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Polysaccharide labelling: impact on structural and biological properties

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Abstract

Three sulphated polysaccharides labelled at the reducing end with three different chemical treatments (2-aminopyridine (2-AP), 8-amino-1,3,6-trisulphonic acid (ANTS) and 6-(biotinyl)-aminocaproyl-hydrazide (BACH)) were compared. Molecular weight, chemical composition, nuclear magnetic resonance features and anticoagulant activity expressed as activated partial thromboplastin time were studied to determine whether structural and biological properties were retained or not after labelling. Partial depolymerisation and reduced biological activity were observed when reductive amination was performed with 2-AP. The initial properties were better retained with ANTS, and BACH did not modify the anticoagulant and structural properties.

Keywords: Sulphated polysaccharide; GAG; Fucan; Fluorescent tagging; Reductive amination; Anticoagulant activity

1. Introduction

In recent years, there has been increasing interest in the isolation and identification of new bioactive polysaccharides, especially sulphated polysaccharides that occur in a great variety of animals, plants, algae and micro-organisms. These polysaccharides are known for their biological activities resulting from carbohydrate/protein interactions, and heparin-binding proteins play a prominent role in the biology and physiology (Conrad, 1998). In fact, heparin, the best known of these compounds, is widely used for its anticoagulant properties. Nonetheless, some other compounds such as fucoidans have been actively studied as anticoagulant/antithrombotic (Mauray et al., 1995), anti-inflammatory (Blondin, Fischer, Boisson-Vidal, Kazatchkine, & Jozefonvicz, 1994), antiviral (Baba, Schols, Pauwels, Nakashima, & De Clercq, 1990), and antiangiogenic (Hahnenberger & Jakobson, 1991) agents. Artificially sulphated polysaccharides are already in use, e.g. pentosan polysulphate (Colwell, Grupe, & Tollefsen, 1999), or are promising candidates, such as bacterial exopolysaccharides (EPSs) (Guezennec et al., 1998). However, methods are needed to facilitate the detection of these low-molecular-weight polysaccharides and allow identification, characterisation and studies of their biological functions.

Carbohydrate derivatisation by reductive amination improves sensitivity and facilitates the analysis of this class of compounds. A wide variety of fluorophores have been described, e.g. 2-aminopyridine (2-AP) (Taga, Yabusako, Kitano, & Honda, 1998) 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS) (Che, Song, Zeng, Wang, & Xia, 1999; Starr, Klock, Skop, Masada, & Guidici, 1994), and some biotinylation reagents such as biotinylated diaminopyridine (Rothenberg, Hayes, Toomre, Manzi, & Varki, 1993). The group of fluorescent tags is useful for purification and analysis of carbohydrates requiring high-performance liquid chromatography or capillary electrophoresis. It is now routinely possible to separate and quantify monosaccharides at the femtomole level (Suzuki & Honda, 1998). The combination of tags with the reducing end of an oligosaccharide is not a common means of studying biological activities, although it offers some advantages over other reactions involving activation of non-terminal residues, i.e. relatively mild conditions that avoid deep modifications and depolymerisation; no preferential labelling of chains, because there is no specific structural requirement (each chain ends by a terminal reducing end); and low risk of modifying the active sequence and inducing a loss of biological activities. Fluorescent tagging by reductive

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amination is especially promising for studies of protein–sugar interaction by affinity chromatography or electrophoresis.

In our study, three sulphated polysaccharides of various origins were labelled with three different reagents: 2-aminopyridine (2-AP), 8-amino-1,3,6-trisulphonic acid (ANTS), and 6-(biotinyl)-aminocaproyl-hydrazide (BACH). The three sulphated polysaccharides were a commercially available heparin, a low-molecular-weight fucan from *Ascophyllum nodosum*, and a sulphated and partially depolymerised EPS secreted by a bacterium (*Alteromonas infernus*) isolated from a hydrothermal deep-sea environment. The chemical characteristics (molecular weight, chemical composition, 1D ^{1}H nuclear magnetic resonance (NMR) spectra) and biological properties (anticoagulant activity) of their derivatives were investigated to determine whether labelled polysaccharides retained the initial properties of free polysaccharides.

2. Experimental

2.1. Polysaccharides

Unfractionated heparin from porcine mucosa (25–35 kDa in low-angle laser measurements by the manufacturer, and 30 kDa as determined in our laboratory (see later)) was from Sigma Chemical (St Louis, MO, USA). The fucan fraction was obtained from *A. nodosum* and prepared as previously described (Nardella et al., 1996). The isolation procedure and characteristics of the GY785 strain (*A. infernus*) were previously reported (Raguenes et al., 1997). The EPS was produced, purified, sulphated and depolymerised according to previously described procedures (Guezennec et al., 1998).

