Carbohydrate Polymers 2 (1982) 115–121

STUDIES ON THE BLOOD-ANTICOAGULANT ACTIVITY OF SULPHATED POLYSACCHARIDES WITH DIFFERENT URONIC ACID CONTENT

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(Received: 9 March, 1982)

ABSTRACT

The anticoagulant activities of sulphated alginic acids, amylose, cellulose, chitosan, curdlan, dextran, guaran, laminaran and locust bean gum have been studied. The alginic acids have been partially reduced and some of the neutral polysaccharides have been partially oxidised at C-6 of the glycopyranosyl residues. The activities of sulphated polymers containing different proportions of uronic acid and neutral sugar residues have been compared.

The results suggest that polysaccharides with an average of at least one sulphate group on each monomeric unit, a molecular weight higher than 10 000 and a high proportion of sulphated primary hydroxyl functions will display activity in the activated, partial thromboplastin time assay (APTT). Sulphated guaran and locust bean gum had the highest activities of the polymers investigated (70 IU/mg, heparin has 130–50 IU/mg). In the concentration range investigated, none of the sulphated polymers showed any significant activity in an anti-factor X_a assay.

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Carbohydrate Polymers 0144-8617/82/0002-0115/$02.75 – © Applied Science Publishers Ltd, England, 1982
Printed in Great Britain

INTRODUCTION

Heparin contains (1→4)-linked α-L-idopyranosyluronic acid residues, 2-amino-2-deoxy-α-D-glucopyranosyl residues, and a small proportion of β-D-glucopyranosyl uronic acid residues. The hexosamine and uronic acid residues are linked alternately to form a polymer. The residues are partially *O*-sulphated; in addition, most of the 2-amino-2-deoxy-D-glucose is *N*-sulphated, the remainder being *N*-acetylated (Lindahl, 1976). Heparin exerts its main blood-anticoagulant activity by potentiating the inhibitory effect of the plasma protein antithrombin III (AT III). This inhibits a number of blood serine proteases, including thrombin and an activated coagulation factor *X* (X_a). The discovery that heparin was a sulphated polysaccharide and that its anticoagulant activity was dependent on the sulphate content initiated studies on a new group of substances called heparinoids. These are sulphated polysaccharides, prepared by sulphation of polysaccharides from plant or animal tissue.

In a previous report (Larm *et al.*, 1979) we demonstrated that an extensively modified alginic acid showed affinity towards AT III and thus resembled heparin in its anticoagulant activity.

EXPERIMENTAL

General Methods

Solutions were concentrated at reduced pressure below 40°C. Nuclear magnetic resonance spectra were recorded on a Jeol FX-90Q instrument. ^{1}H n.m.r. spectra were recorded at 90 MHz in D_2O at 85°C. To eliminate the solvent peak, a 180°-*t*-90° pulse sequence ($t = 2$-4 s) with a recycle time of 5 s was used. ^{13}C n.m.r. spectra were recorded at 22 MHz in D_2O at 70°C. Uronic acids were analysed quantitatively by decarboxylation (Bylund & Donetzhuber, 1968) or by ^{13}C n.m.r. spectroscopy by using an inversely gated decoupling technique (90° pulse; 2 s pulse delay) to suppress n.O.e. (the nuclear Overhauser effect) (Ganzow & Schittenhelm, 1971). Derivatives of cellulose were fractionated in 0·25 M NaCl on Bio-Gel P30 (fractionation range 2500–40 000 Daltons). Fractions with the same elution volume were collected, representing a molecular weight of 15 000–25 000 compared to dextran standards.

Preparation of 6-Oxo Derivatives (Painter, 1977)

The polysaccharide (2·5 g) was dissolved in orthophosphoric acid (85% w/w; 50 ml) at 0°C by thorough grinding in a large mortar. Sodium nitrite, ground to a very fine powder, was added in portions during 30 min continuous mixing and the reaction was left overnight at room temperature. Formic acid (90% w/w; 2 ml) was added to destroy excess reagent. Ice-cold ether (150 ml) was added and the precipitate formed was repeatedly extracted by grinding with ice-cold ether in the mortar. Water (20 ml) was added, the pH adjusted to 6·0 (1 M NaOH) and the solution dialysed against three

changes of tap water and three changes of distilled water and then freeze-dried. Oxidation of polysaccharide (2·5 g) with sodium nitrite (1·25 g) gave a material containing about 20% uronic acid residues while oxidation with 2·5 g nitrite gave a material with about 40% uronic acid residues. When two more portions of sodium nitrite (2·5 g) were added, 5 h and 10 h after the first portion, a polysaccharide containing about 80% uronic acid was obtained.

