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Note

Characterization of cross-linked 6% agarose (Sepharose CL-6B) as a medium for gel filtration of proteins in the presence of guanidine hydrochloride

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In concentrated solutions of guanidine hydrochloride (Gdn·HCl), polypeptide chains behave as linear random coils[1] and, as a consequence, the hydrodynamic radii vary in a predictable manner with the molecular weights[2]. Because in gel filtration experiments the distribution coefficients of proteins depend on the hydrodynamic radii[3], gel filtration in the presence of 6 *M* Gdn·HCl is suitable for the determination of molecular weights[2,4,5].

Sepharose 6B (Pharmacia, Uppsala, Sweden) or Bio-Gel A-5m (Bio-Rad Labs., Richmond, Calif., U.S.A.), both containing 6% agarose, have been used as filtration media in most of the studies described. Recently it has been reported that soft (non-cross-linked) agarose gels are not stable in strong denaturants such as 6 *M* Gdn·HCl, whereas under the same conditions cross-linked gels (*e.g.*, Sepharose CL-6B) are highly stable[6].

The present study confirms and further extends this observation. Proteins have been fractionated on a column of Sepharose CL-6B in 6 *M* Gdn·HCl. The mean effective pore radius of the gel has been determined from data obtained with proteins of known hydrodynamic radii in 6 *M* Gdn·HCl. The mean effective pore radius of the cross-linked gel was found to be almost twice that of the non-cross-linked gel.

Calculation of the fraction of the gel matrix that is involved in protein chromatography showed that Sepharose CL-6B in 6 *M* Gdn·HCl has a variable gel porosity. In agreement with this result it was found that a non-linear relationship exists between the hydrodynamic radii (R_s) of proteins and the inverse of the error function complement of the distribution coefficients ($\text{erfc}^{-1}\ K_d$).

The column described has been used for the determination of the hydrodynamic radii of the polypeptides of Rauscher murine leukaemia virus.

MATERIALS AND METHODS

Samples of standard molecular weight proteins were dissolved in 6 *M* Gdn·HCl containing 13% (v/v) of 2-mercaptoethanol and 20 m*M* of EDTA[8], and incubated for 2–4 h at 37°. The pH was adjusted to 5–6 with acetic acid and sucrose was added to a final concentration of 10% (w/v). A column (166 × 1.6 cm) of Sepharose

CL-6B (Lot No. 2006, Pharmacia) was prepared according to Green and Bolognesi[8]. The elution buffer contained 6 *M* of Gdn·HCl, 50 m*M* of sodium acetate and 0.005% (v/v) of 2-mercaptoethanol and had a pH of 5.0. All chromatographic experiments were carried out at 4°. Mixtures of three or four proteins (3–5 mg of each) were run through the column at one time. The column was operated at a pressure drop of 50 cm, resulting in a flow-rate of 2 ml/h (1 ml per cm^2 of column cross-section per hour). Fractions of about 1.5 ml were collected and monitored by measuring the absorbance at 280 nm. All fractions collected were weighed. Weight instead of volume was used as the measure of elution positions[2]. The density of the elution buffer remained constant, so that elution weights are proportional to elution volumes (V_e).

The distribution coefficients (K_d) were calculated according to equation $K_d = V_e - V_0/V_i$. The void volume (V_0) was determined by chromatography of a sample of Dextran Blue 2000 (Pharmacia) while the volume of solvent contained within the gel (V_i) was determined by subtracting the value of V_0 from the elution volume of 2-mercaptoethanol. For the column described above, V_0 and V_i were 117 and 220 ml, respectively.

RESULTS AND DISCUSSION

A single column of Sepharose CL-6B in 6 *M* Gdn·HCl was calibrated with molecular weight marker proteins. The distribution coefficients (K_d) of these proteins are given in Table I. Hydrodynamic radii (or Stokes radii, R_s) are given for those proteins for which values of R_s in 6 *M* Gdn·HCl are available from intrinsic viscosity measurements[9]. Values of K_d are the means of three or more determinations. The origin of the protein designated p170 in Table I has been described elsewhere[10]. The column was used over a period of 2 years at 4°, and no change was observed in the elution positions of the molecular weight marker proteins.

TABLE I

PROTEINS USED FOR THE CALIBRATION OF A COLUMN OF SEPHAROSE CL-6B IN 6 *M* GUANIDINE HYDROCHLORIDE

Protein	*Molecular weight*	R_s (Å)	K_d
p170	170,000		0.179
Phosphorylase a	94,000		0.255
Conalbumin	86,200		0.294
Transferrin	76,600	86.8	0.308
Bovine serum albumin	68,000	83.0	0.322
Catalase	60,000		0.350
Rabbit γ-globulin, heavy chain	49,500	68.2	0.380
Aldolase	40,000		0.400
Carbonic anhydrase	29,000		0.478
Chymotrypsinogen	25,700	47.8	0.488
β-Lactoglobulin	18,400	40.5	0.543
Lysozyme	14,300		0.601
α-Lactalbumin	14,200	34.0	0.601

The relationship between molecular weight (M) and K_d was calculated by linear regression analysis to be $\log M = 5.652 - 2.523\ K_d$. The exclusion limit ($K_d = 0$) of the gel is approximately 450,000. For a non-cross-linked 6% agarose gel (Sepharose 6B or Bio-Gel A-5m) the exclusion limit lies in the range 100,000–120,000[2,4,5].

