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Short Communication

Determination of the average molecular size of glycosaminoglycans by fast protein liquid chromatography

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ABSTRACT

The determination of the molecular masses of glycosaminoglycans (GAGs) has in the past been hampered by a lack of readily available standards. In the present study methods were determined for the fractionation of commercially available bovine tracheal chondroitin sulphate A into essentially mono-disperse GAG pools which were shown to be suitable as standards for the calibration of Superose 6 and 12 fast protein liquid chromatograph (FPLC) columns. Superose 6 FPLC was particularly suitable for assessing the size distribution of GAGs of M_r 10 000–40 000 and the relatively high flow-rates possible with this support enabled a considerably faster analysis of samples compared to the soft gels previously used for this purpose.

INTRODUCTION

In 1971 Wasteson [1,2] published details of the fractionation and characterisation of glycosaminoglycans (GAGs) from ox nasal septa using Sephadex G200 gel permeation chromatography [1,2]. Despite the relatively polydisperse nature of the GAGs examined, discrete essentially mono-disperse pools were nevertheless prepared which were shown to be suitable as standards for assigning size distributions to unknown GAG samples. However, the soft gel matrices employed which necessitated slow flow-rates precluded the rapid analysis of samples. In the current study, the original methodology of Wasteson was employed to prepare chondroitin sulphate standards from bovine tracheal chondroitin sulphate A, a commercially available source, and these were subsequently used to calibrate Superose 6 and 12 fast protein liquid chromatography (FPLC) columns. The molecular mass values obtained by FPLC were comparable to those obtained earlier with softer gel matrices [1,2], however a considerably faster analysis of samples was possible (*ca.* 1 h per sample), since a relatively fast flow-rate was possible with these FPLC matrices.

EXPERIMENTAL

Reagents

Sephadex G200, Superose 6 and 12 pre-packed HR 10/30 FPLC columns were obtained from

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Pharmacia Australia (N. Ryde, Australia). Chondroitin sulphate A from bovine trachea was obtained from Calbiochem (Alexandria, Australia). Dimethyl methylene blue was an Aldrich product (Castle Hill, Australia). Four chondroitin sulphate standards (designated II, IV, VI and IX in this manuscript) prepared from bovine nasal cartilage, whose molecular masses had been calculated from intrinsic viscosity measurements [1,3] (see Table I), and human intervertebral disc chondroitin sulphate (CS) and keratan sulphate (KS) [3] were gifts provided by Professor R.H. Pearce, University of British Columbia, Vancouver, Canada). The semi-synthetic polysulphated polysaccharides, glycosaminoglycan polysulphate ester (GPSE, Arteparon) and pentosan polysulphate (PPS, SP-54) were products of Luitpold-Werk and Benechemie respectively, both of Munich, Germany.

Methods

Analysis of chromatographic fractions for glycosaminoglycan. The GAG content of chromatographic fractions from the analytical runs were determined by reaction with the metachromatic dye 1,9-dimethyl methylene blue [4,5]. The carbazole method of Bitter and Muir [6] was used to measure the hexuronic acid contents of fractions from the preparative chromatography runs.

Preparation of the chondroitin sulphate standards. A solution of bovine tracheal CS 2.0 g in 0.2 *M* NaCl (20 ml) was chromatographed on a column of Sephadex G200 (95 × 5.0 cm I.D.) and eluent fractions collected and pooled as described earlier [1,2]. Chondroitin sulphate pools corresponding to the hydrodynamic size-range covered by CS pools 1–6 of Wasteson's methodology [1,2] were collected, concentrated by diafiltration (2000 cut off membrane), dialysed against distilled water and freeze dried. Samples of these CS pools (300 μg GAG) were re-chromatographed on an analytical column of Sephadex G200 (90 × 1.6 cm I.D.) eluting with 0.2% NaCl at a flow-rate of 6 ml/h (Fig. 1).

Superose 6 and 12 FPLC of CS samples.

TABLE I

CHARACTERISTICS OF THE GLYCOSAMINOGLYCAN SAMPLES USED IN THIS STUDY

Sample	K_{av} [a] Sephadex G200	K_{av} [a]		Assigned M_r [b]
		Superose 6	Superose 12	
CS1	0.093	0.456	0.151	36 000
CS2	0.237	0.550	0.210	23 200
CS3	0.300	0.600	0.265	20 000
CS4	0.405	0.673	0.340	14 500
CS5	0.467	0.725	0.437	11 800
CS6	0.533	0.775	0.490	10 700
CS7	0.633	0.900	0.617	7300
II	nd	0.400	0.122	40 000[c]
IV	nd	0.605	0.250	20 000[c]
VI	nd	0.635	0.337	18 000[c]
IX	nd	0.825	0.572	10 000[c]
Disc KS	nd	0.837	0.525	8000–9000
Disc CS	nd	0.675	0.280	17 000–19 000
Arteparon	nd	nd	0.725	5200
SP-54	nd	nd	0.818	4800

[a] K_{av} = Average distribution coefficient described by the formula $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e represents the elution volume of the solute of interest and V_0 and V_t represent the void and total volumes of the column, respectively. nd = Not determined.

