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Short Communication

Glycosaminoglycans and proteins: different behaviours in high-performance size-exclusion chromatography

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ABSTRACT

The influence of the conformation of globular proteins and glycosaminoglycans in high-performance size-exclusion chromatography (HPSEC) was studied. Glycosaminoglycans (heparin, chondroitin sulphate and dermatan sulphate) with different primary structures, sulphate-to-carboxyl ratios and physico-chemical properties were extracted and purified. Their physico-chemical properties and purity were evaluated by several analytical techniques. Glycosaminoglycans with different relative molecular masses (M_r) were prepared by a chemical depolymerization process. These heteropolysaccharides were evaluated by HPSEC and compared with globular proteins of known relative molecular mass. The two third-degree polynomial regression curves for proteins and glycosaminoglycans have different coefficients and the columns present different exclusion limits. In particular, under the experimental conditions, the M_r exclusion limits for high M_r are 44 000 for glycosaminoglycans and 240 000 for globular proteins. In contrast, the behaviours of these two classes of macromolecules are similar for lower M_r. In fact, the two third-degree polynomial curves show the same regression below about M_r = 1000. The behaviour in HPSEC is discussed in relation to the different steric conformations for proteins and glycosaminoglycans with different relative molecular masses.

INTRODUCTION

Size-exclusion chromatography (SEC) (also termed gel filtration, gel permeation or molecular sieving) involves a simple principle of separation. Unlike other separation systems, such as ion-exchange, reversed-phase or affinity chromatography, SEC generally obviates chemical interactions between the sample and stationary phase. The elution order depends mainly on the molecular size dissolved in the eluent and also on hydrodynamic volume [1].

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For linear polymers, which are present in solution as random coils, the size can be directly related to relative molecular mass (M_r). For molecules with an ordered structure the usual plot of the log M_r *versus* elution volume does not properly describe their behaviour in SEC properly [2].

Glycosaminoglycans (GAGs) are complex, polydisperse, sulphated polysaccharides. They are alternating copolymers of uronic acids and amino sugars; the structures are commonly represented by their prevalent disaccharide sequences obtained by enzymatic cleavage. They are very heterogeneous polysaccharides in terms of relative molecular mass, physico-chemical properties and biological activities [3].

Heparin (Hep) has a heterogeneous structure **due**

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to the presence of variously sulphated regions distributed along the chain. It is a polysaccharide composed of alternate sequences of differently sulphated residues of uronic acid (β-D-glucuronic acid and α-L-iduronic acid) and α-D-glucosamine linked by a(1→4) bonds [4].

Polysaccharide chains of dermatan sulphate (DS) consist of a prevailing disaccharide unit [(1→4)-O-(cl-idopyranosyluronic acid)-(1→3)-O-(2-acetamido-2-deoxy-β-D-galactopyranosyl 4-sulphate)] [5].

Chondroitin sulphate (CS) is a heteropolysaccharide composed of alternate sequences of differently sulphated residues of uronic acid (β-D-glucuronic) and a-D-N-acetylgalactosamine linked by β(1→3) bonds [6]. The regular disaccharide sequence of chondroitin sulphate A (CS-A), chondroitin-4-sulphate, is constituted by (1→4)-O-(β-D-glucopyranosyluronic acid)-(1→3)-O-(2-acetamido-2-deoxy-β-D-galactopyranosyl-4-sulphate). Chondroitin sulphate C (CS-C), chondroitin-6-sulphate, is mainly composed of a disaccharide unit [(1→4)-O-(β-D-glucopyraposyluronic acid)-(1→3)-O-(2-acetamido-2-deoxy-β-D-galactopyranosyl-6-sulphate)] [6].

In this paper we report the different chromatographic behaviours of GAGs and proteins in high-performance size-exclusion chromatography (HPSEC). The different chromatographic behaviours of these two classes of macromolecules depending on relative molecular mass, are discussed in relation to their different secondary and tertiary structure and different conformation. Moreover, these differences should be considered in those processes influenced by steric conformation.