Partial Reduction of Alginic Acids (Taylor & Conrad, 1972)

Partial reduction of the samples was performed by treatment with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) at pH 4·75 followed by reduction with sodium borohydride in excess (50 times the amount of EDC) at pH 7. The molar ratios of carbodiimide to anhydrouronide necessary for achieving 25, 50 and 80% reduction were 0·5, 1·0 and 2·0, respectively. After reduction, the polysaccharides were dialysed three times each against tap water and distilled water and freeze-dried. No degradation of the alginates by β-elimination reactions occurred (Larm & Larsson, to be published).

Sulphation

The polysaccharide (0·5 g) was dissolved in dimethyl sulphoxide (4 ml), a sulphur trioxide-pyridine complex (3 g) added and the mixture stirred at 40°C for 1 h. Crushed ice (10 g) and water (5 ml) were added and the solution was neutralised to a phenolphthalein end-point with sodium hydroxide (2 M). A 5% excess of alkali was added. The product was precipitated with ethanol, collected by centrifugation, dissolved in sodium chloride (0·25 M), dialysed as above and freeze-dried. The degree of sulphation was determined by elemental analysis of the sodium salts.

Anticoagulant Activity Tests

The anticoagulant activity of the sulphated polysaccharides was determined using the activated partial thromboplastin time (APTT) assay (Andersson *et al.*, 1976). The effect of the sulphated polymers on the inhibition of coagulation factor X_a was studied in plasma utilising the chromogenic substrate S-2222 (Bz-Ile-Glu-Gly-Arg-pNA) (Andersson *et al.*, 1979).

RESULTS AND DISCUSSION

The sulphated polysaccharides contained an average of about one sulphate group on each glycosyl residue (d.s. $\simeq$ 1). Sulphated polysaccharides with a d.s. value less than 0·8 show activities in the APTT test that are lower than 15 IU/mg (for heparin the value is 130–50 IU/mg).

Alginic acid contains (1 → 4)-linked β-D-mannopyranosyluronic (*M*) and α-L-gulopyranosyl uronic acid (*G*) residues, arranged in a blockwise fashion (Haug *et al.*, 1967;

Grasdalen *et al.*, 1981). The *M/G* ratios in alginic acids and the relative proportion of the four possible dimers *M—M*, *M—G*, *G—G* and *G—M* can be determined by ^{1}H n.m.r. spectroscopy (Grasdalen *et al.*, 1979). By comparing a number of different alginic acids with this technique, polymers with different *M/G* ratios and different proportions between alternating and homologous blocks were selected (Table 1). There was no significant difference between the activities of alginates with comparable sulphur contents but different block compositions or *M/G* ratios (Tables 1 and 2). Sulphated amylose, cellulose and curdlan, which are high molecular, linear glucans with different types of glycosidic linkages, had similar activities in the APTT test (Table 3). Guaran and locust bean gum consist of linear chains of (1→4)-linked β-D-mannopyranosyl units with α-D-galactopyranosyl units (36% and 24%, respectively) attached to the 6-position of the D-mannopyranosyl units (Goldstein *et al.*, 1973; Rol, 1973). Sulphated guaran and locust bean gum showed the highest activities of the polymers investigated (Table 3). The results indicate that neither the nature of the monomer, nor the type of linkage between them can be correlated to the APTT activity of sulphated polysaccharides.

Sulphated curdlan ($DP_n \simeq 550$ before sulphation) (Harada *et al.*, 1968) had a higher activity than the structurally similar but lower molecular weight laminaran ($DP_n \simeq 20$ before sulphation) (Table 3). Sulphated cellulose and partially oxidised (C-6) and sulphated cellulose were fractionated on a Bio-Gel P30 column and fractions with different molecular weights were collected. As no proper reference polysaccharides were available, the molecular weights were approximately estimated by reference to dextran standards. The fractions with DP_n values higher than about 75 showed the same activities in the APTT test as the starting materials. The more low-molecular fractions ($DP_n < 35$) had lower activities.

The activity of the partially reduced alginates in the APTT test increased with increasing amounts of neutral sugar residues (Table 2). The activities of oxidised (C-6),

TABLE 1
M/G Ratio and Doublet Frequencies[a] of the Alginates as Determined by ^{1}H n.m.r.

