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Calibration of the relative molecular mass of proteoglycan subunit by column chromatography on Sepharose CL-2B

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Calibration relationships were derived for cartilage proteoglycan subunit (PGS) that relate the inverse *z*-average hydrodynamic radius (R_s) and the weight-average M_r (M_w) to the partition coefficient ($K_{av.}$) for PGS when chromatographed on a Sepharose CL-2B column. PGS isolated from chick limb-bud chondrocyte cell cultures was fractionated chromatographically into eight pools, for which M_w and R_s were determined by total-intensity and dynamic light-scattering measurements. These data were found to be related to $K_{av.}$ through the following empirical equations:

$$\log \overline{M}_w = -(1.65 \pm 0.27) K_{av.} + (6.58 \pm 0.08)$$

$$\log R_s = -(0.69 \pm 0.04) K_{av.} + (2.75 \pm 0.01)$$

Application of these relationships to the chromatographic data led to $M_w = 1.48 \times 10^6$ and $R_s = 38.7$ nm (387 Å) for the unfractionated specimens compared with values of $M_w = 1.46 \times 10^6$ and $R_s = 38.2$ nm (382 Å) determined by light-scattering. Our results were found to be consistent with previously proposed phenomenological models for the gel-filtration mechanism. Application of these calibration relationships to $K_{av.}$ for several unfractionated specimens led to predicted values of M_w and R_s that are accurate to within 10%.

INTRODUCTION

We are using light-scattering and other physical-chemical techniques to investigate the structure and properties of cartilage proteoglycans in solution. In previous papers [1, 2] we have reported a weight-average M_r (M_w) $= 1.42 \pm 0.30 \times 10^6$ and a hydrodynamic radius $R_s = 37.6 \pm 2.0$ nm (376 ± 20 Å) for PGS isolated from day-8 chick limb-bud chondrocyte cell cultures. These data are for PGS isolated in 4 M-GdnHCl and separated as the bottom one-fourth of a CsCl gradient (fraction D1). The size of these PGS molecules is typical of those seen for other cartilage proteoglycans, for which we have measured M_r values in the range 0.5×10^6–4.0×10^6 [3, 4]. The present knowledge of the structure of proteoglycans is reviewed elsewhere [5–8].

Although light-scattering is the method of choice for absolute M_r determinations, biochemical analyses often employ chromatographic methods to make qualitative comparisons of PGS size and size distribution. This is usually done in terms of the partition coefficient, $K_{av.}$, which is defined as:

$$K_{av.} = \frac{V_e - V_v}{V_{tot.} - V_v} \quad (1)$$

where V_e, V_v and $V_{tot.}$ are the elution, void and total volumes respectively. Typical values of $K_{av.}$ for cartilage PGS on Sepharose CL-2B are in the range 0.1–0.4. For many synthetic polymers and biopolymers, calibrations have been established for determination of M_r values from the $K_{av.}$ values. These calibration relationships generally take the form:

$$\log M_w = a \cdot K_{av.} + b \quad (2)$$

where *a* and *b* are constants. A similar type of relationship is to be expected between $K_{av.}$ and the hydrodynamic radius, R_s. Note that these empirical relationships are found to be accurate only when $K_{av.}$ is not close to 0 or 1.

The aim of the present paper is to derive such calibration relationships for cartilage PGS. It should be noted that Schwartz *et al.* [9] and Iozzo *et al.* [10] have recently used h.p.l.c. to estimate M_w of PGS on the basis of the retention times. However, it is more useful to compare samples in terms of $K_{av.}$, in order to correct for experimental factors such as the column filler, the column length and the flow rate. In view of the frequency of reported characterization of PGS in terms of $K_{av.}$ on Sepharose CL-2B, we have determined $K_{av.}$, M_w and R_s for a series of fractionated chick limb-bud chondrocyte PGS specimens, and have derived calibration relationships therefrom. Our work also provides a physical rationale for the validity of using eqn. (2) for determining M_r values of PGS by exclusion chromatography.

MATERIALS AND METHODS

Chick limb-bud chondrocyte PGS

The PGS were prepared from day-8 and day-21 chick limb-bud chondrocyte cell cultures [11, 12] as described

Abbreviations used: PGS, proteoglycan subunits; GdnHCl, guanidinium chloride.