EXPERIMENTAL

Materials

Proteins of different molecular mass were obtained from Sigma: catalase from bovine liver (C-10) (M_r = 240 000); β-galactosidase from *Escherichia coli* (G-851 1) (M_r = 116 000); bovine serum albumin (A-6793) (M_r = 66 000); egg albumin (A-7642) M_r = 45 000); carbonic anhydrase from bovine erythrocytes (C-2273) (M_r = 29 000); trypsinogen from bovine pancreas (T-9011) (M_r = 24 000); trypsin inhibitor from soybean (T-9767) (M_r = 20 100); cl-lactalbumin from bovine milk (L-6385) (M_r = 14 200); insulin from bovine pancreas (I-5500) (M_r = 6000); and glutathione from cell culture (G-4251) (M_r = 310).

GAGs of different relative molecular mass were prepared by chemical depolymerization of native heteropolysaccharides, as reported below. An unsaturated disaccharide with known relative molecular mass (503) was from Sigma (C-4045).

All reagents and solutions were of analytical-reagent grade.

Extraction and purification of glycosaminoglycans: heparin, dermatan sulphate and chondroitin sulphate

Hep (from beef mucosa), DS (from pig skin) and CS (from bovine trachea) were isolated and purified according to Taniguchi [7]. CS was also purified from shark cartilage.

Preparation of glycosaminoglycans of different relative molecular mass

Native Hep, DS and CS from bovine trachea were depolymerized by a controlled chemical reaction, according to Volpi and co-workers [8–10].

GAGs of different relative molecular mass were obtained by a controlled chemical depolymerization process induced by free radicals. A 5-g amount of Hep or DS or CS and 0.2 g of copper acetate monohydrate (0.02 mol/l) were dissolved with 50 ml of water into a reaction vessel fitted with a thermostated bath, stirrer, calibrated dropping funnels and a thermometer. The temperature was kept constant at 60°C and the pH was adjusted to 7.5 by addition of 1 *M* NaOH solution. Hydrogen peroxide solution (9%) was added at a rate of 10 ml/h. The reactions for Hep, DS and CS were stopped at different times, and at the end of the reaction the chelating resin Chelex 100 (Bio-Rad) was utilized to remove copper contaminant from the product and a strong anion-exchange resin in the OH^- form was used to remove acidic contaminants. The pH of the percolate was adjusted to 5.5 with acetic acid, then two volumes of acetone were added. The precipitate, collected by filtration and washed with acetone, was dissolved in 100 ml of water. A 5-g amount of sodium acetate was added to this solution, and then low-M, GAG sodium salt was precipitated with two volumes of acetone. The precipitate was collected and dried.

The relative molecular masses of the various GAGs obtained (Hep from 12 000 to 2000, DS

from 35 000 to 5000 and CS from 30 000 to 5000) were evaluated by ultracentrifugation according to Nieduszinski [11] and by HPSEC utilizing a calibration graph constructed using GAGs of known M_r.

High-performance size-exclusion chromatography (HPSEC)

The HPLC system from Jasco consisted of a Model 880 PU pump, a Model 801 SC system controller, a Model 880-02 ternary gradient unit, a Rheodyne injector equipped with a 100-μl loop and a Model 875 UV detector. The mobile phase was 125 m*M* Na_2SO_4–2 m*M* NaH_2PO_4 adjusted to pH 6.0 with 0.1 *M* NaOH. The flow-rate was 0.9 ml/min with a back-pressure of 25 kg/cm^2. Proteins and GAGs of different relative molecular mass were dissolved in the mobile phase at a concentration of 5 mg/ml and volumes of 10 μl (50 μg) were injected into the HPLC system.

Protein Pak 125 and 300 columns (Waters) were assembled in series. The Protein Pak 125 column (30 cm × 7.8 mm I.D.) had M_r ranges of native globular 2000-80 000 and random coil 1000–30 000 and the Protein Pak 300 column (30 cm × 7.5 mm I.D.) had M_r ranges of native globular 10 000–400 000 and random coil 2000–150 000.

The retention times were plotted against log M_r for proteins and GAGs. The curve that fitted the experimental data was a third-degree polynomial $y = -ax^3 + bx^2 - cx + d$, performed with a Macintosh computer program.