In order to illustrate further the different behaviour of the cross-linked and the non-cross-linked gels towards a strong denaturant such as 6 *M* Gdn·HCl, the mean effective pore radii, r, have been calculated. Values of r were calculated according to Ackers[11] from the K_d values of proteins with known hydrodynamic radii in 6 *M* Gdn·HCl[9]. For Sepharose CL-6B r was found to be 33 nm. However, different lots of a particular gel can be expected to vary with respect to their physical properties. The only other batch of Sepharose CL-6B (Lot No. 2108) used during the course of this study had a mean effective pore radius of 31 nm, close the value given above.

From the data of Fish *et al.*[2], Bryce and Crichton[4] and Tung and Knight[5], values of r for non-cross-linked 6% agarose gels were calculated to be 17, 19 and 19 nm, respectively. From these values, it can be inferred that the non-cross-linked gel, as compared to the cross-linked gel, is subject to shrinkage in 6 *M* Gdn·HCl.

Bryce and Crichton[4] found that two gel porosities are involved in the separation of proteins and peptides by Sepharose 6B in 6 *M* Gdn·HCl. The gel population with larger pore size is involved in protein chromatography and that with smaller pore size in peptide chromatography. The fraction of the total gel matrix that is involved in protein chromatography was found to be 0.742 (ref. 4). The data listed in Table I were used to calculate similar data for Sepharose CL-6B in 6 *M* Gdn·HCl according to Bryce and Crichton[4]. The fraction of the gel matrix that is responsible for the fractionation of proteins was calculated to be 0.751, close to the value found by Bryce and Crichton for the non-cross-linked gel.

Ackers[3,7] has shown that for many filtration media there is a linear relationship between hydrodynamic radii (or Stokes radii, R_s) of proteins and the inverse of the error function complement of the distribution coefficients ($\mathrm{erfc}^{-1}K_d$), whereas for a column with different gel porosities the plot of R_s against $\mathrm{erfc}^{-1}K_d$ is not linear. For Sepharose CL-6B in 6 *M* Gdn·HCl the plot of R_s against $\mathrm{erfc}^{-1}K_d$ has been constructed using the data in Table I for those proteins for which values of R_s in 6 *M* Gdn·HCl were available. The plot of R_s *versus* $\mathrm{erfc}^{-1}K_d$ is shown in Fig. 1. The points show a deviation from linearity that is characteristic of a column with different gel porosities[7].

The column has been used for the determination of the hydrodynamic radii of Rauscher murine leukaemia virus proteins. R_s values in 6 *M* Gdn·HCl were found to be 74 Å for gp70, 51 Å for p30, 36 Å for p15, 29 Å for p12 and 24 Å for p10. The nomenclature of these proteins is according to August *et al.*[12].

Summarizing, a column of Sepharose CL-6B in 6 *M* Gdn·HCl can be used over a long period of time for the determination of molecular weights and Stokes radii of proteins. More than two calibration standards should be used for calibration of the column according to Ackers[3], because the relationship between R_s and $\mathrm{erfc}^{-1}K_d$ is not linear.

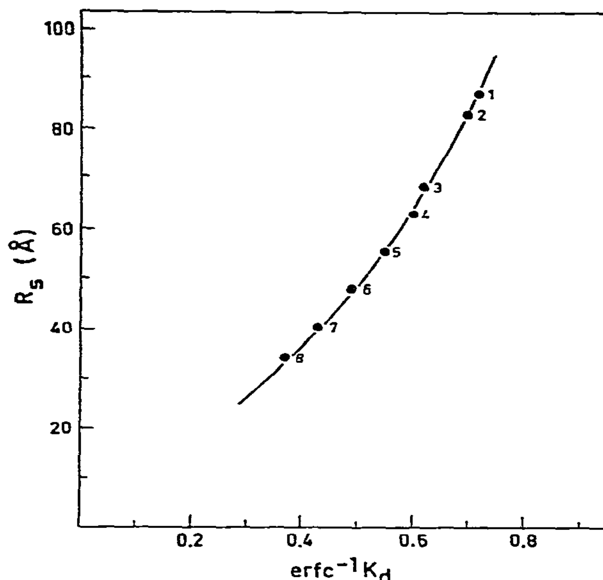

Fig. 1. Calibration plot of Stokes radii (R_s) against erfc^{-1} K_d for Sepharose CL-6B in 6 M Gdn·HCl. The following proteins with known R_s in 6 M Gdn·HCl[9] were used for constructing the plot: 1 = transferrin (86.8 Å); 2 = bovine serum albumin (83.0 Å); 3 = γ-G heavy chain (68.2 Å); 4 = ovalbumin (62.8 Å); 5 = lactate dehydrogenase (55.3 Å); 6 = chymotrypsinogen (47.8 Å); 7 = β-lactoglobulin (40.5 Å); 8 = α-lactalbumin (34.0 Å).

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