Name	Particle size / μm	Water content / $cm^3\ g^{-1}$	$M_w^{1/3}$	Exclusion limit
Perlose MT 100 M	100—250	9.90	53.72 ± 3.08	155 000 ± 28 000
Perlose MT 200 M	100—250	14.90	64.08 ± 5.63	263 000 ± 75 000
Perlose MT 500 M	100—250	19.70	81.80 ± 3.56	547 000 ± 75 000
Perlose MT 5L low porous		3.17		< 12 400

Results represent mean approximated values ± standard error for n = 3—5.

Gel chromatography

Stable flow (2 $cm^3\ min^{-1}$) of mobile phase consisting of 0.05 M phosphate buffer (pH = 7.6) containing 0.2 M-NaCl and 0.1 mass % sodium dodecyl

sulfate (SDS) through the column (28 mm × 300 mm; Pharmacia, Uppsala) was supplied by a box pump SC4 (Developmental shops of the Czechoslovak Academy cf Sciences, Prague). Flow out of the mobile phase from the column was detected by means of spectrophotometer UVM 4 (the latter producer).

Values of available distribution coefficients K_{av} were calculated from the equation

$$K_{av} = \frac{V_r - V_0}{V_t - V_0} \tag{1}$$

where V_r represents the retention volume of the given protein, V_0 the dead volume and V_t the total volume of the column.

The course of relationship between K_{av} and M_w was evaluated with the aid of the classical equation for size-exclusion chromatography

$$K_{av} = a + b \log M_w \tag{2}$$

as well as using the following equation

$$K_{av} = a' + b' M_w^{1/3} \tag{3}$$

Exclusion limits were established by extrapolation from eqns (*2*) and (*3*) as the values of $\log M_w$ or $M_w^{1/3}$ for $K_{av} = 0$.

Results

Gel chromatography of protein standards on Perloses with mobile phase not containing SDS showed considerably long retention times. The sample re-

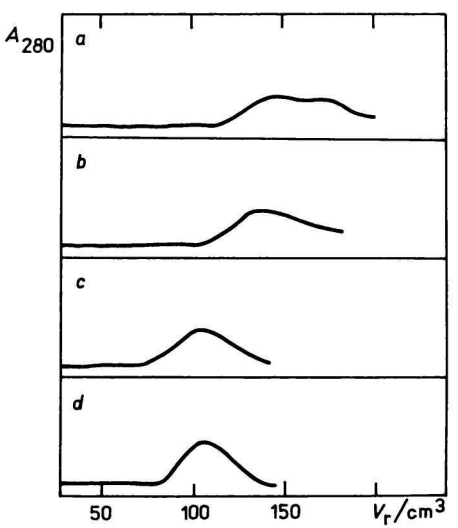

Fig. 1. Dependence of elution profiles of bovine serum albumin on the content of sodium dodecyl sulfate in the mobile phase during chromatography on Perlose MT 100 M. *a*) Without SDS; *b*) 0.005 mass % SDS; *c*) 0.1% SDS; *d*) 0.2% SDS.

mained on beginning of the column and the elution profile was smudgy as it is demonstrated in the case of chromatography of BSA on Perlose MT 100 M (Fig. 1). Increasing content of SDS in the mobile phase had depressive effect on smudging of elution profiles of proteins. At the same time retention volume was also decreased as shown on examples of BSA and myoglobin chromatographed on Perlose MT 100 M (Fig. 2). The relationship between retention volume and mass fraction of SDS revealed that 0.1% SDS added to the mobile phase secures already an exact estimation of the retention volume avoiding thus any mistakes brought by smudging of the elution profile.

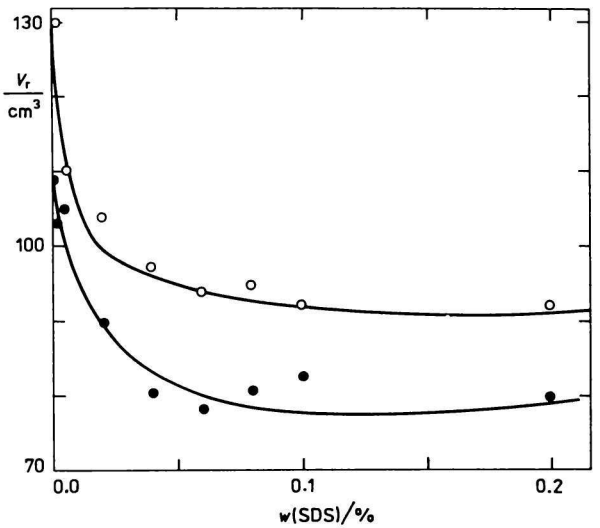

Fig. 2. Dependence of retention volume of bovine serum albumin (●) and myoglobin (○) on the content of sodium dodecyl sulfate in the mobile phase during chromatography on Perlose MT 100 M.