Determination of the purity and physico-chemical properties of glycosaminogiycans

Sulfate and carboxyl groups were determined by potentiometric titration with 0.1 A4 NaOH [12] in waterdimethylformamide (Merck) (50:30) of heparinic, dermatanic and chondroitinic acids obtained by removing the cations using strong ion-exchange resins (Amberlite IRA-400, strongly basic polystyrene gel-type resin, and Amberlite IRA-120, strongly acidic polystyrene gel-type resin; Rohm & Haas). The sulphate-to-carboxyl ratio was also determined by enzymatic degradation (by heparinases for Hep and chondroitinases for DS and CSs) after HPLC separation of the constituent disaccharides [8–10]. The ratio was calculated by considering the percentage and the presence of carboxyl and sulphate groups for each disaccharide.

The presence of possible GAGs as contaminants in the preparations and the ratio of "slow-moving" component (constituted by the most highly sulphated and higher-M_r species) to "fast-moving" component (less sulphated and lower-M_r species) of heparin was determined by electrophoresis on agarose in barium acetate/l ,2-diaminopropane according to Cassaro and Dietrich [13].

The specific optical rotation was determined at 25°C at a concentration of 5% (w/v) of GAGs in water using a polarimeter.

The different physico-chemical properties of purified native heparin, DS and CS (from bovine trachea and shark cartilage) are reported in Tables I and II.

The different M_r values and the log M_r *versus* retention time for GAGs obtained by chemical depolymerization are given in Table III, which also reports the standard errors. Table IV gives the values of M_r and log M_r *versus* retention time with standard errors for proteins.

RESULTS AND DISCUSSION

The extracted and purified GAGs had a purity of 100% as tested by agarose electrophoresis, specific optical rotation and HPSEC. Heparin has M_r 11 600, DS 32 110, bovine trachea CS 26 140 and shark cartilage CS 44 120.

TABLE I

PHYSICO-CHEMICAL CHARACTERISTICS OF PURIFIED BEEF MUCOSA HEPARIN

Parameter	Value	
Peak M_r	11600	
Sulphate/carboxyl ratio (titrimetric)	2.19	
Sulphate/carboxyl ratio (enzymatic cleavage)	2.42	
Optical rotation	+50	
Agarose electrophoresis:	Fast moving	Slow moving
Mobility (%)	48	18
Glycosaminoglycans (%)	80	20

TABLE II

PHYSICO-CHEMICAL CHARACTERISTICS OF PURIFIED PIG SKIN DERMATAN SULPHATE, AND BOVINE TRACHEA (BT) AND SHARK CARTILAGE (SC) CHONDROITIN SULPHATES

Parameter	DS	CS (BT)	cs (SC)
Peak M_r (× 1000)	32.11	26.14	44.12
Sulphate/carboxyl ratio (titrimetric)	1.12	0.99	1.13
Sulphate/carboxyl ratio (enzymatic cleavage)	1.09	0.97	1.15
Optical rotation	- 5 5	- 2 0	− 14
Agarose electrophoresis:			
Mobility (%)	62	75	75
Glycosaminoglycans (%)	100	100	100

TABLE III

RELATIVE MOLECULAR MASSES, LOGARITHMS OF RELATIVE MOLECULAR MASSES, RETENTION TIMES AND STANDARD ERRORS FOR STANDARD PROTEINS

Protein standard	M_r (× 1000)	Log M_r	Retention time (min)	Standard error
Catalase	240.0	5.38	16.82	0.21
β-Galactosidase	116.0	5.06	16.98	0.28
Bovine serum albumin	66.0	4.82	17.58	0.19
Egg albumin	45.0	4.65	18.86	0.25
Carbonic anhydrase	29.0	4.46	20.26	0.27
Trypsinogen	24.0	4.38	20.42	0.32
Trypsin inhibitor	20.1	4.30	20.92	0.29
a-Lactalbumin	14.2	4.15	21.02	0.34
Insulin	6.0	3.78	22.96	0.32
Glutathione	0.3	2.49	24.37	0.30

TABLE IV

RELATIVE MOLECULAR MASSES, LOGARITHMS OF RELATIVE MOLECULAR MASSES, RETENTION TIMES AND STANDARD ERRORS FOR STANDARD GLYCOSAMINOGLYCANS