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previously [13]. The tissue was extracted in 4 M-GdnHCl containing proteinase inhibitors. Solid CsCl was added directly to the solution to produce a density of 1.5 g/ml, and centrifuged to the equilibrium at 35000 rev./min in a Beckman L5-50 centrifuge (50 Ti rotor), for 6 h at 10 °C. The resultant gradients were cut into four approximately equal fractions. The densest fraction (D1), which contained about 80% of the uronic acid [14], was further purified by chromatography on a preparative column (110 cm × 2.5 cm) of Sepharose CL-2B with 4 M-GdnHCl as eluent. Solutions containing 10–20 mg of PGS were prepared in 4 M-GdnHCl containing proteinase inhibitors and buffered with 0.05 M-sodium acetate to pH 5.8. Fractions (6 ml) were collected and assayed for uronic acid [14]. The fractions were combined as eight pools, as shown in Fig. 1. These were dialysed against distilled water at 4 °C, freeze-dried and stored at −20 °C. In some cases the dialysed samples were concentrated without being taken to dryness. While still in the dialysis bag, these samples were immersed in Sephadex G-50 at 4 °C and allowed to stand so that most of the water would be drawn out by the resin. After the samples reached the desired concentration, as determined by uronic acid assay, they were stored at 4 °C.

Human articular cartilage PGS

PGS extracted from human articular cartilage were gifts from Dr. Peter J. Roughley of Shriners Hospital for Crippled Children, Montreal, Que., Canada, and had been extracted and characterized as described in ref. [15].

Light-scattering

Dynamic and total-intensity light-scattering data were obtained by using the equipment described previously [1–4]. The solutions of PGS in 4 M-GdnHCl were filtered through 0.45 μm-pore Millipore HAWP filters, sealed in light-scattering cells and centrifuged at 8000 g for 30 min before light-scattering measurements. The weight-average M_r values, $\overline{M}_w$, were determined from Zimm plots of the total-intensity data. The refractive-index increment, dn/dc, for PGS in 4 M-GdnHCl at 25 °C was determined to be 0.141 ml/g with a Woods model RF-600 differential refractometer. The apparent diffusion coefficients were measured at 30°, 50° and 60° scattering angles and extrapolated to zero scattering angle. This extrapolated value was taken as $D^0_{t,z}$, the z-average zero concentration translational diffusion coefficient, since the apparent diffusion coefficient was independent of concentration at low concentrations. The hydrodynamic radius, R_s, was calculated from $D^0_{t,z}$ via the Stokes–Einstein equation. This calculation for polydisperse specimens generates an average that is actually the inverse of the z-averaged reciprocal hydrodynamic radius, $\langle 1/R_s \rangle_z$.

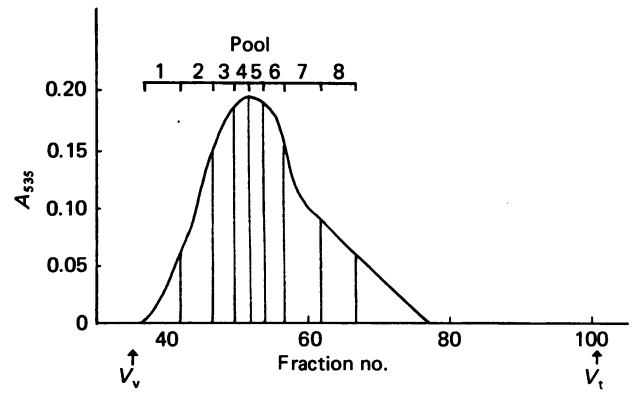

Fig. 1. Sepharose CL-2B chromatogram of day-8 chick limb-bud chondrocyte PGS, showing separation into eight pools

For experimental details see the text.

RESULTS AND DISCUSSION

Fig. 1 is the Sepharose CL-2B column profile for day-8 chick limb-bud chondrocyte culture PGS. The specimen was separated into eight pools as shown, and the $K_{av.}$, M_w and R_s data for these pools are listed in Table 1. Table 2 shows the equivalent data for unfractionated day-8 and day-21 chick limb-bud chondrocyte PGS, and for a previously analysed day-8 specimen [1] that had been fractionated into three pools. Data for unfractionated human foetal and adult articular-cartilage PGS are also included.