Figs. 3—5 represent calibration relationships for diverse Perlose fillings. Exclusion limits ascertained by extrapolation from eqn (*2*) exhibited very high and unrealistic values. This may be ascribed to the good visible concave course of the relationship $K_{av} = f(\log M_w)$ (Figs. 4 and 5). For latter reason the values of exclusion limits were established by extrapolation from eqn (*3*) which is expressing the events occurring on the column more exactly. Exclusion limits read up from calibration curves for different Perloses are presented in Table 1.

In the case of low porous Perlose MT 5L the exclusion limits for diverse proteins were impossible to establish experimentally because they are scaled between the relative molecular masses of 255 to 12 400. Hence, all the protein standards applied have passed the column within its dead volume V_0 (Fig. 6).

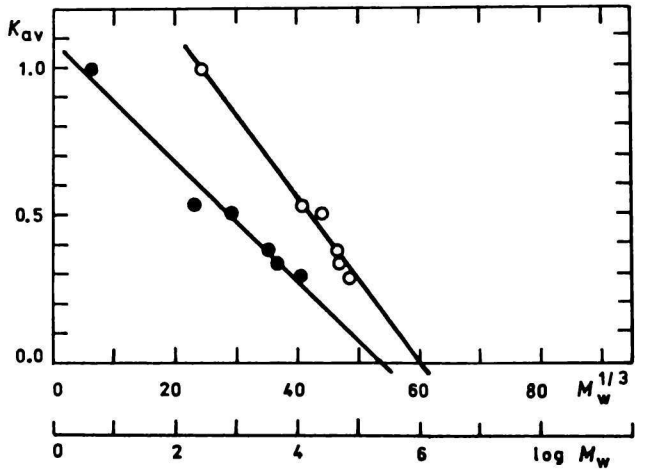

Fig. 3. Calibration curves for Perlose MT 100 M.
○ the relationship obtained by means of eqn (*2*) (parameters: $a = 1.693 \pm 0.077$; $b = -0.283 \pm 0.018$; $r = 0.992$), ● the relationship obtained by means of eqn (*3*) (parameters: $a' = 1.090 \pm 0.053$; $b' = -(2.031 \pm 0.172) \times 10^{-2}$; $r = 0.986$).

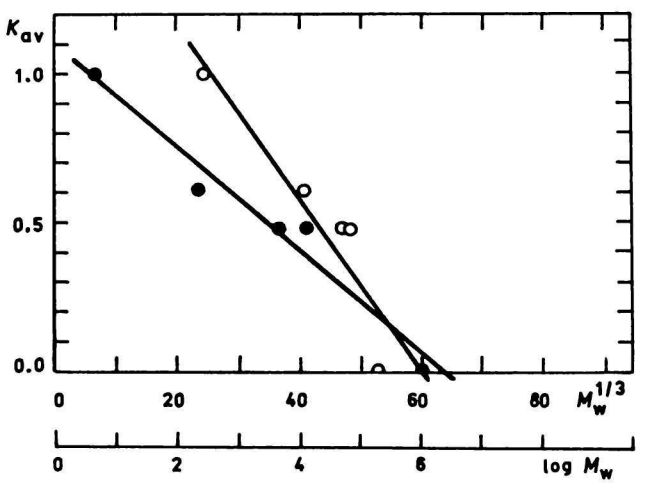

Fig. 4. Calibration curves for Perlose MT 200 M.
○ the relationship obtained by means of eqn (*2*) (parameters: $a = 1.750 \pm 0.293$; $b = -0.287 \pm$ ± 0.067; $r = 0.928$), ● the relationship obtained by means of eqn (*3*) (parameters: $a' = 1.094 \pm$ ± 0.075; $b' = -(1.710 \pm 0.197) \times 10^{-2}$; $r = 0.981$).