GAG standard	M_r (× 1000)	Log M_r	Retention time (min)	Standard error
cs 45	44.12	4.64	13.50	0.31
DS 32	32.11	4.51	13.89	0.38
CS 26	26.14	4.42	14.29	0.35
cs 17	17.02	4.23	15.13	0.35
DS 16	15.64	4.19	15.22	0.37
DS 13	12.78	4.11	16.11	0.32
Hep 9	8.70	3.94	16.92	0.38
CS 6	6.26	3.80	19.27	0.42
DS 6	5.83	3.17	19.76	0.42
Hep 4	3.70	3.57	21.52	0.45
Hep 2	2.13	3.33	22.90	0.47
Δ-Di-4s	0.50	2.70	23.85	0.43

GAGs of different and lower M_r than the native molecule can be produced by various processes. such as enrichment of low-M, fractions of commercial heteropolysaccharides or their chemical depolymerization products. Recent methods involve controlled chemical depolymerization by nitrous acid, chemical p-elimination and utilization of peroxides and redox systems [14]. Chemical synthesis [15] and enzymatic depolymerization [16] represent other methods of preparation. The radical depolymerization process we used to produce GAGs of different M_r [8,9] was controlled by means of the reaction kinetics, calculated by withdrawing the specimens at different times and evaluating their relative molecular masses by HPSEC. The relative molecular mass decreases with time according to an exponential-like function, as shown in Fig. 1, which shows the reaction kinetics of beef lung heparin, pig skin DS and bovine trachea CS. The GAGs of different M_r produced by the chemical process were used to construct a calibration graph in HPSEC and to evaluate the differences compared with proteins.

The third-degree polynomial calibration graph was constructed using GAGs with different primary structure. The backbones of CS, DS and Hep present different uronic acids, different hexosamines and 0-sulphate groups in different amounts and O-linked in different positions. The prevailing monosaccharides identified in DS, CS and Hep are α-L-iduronic and β-D-glucuronic acids, N-acetyl-α-D-glucosamine, N-sulpho-a-D-glucosamine and N-acetyl-a-D-galactosamine. Sulphate groups can be O-linked in position 2 of uronic acids, in positions 4 and 6 of N-acetyl-a-D-galactosamine, in position 6 of N-acetyl-α-D-glucosamine and in positions 3 and 6 of N-sulpho-a-o-glucosamine [4, 5, 6]. However, the secondary and tertiary structures are relatively similar for GAGs with different monosaccharide units and charge density solubilized in the same aqueous medium [17,18]. In fact, these linear polyelectrolytes were assumed to be long cylinders in aqueous solutions with a continuous distribution of point charges upon them [19]. In these conditions, a difference in charge density, about l-l. 1 for CS and DS and about 2.2-2.4 for heparin, has a minimum influence on HPSEC. The calibration graph was constructed using different GAGs to check high and very low relative molecular masses. In fact, natural CS from shark cartilage has $M_r \approx 44\,000$, natural DS from pig skin cu. 32 000 and natural CS from bovine trachea cu. 26 000. On the other hand, the chemical depolymerization process of heparin is controlled easier than the degradation of CS or DS to obtain very-low-M, fractions (Table IV).

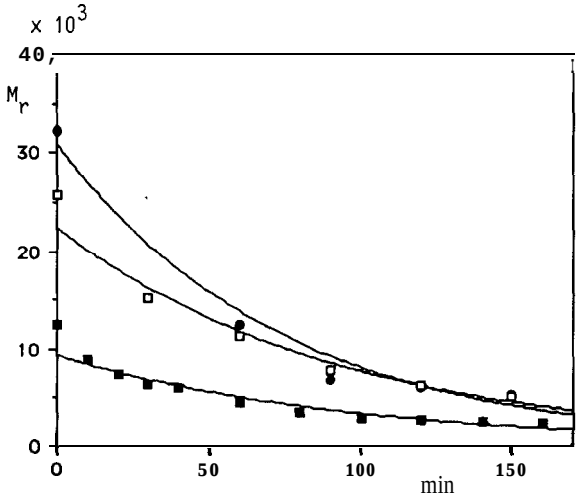

Fig. 1. Chemical process of depolymerization of glycosaminoglycans. Relative molecular mass *versus* time in min of chemical reaction is reported. Coefficients of exponential regression and correlation coefficients are as follows: for DS (●), $y = 30.8\ 10^{-5.7\ e^{-3x}}$, $R = 0.930$; for CS (0), $y = 22.3\ 10^{-4.5\ e^{-3x}}$, $R = 0.971$; and for heparin (■), $y = 9.4 \cdot 10^{-4.3\ e^{-3x}}$, $R = 0.935$.