2.2. Fluorescence labelling

2-AP labelling was performed according to Hase, Ibuki, and Ikenaka (1984), with the following modifications to avoid an excess of reagents: reduction of the $NaCNBH_3$/polysaccharide ratio to 1000, with a 100-fold excess of 2-AP, and purification of 2-AP-polysaccharides by dialysis against ammonium bicarbonate until no residual UV absorbance was detected at 230 nm in the last outside solution. A 2-AP solution prepared by dissolving 1 g of 2-AP (Sigma) in 760 μl of hydrochloric acid 37% (Panreac) could be stored at −20 °C for several months. Aqueous sodium cyanoborohydride ($NaCNBH_3$) was prepared just before use by dissolving 200 mg of $NaCNBH_3$ (Sigma) in 200 μl of distilled water. Fifty milligrams of dry polysaccharide (3.7×10^{-6} mol) were then mixed with 40 μl of the 2-AP solution in a Teflon-lined screw-capped tube and incubated at 65 °C for 17 min. Two hundred microlitres of the aqueous solution of $NaCNBH_3$ were added, and the preparation was vortex-mixed and heated for 2 h at 65 °C. After the tube was opened under effective ventilation, the reaction mixture was diluted with 10 ml of water, dialysed three times against a 50 mM ammonium bicarbonate solution, using a molecular weight cut-off membrane (3500, Spectra/Por), and finally freeze-dried.

Derivatisation of the polysaccharides with ANTS was performed according to Che et al. (1999). The $NaCNBH_3$/polysaccharide ratio was 20 with a 10-fold excess of ANTS (Fluka). Polysaccharides (3.7×10^{-6} mol) were suspended in 300 μl of a 0.15 M ANTS solution in 30% aqueous acetic acid and heated for 15 min at 45 °C in a Teflon-lined screw-capped tube. One hundred microlitres of aqueous $NaCNBH_3$ (0.79 M) were added, and the reaction mixture was heated for 25 h at 40 °C. The reaction was stopped by addition of 10 ml of water, and the solution was dialysed and freeze-dried as earlier.

BACH derivatives were prepared according to Shinohara et al. (1995), with a BACH (Sigma)/polysaccharide ratio of 10. Fifty milligrams of dry polysaccharides (3.7×10^{-6} mol) were mixed with BACH (5 mM in 30% aqueous acetonitrile). This solution was evaporated to dryness under nitrogen and then dissolved in 1.8 ml of methanol/water/acetic acid, 95:4:1 v/v/v. The reaction mixture was heated for 15 h at 60 °C and evaporated to dryness. Ten millilitres of water were added, and the solution was dialysed and freeze-dried as earlier.

2.3. Molecular weight determination

Molecular weights were determined, as previously described (Nardella et al., 1996), by high-performance steric exclusion chromatography (HPSEC) in 0.1 M ammonium acetate, using a 25 cm × 0.4 cm i.d. Lichrospher 300 Å Diol 7 UM column (Merck, S.A., France) and a 25 cm × 0.46 cm i.d. HEMA SEC BIO 40 10 U column (Alltech, France) connected in series. Area measurements and calculations of M_w (weight-average molecular weight) and I (polydispersity) were performed using ARAMIS software (JMBS Développements, Le Fontanil, France). The columns were calibrated with standard pullulans. As pullulans are neutral glucans and the studied polysaccharides were highly negatively charged, such calibration does not allow exact measurement of molecular weight and was only used for relative comparisons.

2.4. Chemical composition

Sulphate content was deduced from sulphur elemental analysis performed for each fraction by the Central Microanalysis Department of the CNRS (Gif sur Yvette, France). The uronic acid content of polysaccharides was determined as previously described (Filisetti-Cozzi & Carpita, 1991). Neutral sugars, after acidic methanolysis of the polymers and subsequent gas chromatography analysis according to Kamerling, Gerwig, Vliegenthart, and Clamp (1975) and Montreuil et al. (1986), were identified as trimethylsilyl derivatives.