The experimental $K_{av.}$, M_w and R_s data in Table 1 were used to derive calibration relationships. Gel filtration separates the molecules according to their hydrodynamic radius, and hence it is more rigorous to derive calibrations in terms of R_s than M_w. Note also that the R_s data are determined with higher precision by dynamic methods than are the M_w data derived from Zimm plots of the total scattered intensity. Fig. 2 shows a plot of $K_{av.}$ versus log R_s for the eight fractions, in which it can be seen

Table 1. Physicochemical parameters of day-8 chick limb-bud chondrocyte PGS fractions

Pool no.	$K_{av.}$	$10^6 \times \overline{M}_w$		R_s (nm)	
		Observed	Calculated	Observed	Calculated
1	0.06	3.00±0.5	3.00	59.3±5.5	51.1
2	0.15	2.42±0.4	2.15	44.2±2.0	44.3
3	0.20	1.95±0.3	1.78	42.5±1.5	40.9
4	0.23	1.33±0.4	1.59	39.0±1.5	39.0
5	0.26	1.43±0.3	1.42	36.0±1.5	37.2
6	0.30	1.05±0.2	1.22	34.6±1.0	34.9
7	0.36	0.88±0.1	0.97	32.5±1.0	31.7
8	0.44	0.83±0.1	0.71	28.0±2.0	28.0
Unfractionated	0.25	1.50±0.3	1.48	38.2±1.5	38.7

Table 2. Physicochemical parameters of several PGS preparations

Sample	$K_{av.}$	$10^{-6} \times M_w$	R_s (nm)	Ref.
Chick day 8*				
Fraction I	0.19	1.96±0.3	41.7±2.0	[1]
Fraction II	0.28	1.42±0.3	38.3±2.0	[1]
Fraction III	0.41	1.05±0.3	33.0±2.0	[1]
Chick day 8	0.25	1.50±0.3	39.2±2.0	
Chick day 21	0.27	1.30±0.3	36.6±2.0	
Human foetal	0.18	1.80±0.3	42.1±2.0	[15]
Human adult	0.33	1.17±0.3	36.3±2.0	[15]

* Data cited from ref. [1].

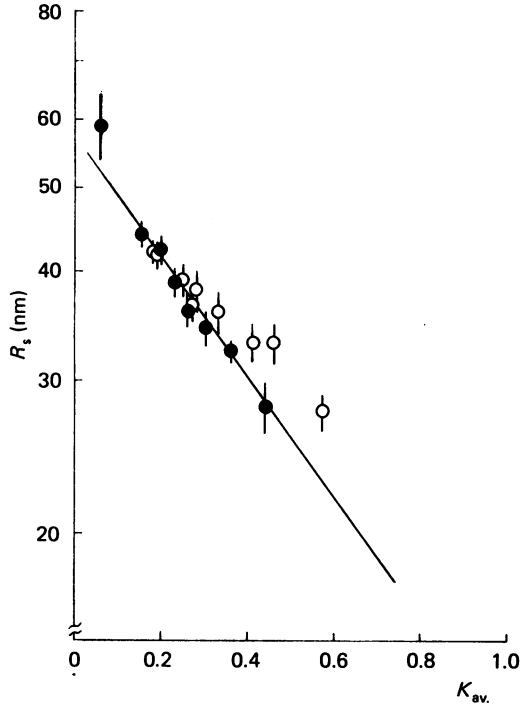

Fig. 2. Plot of $\log R_s$ versus $K_{av.}$ for fractionated day-8 chick limb-bud chondrocyte PGS (●)

The data for unfractionated PGS are shown as ○ symbols and were not used to determine the least-squares fit to a straight line shown.

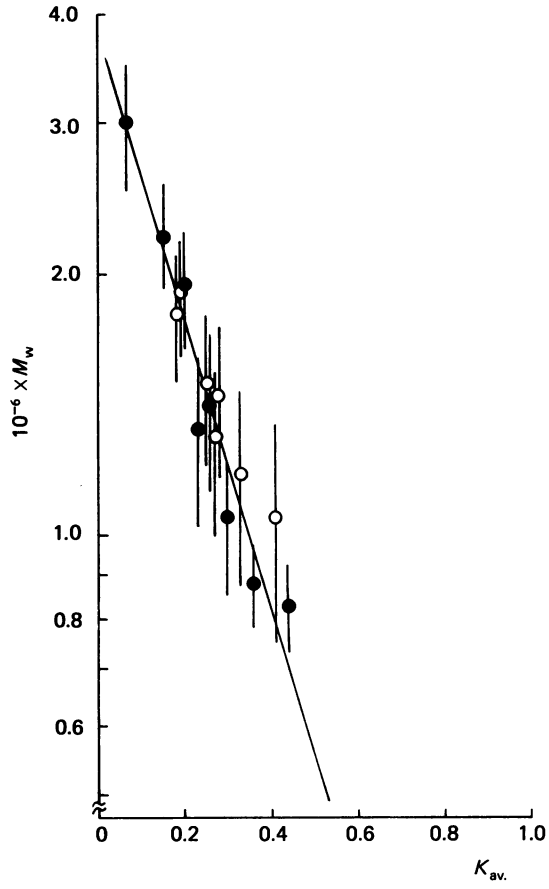

Fig. 3. Plot of $\log M_w$ versus $K_{av.}$ for fractionated day-8 chick limb-bud chondrocyte PGS (●)