HPSEC gives different upper limits of M_r for proteins and GAGs, as in Fig. 2. Under our experimental conditions, the M_r exclusion limits for higher M_r are 240 000 for globular proteins and 44 000 for GAGs. In contrast, the behaviour for these two classes of macromolecules are similar for low M_r. In fact, the two third-degree polynomial curves show the same regression below about M_r = 1000. This effect stems from the loss of globular conformation of lower-M_r proteins due to the decreased number of hydrogen bonds [20]. In this condition, the proteins in solution present a random coil, a conformational state which is similar to that of GAGs.

Proteins utilized to evaluate the behaviour in HPSEC have a generally globular conformation. The secondary structure is stabilized by hydrogen bonds between N–H and C = 0 groups involved in peptidic linkages [20]. The tertiary structure is stabilyzed by hydrogen bonds of amino acid side-chains, by hydrophobic interactions and by disulphur (co-

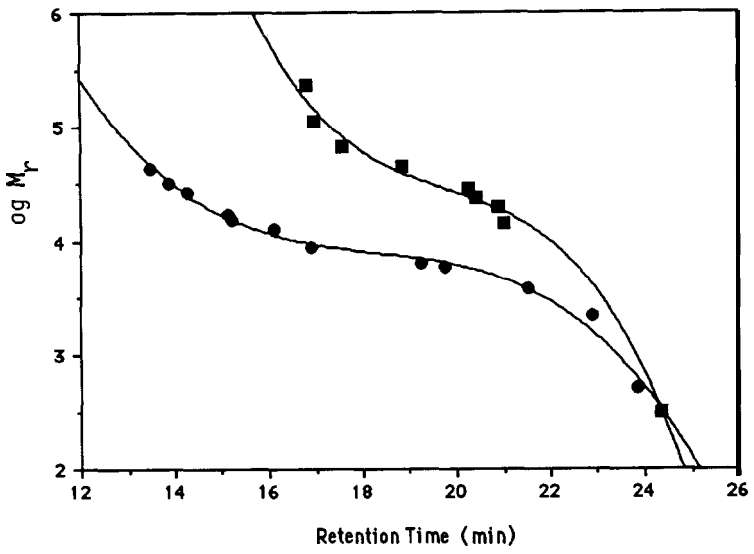

Fig. 2. Comparison of the different third-degree grade polynomial curves for standard (■) proteins and (●) glycosaminoglycans in HPSEC. For proteins, $J = -0.018x^3 + 1.07x^2 - 21.54x + 149.08$, $R = 0.993$; for glycosaminoglycans, $y = -0.0051x^3 + 0.29x^2 - 5.22x + 36.29$, $R = 0.993$.

valent) bridges [20]. The globular conformation is imposed to achieve a stable state with minimum energy and entropy. Thus, proteins in aqueous solution are generally closely packed, although some degree of flexibility is possible [20]. Therefore, high-M_r globular proteins have a very compact conformation.

Heteropoly acids, in contrast, do not present a well defined packed conformation. The absence of covalent bridges and the irregular location and direction of hydrogen bonds produce very deformable saccharidic chains. The presence of numerous negative groups extends the saccharidic frame, depending on the nature and concentration of the ions present in the solution. Although the flexibility of the chains is different for different GAGs owing to the presence of heterogeneous monosaccharides with various conformations [4, 21], the GAGs present extended chains in aqueous solution [22]. GAGs assume the form of a "small elongated stick" surrounded by water molecules [23], which gives these heteropoly acids a larger steric conformation than proteins with the same relative molecular mass.

In particular, this work confirms the importance of steric conformation in HPSEC experiments performed to evaluate relative molecular masses and also in preparative processes to produce purified GAGs. Moreover, we should stress that GAGs, in addition to their polyanionic nature, behave as high-M_r proteins under conditions of steric hindrance, such as in gel filtration and dialysis.

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