[b] M_r = Average molecular mass [1,2].

[c] Average molecular mass $M_{[\eta]}$ determined from intrinsic viscosity measurements.

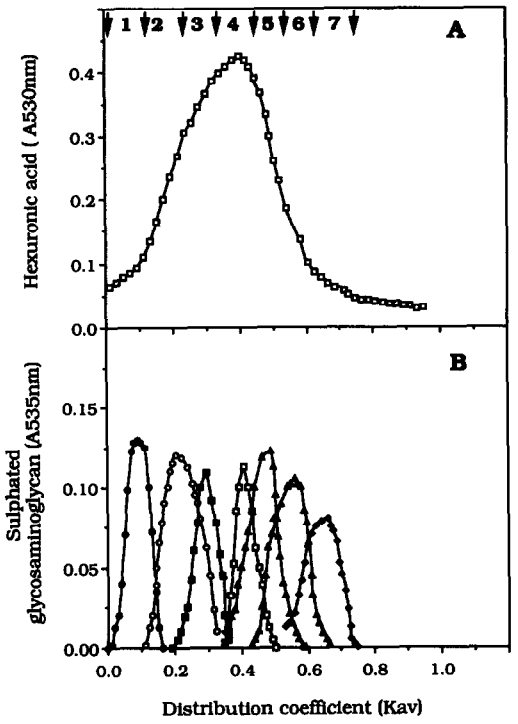

Fig. 1. (A) Preparative chromatography on Sephadex G200 of bovine tracheal CS demonstrating the pooling protocol used for CS pools 1–7 which were subsequently examined by (B) analytical chromatography on the same gel matrix. CS pools 1 = ●; 2 = ○; 3 = ■; 4 = □; 5 = ▲; 6 = △; 7 = ◆. (See Table I for details of the CS pools). In A aliquots of fractions were assayed for hexuronic acid [6] to assess the distribution of GAG species. In B aliquots of fractions were reacted with 1,9-dimethyl methylene blue to assess the distribution of GAG species [4,5].

Samples of CS pools 1–7; CS standards II, IV, VI, IX; Intervertebral disc CS and KS; GPSE and PPS (200–300 μg GAG dry weight) were chromatographed on Superose columns at a flow-rate of 24 ml/h using 0.5 *M* sodium acetate buffer pH 7.0 containing 0.05% (v/v) Tween 20 as eluent (Figs. 2 and 3). Fractions (0.25 ml) were collected directly into 96-well flat bottom microplates using a Gilson Model 201 fraction collector, GAG was determined with 1,9-dimethyl methylene blue [4,5]. The void and total volumes of these columns were determined using ^{35}S-labelled PG aggregate and free ^{35}S, respectively, using liquid scintillation spectrometry.

RESULTS

Re-chromatography of CS samples on Sephadex G200 (Fig. 1), indicated a similar level of polydispersity to that obtained by Wasteson [1,2], and these CS pools were assigned tentative average molecular mass values on the basis of these earlier studies [1,2] (Table I). FPLC of CS pools 1–7 resolved them as well defined peaks and a linear relationship was evident between their average hydrodynamic sizes and assigned molecular masses (Fig. 2). Four CS standards, whose molecular masses had been independently verified by intrinsic viscosity measurements (II, IV, VI, IX, see Table I) were also chromatographed under identical conditions. These samples eluted with hydrodynamic sizes calculated from the elution volume *vs.* assigned molecular mass calibrations (Fig. 2) similar to their sizes by intrinsic viscosity measurements and this was taken as validation of the assigned molecular masses given to the CS pools 1–7 (Table I). Human intervertebral disc KS and CS eluted with assigned average molecular masses of 8000–9000 and 17 000–19 000, respectively, by Superose FPLC which was in close agreement to their literature values [3]. Commercial preparations of the polysulphated polysaccharides GPSE and PPS were also examined by Superose 12 FPLC and were assigned average molecular masses of 5200 and 4800, respectively (Table I), these values however may be under-estimated since they were at the lower limit of the calibration (Fig. 3). Average molecular mass values of 6000–7000 and 5700, respectively, have been reported [7] elsewhere for these polymers.