Data for unfractionated PGS are shown as ○ symbols and were not used to determine the least-squares fit to a straight line shown.

that points for pools 2–8 fall on a straight line, but that that for pool 1 is displaced to higher R_s. It should be noted that fraction 1 is from the high-M_r part of the distribution close to the void volume and has $K_{av.} = 0.05$, which is in the region where the calibration relationship is not expected to hold. The relationship derived from the points for pools 2–8 is:

$$\log R_s = -(0.69 \pm 0.04) K_{av.} + (2.75 \pm 0.01) \quad (3)$$

The equivalent plot of $K_{av.}$ versus $\log M_w$ is shown in Fig. 3. The straight line derived by linear regression for the points for pools 2–8 is:

$$\log M_w = -(1.65 \pm 0.27) K_{av.} + (6.58 \pm 0.08) \quad (4)$$

The higher experimental errors in the M_w data are apparent, and the point for pool 1 also conforms to eqn. (4).

Combination of eqns. (3) and (4) gives:

$$\log M_w = 2.39 \log R_s - 0.09 \quad (5)$$

which compares reasonably well with our empirical relationships for R_s and M_w derived previously [4] for PGS and proteoglycan aggregate:

$$\log M_w = 2.65 \log R_s - 0.67 \quad (6)$$

in which R_s is expressed in 0.1 nm (Å) units. The last equation was derived for unfractionated specimens including high-M_r proteoglycan aggregate (for which the determined M_w values have higher experimental errors) and this probably accounts of the small differences in the empirical constants. These relationships suggest that, as a result of their branched structure, PGS molecules behave like highly solvated spheres of uniform segment density, for which:

$$\frac{4}{3}\pi R_s^3 = \frac{M_w}{N_A}(\bar{v}_2 + \delta_1 v_1^0) \quad (7)$$

where $\bar{v}_2$ is the partial specific volume of the solute, v_1^0 is the molar volume of solvent, δ_1 is the ratio of the concentrations of solute and solvent in the solvated particles and N_A is Avogadro's number. Given that both

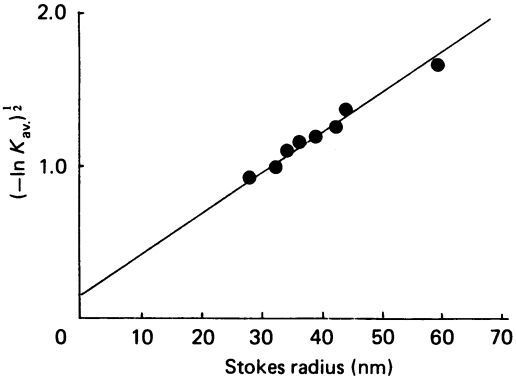

Fig. 4. Plot of $(-\ln K_{av.})^{\frac{1}{2}}$ against R_s for eight fractions of day-8 chick limb-bud chondrocyte PGS

The straight line is a fit to eqn. (11).

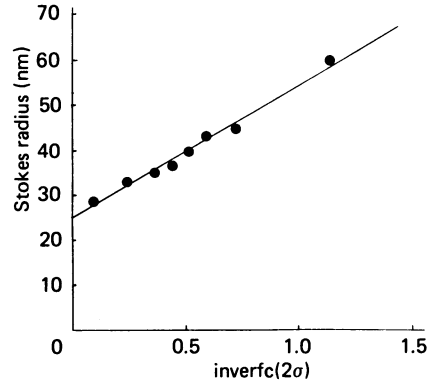

Fig. 5. Plot of inverfc(2σ) against R_s for eight fractions of day-8 chick limb-bud chondrocyte PGS

The straight line is a fit to eqn. (14).

PGS and proteoglycan aggregate molecules form spheres containing approx. 99% water [2], $\delta_1 v_1^0 \gg \bar{v}_2$ for these solutions, and hence:

$$M_w \propto R_s^3 \tag{8}$$

The exponents of 2.39 and 2.65 from eqns. (5) and (6) probably reflect deviations from the homogeneous spherical conformation.