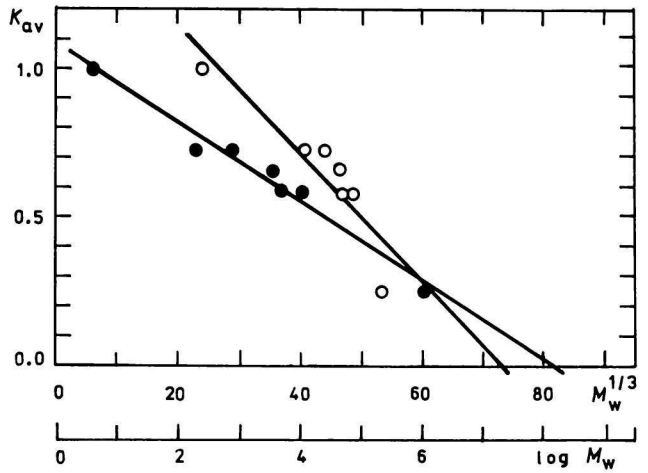

Fig. 5. Calibration curves for Perlose MT 500 M.
○ the relationship obtained by means of eqn (*2*) (parameters: $a = 1.591 \pm 0.205$; $b = -0.216 \pm 0.046$; $r = 0.902$), ● the relationship obtained by means of eqn (*3*) (parameters: $a' = 1.094 \pm 0.037$; $b' = -(1.337 \pm 0.101) \times 10^{-2}$; $r = 0.986$).

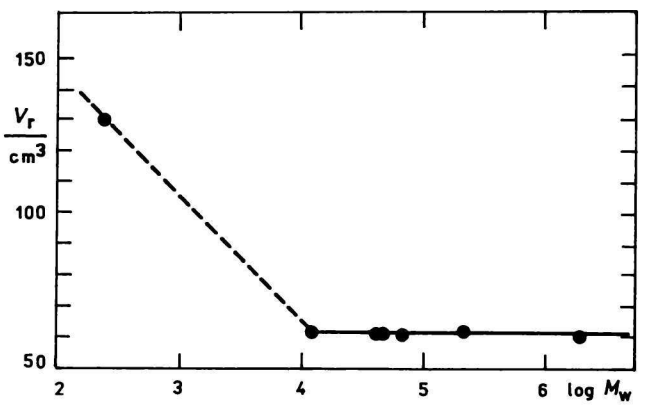

Fig. 6. Dependence of retention volumes on M_w values of protein standards during chromatography on low porous Perlose MT 5L.

Discussion

Perloses investigated proved to be suitable for size-exclusion chromatography of proteins. Nevertheless, their use requires unconditionally the application in the mobile phase of some substance with detergent properties. This

requirement is justified by adsorption of proteins onto Perloses probably *via* electrostatic interactions. Already relatively low content of SDS (0.1%) present in the mobile phase is capable to depress considerably the above interactions. Prevention of adsorption of proteins onto Perloses by increasing the content of SDS in the mobile phase manifested itself in changes of the shape of elution profiles of BSA (Fig. 1) or in changes of retention volume of BSA and myoglobin (Fig. 2) on the Perlose MT 100 M. Relationship between the available distribution coefficient K_{av} and the third root of relative molecular mass of proteins M_w was found to be linear for all three macroporous Perloses investigated (Figs. 3—5). Assuming that for globular proteins the third root of relative molecular mass is proportionate to the radius of the protein ball [12] it would be possible to assume that in given conditions the distribution of protein between mobile and stationary phases depends exclusively on the radius of the protein ball. The latter assumption enabled the reliable estimation of upper exclusion limits for Perloses (Table 1) by extrapolation from eqn (*3*) for $K_{av} = 0$. Constant a' in eqn (*3*) represents the value of K_{av} for a substance with $M_w = 0$ if the value of K_{av} for DNP-alanine is considered arbitrarily as 1. Then, for all three macroporous Perloses the relative retention of DNP-alanine in relation to a fictive substance with relative molecular mass near to zero will represent 91.7% approximately. Constant b' from eqn (*3*) is a measure of dependence of available distribution coefficient on M_w. It is indicating also the sensitivity of M_w estimation by means of size-exclusion chromatography. Therefore it is comprehensible that with upper exclusion limits of Perloses increasing within the range between MT 100 M to 500 M the sensitivity of M_w estimation decreases.

Conclusion

Perloses are suitable for gel chromatography of proteins, but when applied, the mobile phase has to contain detergents which are minimalizing the adsorption of proteins onto the carrier during their passage through the column. The latter property makes the application of Perloses for analytical and preparatory purposes disadvantageous in comparison to carriers such as Sephadex. However, because of their price being one order below that for other carriers for gel chromatography the application of Perloses seems to be still possible particularly in prepurification procedures used in half preparative and preparative scale.

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