Alginate	*M/G ratio*	*Doublet frequencies*		
		MM	*GG*	*GM=MG*
1[b]	1·73	0·49	0·23	0·14
2[c]	1·63	0·39	0·15	0·23
3[d]	1·33	0·34	0·20	0·23
4[e]	0·54	0·18	0·48	0·17

[a] Given as mole fractions of *—M—M—*, *—G—G—*, *—G—M—* and *—M—G—* dimers in the polymer structure.
[b] Commercial sample from Alginate Industries Ltd, England.
[c] Commercial sample from Sigma, USA.
[d] Commercial sample from Fluka, Switzerland.
[e] A gift from Protan & Fagertun, Norway.

TABLE 2
Activity in APTT of Partially Reduced and Sulphated Alginic Acids

Alginate	*Mol % uronic acid residues*	*Sulphur content (%)*	*Sulphate groups (d. s.)*	*Activity in APTT (IU/mg)*
1	100	10·6	1·0	15
	75	10·7	1·0	25
	45	10·6	0·9	24
	20	13·4	1·1	55
2	100	11·1	1·0	14
	80	10·6	1·0	5
	50	12·1	1·1	28
	20	9·7	0·8	27
3	100	11·1	1·0	10
	70	10·6	1·0	12
	50	11·1	1·0	11
	20	12·0	1·0	47
4	100	10·8	1·0	13
	80	10·6	1·0	15
	50	11·1	1·0	25
	20	12·2	1·0	47

sulphated locust bean gum, guaran and amylose decreased with increasing proportions of uronic acid residues. C-6 oxycellulose was prepared from cotton wool cellulose by the method used in this report. It contained 87·5% D-glucuronic acid residues and had a DP_n of about 66 (Painter, 1977). By analogy with this, the oxidised (C-6) locust bean gum, guaran and amylose should have DP_n values higher than 75. With the method used for sulphation, depolymerisation is minimal (Whistler & Spencer, 1964; Whistler, 1972).

Primary alcohol functions are more easily sulphated than secondary alcohol functions (Whistler *et al.*, 1963). For polysaccharides with similar sulphate contents but different glycuronic acid contents, the polymers with a low uronic acid content have a higher proportion of sulphated primary alcohol functions. Dextran NRRL B-512 contains only about 5% glucopyranosyl residues with primary hydroxyl functions (Lindberg & Svensson, 1968). This polysaccharide showed the lowest activity of the glucans investigated.

In conclusion, the results suggest that polysaccharides with an average of at least one sulphate group on each monomeric unit, a DP higher than about 60 and a high proportion of sulphated primary hydroxyl functions will display significant activity in the APTT test. The high activities of sulphated guaran and locust bean gum indicate that the arrangement of the sulphate groups along the polymer backbone might be of importance for activity.

TABLE 3
Activity in APTT of Partially C-6 Oxidised and Sulphated Polysaccharides

Polysaccharides	*Mol % uronic acid residues*	*Sulphur content (%)*	*Sulphate groups (d.s.)*	*Activity in APTT (IU/mg)*
Amylose[a]	0	14·5	1·2	35
	10	15·0	1·3	31
	20	14·3	1·2	24
Cellulose[b]	0	14·0	1·2	42
	20	10·7	0·9	27
	40	13·0	1·1	54
	60	9·6	0·9	13
Dextran[c]	0	12·1	1·0	22
Curdlan[d]	0	15·6	1·3	35
Laminaran[e]	0	15·9	1·3	26
Chitosan[a]	0	11·4	0·9	6
Guaran[a]	0	14·2	1·2	70
	40	13·1	1·1	40
Locust bean gum[a]	0	12·1	1·0	70
	45	13·0	1·1	36
	60	11·5	1·0	19

[a] Commercial sample from Sigma, USA.
[b] Commercial sample from W & R Balston Ltd, England.
[c] Dextran T40, supplied by Pharmacia Fine Chemicals, Sweden.
[d] A kind gift from Takeda Chemical Industries, Japan.
[e] A kind gift from Professor Björn Larsen, Trondheim, Norway.

None of the heparinoids showed significant activity in the anti-factor X_a assay (Andersson *et al.*, 1979) in the concentration range investigated.

ACKNOWLEDGEMENTS

The authors thank Professor O. Theander for his interest. The work was financially supported by the Swedish Board for Technical Development (STU).

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