Eqns. (3) and (4) can now be used to derive R_s and M_w for the unfractionated day-8 specimen from the following definitions:

$$M_w = \frac{\Sigma c_i M_i}{\Sigma c_i} \tag{9}$$

$$R_s^{-1} = \frac{\Sigma c_i M_i R_{s,i}^{-1}}{\Sigma c_i M_i} \tag{10}$$

where c_i is the concentration of PGS in the ith fraction, and the summations are over all eight fractions. The concentrations were determined from the areas under the respective sections of the chromatogram. The latter are estimates of the uronic acid content, and were adjusted for a protein content of 7%. Calculated values of R_s and M_w for each fraction were derived from $K_{av.}$ by using eqns. (3) and (4), and the results are listed in Table 1. These lead to $M_w = 1.48 \times 10^6$ and $R_s = 38.7$ nm (387 Å) for the unfractionated specimen, compared with observed values of $M_w = 1.46 \times 10^6 \pm 0.30 \times 10^6$ and $R_s = 38.2 \pm 2.0$ nm (382 ± 20 Å) determined by light-scattering.

As a further test of self-consistency, insertion of $K_{av.} = 0.245$ for the maximum in the chromatograph (Fig. 1) into eqns. (3) and (4) yields $R_s = 38.1$ nm (381 Å) and $M_w = 1.50 \times 10^6$. These values are almost indistinguishable from those calculated from the data for the fractions and are well within experimental error of the observed R_s and M_w from light-scattering. The equivalent data for four unfractionated PGS specimens and for three broader fractions of day-8 chick limb-bud PGS are plotted in Figs. 2 and 3 as ○ symbols. It can be seen that these data points all fall close to the lines for the fractionated specimens. In each case there are positive deviations in that the M_w and R_s values are larger than would be expected from the calibration, probably owing to the fact that the effect of polydispersity is to bias the measured averages towards the larger molecules. Hence prediction of R_s and M_w from the value of $K_{av.}$ for an unfractionated specimen leads to values that are lower than the true values. Nevertheless this deviation is less than 10% for all seven specimens.

Finally, it is of interest to test our experimental results against the phenomenological models that have been used to correlate $K_{av.}$ and R_s data over the entire range of $K_{av.}$. Laurent & Killander [17] defined a model for agarose gels made up of randomly oriented rodlike chains, and derived the following relationship:

$$(-\ln K_{av.})^{\frac{1}{2}} = (\pi L)^{\frac{1}{2}} \cdot (R_s + R_r) \tag{11}$$

where L is the concentration of rods in cm of rod per cm^3 of gel and R_r is the rod radius. Fig. 4 shows a plot of our data as $(\ln K_{av.})^{\frac{1}{2}}$ against R_s. After omission of the point for pool 1 in view of the high uncertainty in $\ln K_{av.}$ ($K_{av.}$ is close to zero), a linear regression gives $R_r = 3.2$ nm (32 Å) and $L = 0.27 \times 10^{-11}$ cm of rods/cm^3. Note that Laurent [18] obtained $R_r = 2.5$ nm (25 Å) for each of four different agarose gels (not including Sepharose CL-2B) on the basis of a larger spread of $K_{av.}$ for globular proteins.

An alternative treatment is based on that due to Ackers [19], who assumed a normal distribution of pore sizes and computed the partition coefficient $\sigma = V_p/V_1$, where V_p is the penetrable volume for a particle of specified size and V_1 is the internal volume. Thus:

$$\sigma = \frac{1}{\sqrt{\pi}} \cdot \int_{-\infty}^{\infty} e^{-x^2} \cdot dx - \frac{1}{\sqrt{\pi}} \cdot \int_{-\infty}^{x} e^{-x^2} \cdot dx \tag{12}$$

where x is a normalized pore-size parameter:

$$x = (R - R_0)/\Delta R$$

in which R_0 is the mean pore size and ΔR is the standard deviation of the pore sizes. Putting $R = R_s$ leads to:

$$2\sigma = 1 - \frac{2}{\sqrt{\pi}} \cdot \int_0^x e^{-x^2} \cdot dx = \text{erfc}\left(\frac{R_s - R_0}{\Delta R}\right) \tag{13}$$

where erfc signifies the error function complement of the normal distribution. Inversion of eqn. (13) gives [20]:

$$R_s = R_0 + \Delta R \text{ inverfc } (2\sigma) \tag{14}$$

Note that this differs from the result of Ackers [19] by a factor of 2 in the argument of the inverse error function complement, signified by inverfc. Fig. 5 shows a plot of R_s against inverfc(2σ) and the data accurately follow a straight line, from which we derive $R_0 = 22.5$ nm (225 Å)

and $\Delta R = 28.6$ nm (286 Å). The former result is remarkably consistent with the supplier's specification (personal communication from Dr. S. Ostrove, Pharmacia, Piscataway, NJ, U.S.A.) of 45.0 nm (450 Å) for the mean pore diameter (2 R_0) in a Sepharose CL-2B gel.

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