DISCUSSION

Glycosaminoglycans have a diverse range of functions in connective tissues and consequently are of interest to a wide range of scientists [8]. However until recently no convenient method existed for the characterisation of their molecular masses. This has been the consequence of both an absence of readily available GAG standards of defined molecular mass and of a fast high-resolution chromatographic system for their size separation. In the present study standards

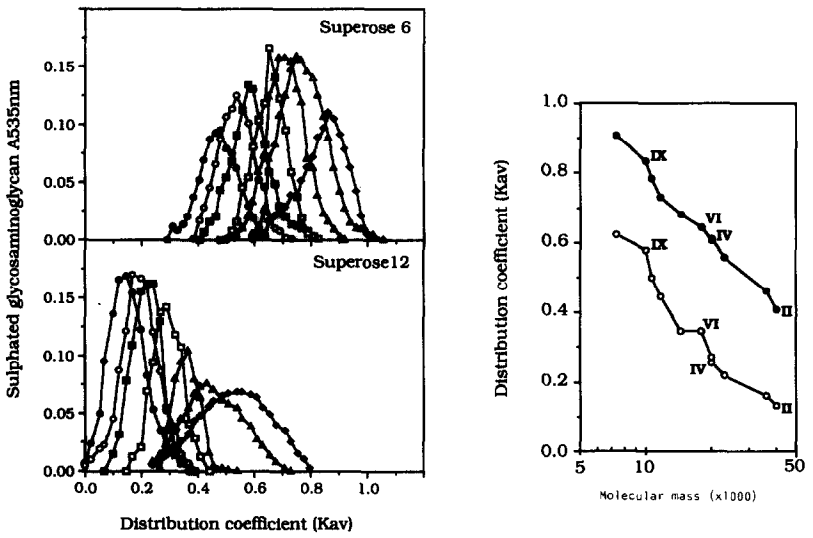

Fig. 2. Superose 6 and 12 FPLC of CS pools 1–7, the symbols used are as depicted in the legend to Fig. 1. Aliquots of fractions were reacted with 1,9-dimethyl methylene blue to assess the distribution of GAG species [4,5]. Linear regression was used to fit the lines of best fit which are indicated at the right-hand side of the figure with the elution positions of CS standards II, IV, VI, IX (see Table I) also indicated. ● = Superose 6: $y = 1.4525 - 0.65400 \log x$ ($R^2 = 0.989$); ○ = Superose 12: $y = 1.2055 - 0.70107 \log x$ ($R^2 = 0.946$).

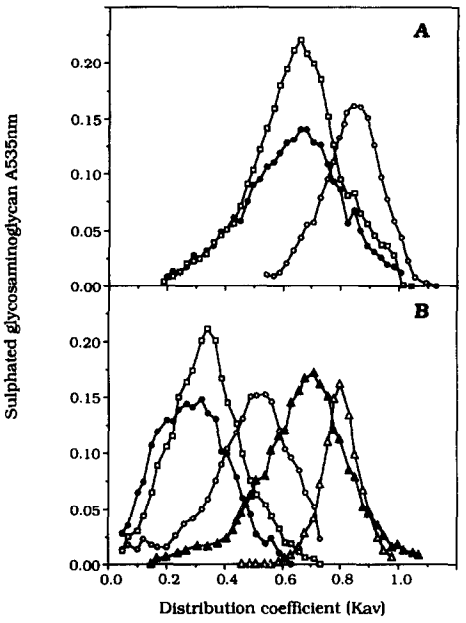

Fig. 3. (A) Superose 6 and (B) Superose 12 FPLC of non-fractionated bovine tracheal CS-A (□) and human intervertebral disc CS (●) and KS (○, 300 μg GAG dry weight each). Arteparon (▲, 300 μg GAG) and SP-54 (△, 300 μg GAG) were also examined by Superose 12 FPLC. Aliquots of fractions were reacted with 1,9-dimethyl methylene blue to assess the distribution of GAG species [4,5].

were prepared using an existing method whereby CS standards of well defined composition and molecular dimensions were obtained [1,2].

Using the methodology described herein, Superose 6 FPLC appeared to be the most suitable means for examination of GAGs of 10 000–40 000 in size. Preparation of smaller CS standards by limited hyaluronidase digestion and fractionation of the products as suggested by Wasteson [2], could however be used to extend the lower end of the calibration for Superose 12 FPLC further increasing the general applicability of FPLC for the examination of the molecular masses of GAGs.

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