Table 1
Molecular mass and sulphur content (% w/w) of labelled and unlabelled low molecular weight polysaccharides

	M_p (g/mol)[a]	I[a]	M_w (g/mol)[a]	M_n (g/mol)[a]	S (%)[b]
Heparin	29,800	1.82	30,400	16,800	9.3
2-AP–heparin	15,500	2.7	13,100	4800	9.7
ANTS–heparin	21,700	2.6	18,500	6900	9.0
BACH–heparin	25,200	2.8	25,300	8800	9.3
LMW-fucan	26,900	2.3	23,600	10,200	11.0
2-AP–LMW-fucan	16,700	2.2	14,500	6700	10.8
ANTS–LMW-fucan	26,600	2.5	24,700	9400	10.0
BACH–LMW-fucan	25,100	2.4	22,800	9400	11.0
LMW-EPS	35,800	1.7	37,500	21,200	13.0
2-AP–LMW-EPS	9100	1.0	9500	9400	11.5
ANTS–LMW-EPS	27,800	1.8	30,600	17,100	12.0
BACH–LMW-EPS	38,600	1.8	41,000	22,700	13.0

[a] M_p (peak-molecular weight), M_n (number-average molecular weight), M_w (weighted-average molecular weight) and I (polydispersity) were measured by HPSEC using pullulans as standards.
[b] Performed using elemental analysis.

2.5. NMR spectroscopy

1D ^{1}H NMR spectra were recorded on a Bruker 500 MHz at a probe temperature of 298 K. Prior to analysis, samples were exchanged twice in D_2O with terminate freeze-drying.

2.6. Tetrazolium blue assay

The content of reducing ends was determined by addition of three volumes of 0.3 M NaOH to one volume of an aqueous suspension of tetrazolium blue (1% w/v, Sigma) and diluted three times. Nine hundred microlitres of tetrazolium blue solution were added to 100 μl of a 5 mg/ml solution of polysaccharide, and the mixture was heated for 30 s at 100 °C. The absorbance at 570 nm was determined after extraction with 1 ml of toluene (Mullings & Parish, 1984).

2.7. Measurement of residual free 2-AP and ANTS

The high-performance liquid chromatograph consisted of a Merck pump (655A-11) and a SM 4000 UV spectrometer (LDC Analytical). AP-polysaccharides and free AP were separated on a column (4.6 mm × 250 mm, LC-318, Supelcosyl™, 300 Å, Supelco) using a 0.125 M citrate buffer (pH 4) containing 1% acetonitrile at a flow rate of 1 ml/min. Free AP and AP derivatives were detected at 228 and 287 nm.

For gel electrophoresis analyses, low-melting-point agarose (1%) from Biorad was dissolved in the electrophoresis buffer (50 mM ammonium acetate and 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) from Sigma, in water). Agarose (19 ml) was poured hot (>70 °C) on a Gel Bond film (Pharmacia) fitted to a Plexiglas casting tray (75 × 100 mm) to obtain a cooled gel ≈ 4 mm thick. In each 66 mm × 1 mm slot, 20 μl of ANTS derivative samples (10 mg/ml) were mixed with 7 μl of a solution containing 480 mg sucrose, 3.2 mg bromophenol blue, and 400 μl CHAPS 10%, completed to 1 ml with electrophoresis buffer. Electrophoresis was performed at 60 V with currents at 300 mA for 3.5 h. Buffer was re-circulated, and a flow of cold water through the coolant ports of the apparatus was used to maintain buffer temperature at 20 °C. After migration, gels were air-dried. For detection of ANTS derivatives, an excitation wavelength of 301 nm and an emission wavelength of 520 nm were used on a Fluor-S™ MultiImager from Biorad.

2.8. APTT activity

Activated partial thromboplastin time (APTT) with the APTT Organon kit (Organon Technika, France) was determined as previously described (Mauray et al., 1995). Free and tagged heparin (0–5 μg/ml), free and tagged fucan fraction (0–50 μg/ml), and free and tagged EPS (0–30 μg/ml) were diluted in human platelet-poor plasma (PPP). These concentrations were chosen to obtain a comparable range of APTT prolongation with all tested products.

3. Results and discussion

All results are given within a 95% confidence interval based on a minimum of three replications for each fraction.

3.1. Molecular weight, sulphate content and chemical composition

The molecular weight and sulphate content of labelled and free polysaccharides are shown in Table 1. In the case of heparin, the three labelling methods induced heparin depolymerisation, increasing its polydispersity and the number of small chains (<10,000 g/mol). Except for 2-AP labelling, the molecular weight and polydispersity of fucans and EPS derivatives were comparable to those of free fucan

Table 2
Uronic acid content of labelled and unlabelled heparin and EPS (uronic acid content was determined according to the procedure described by Filisetti-Cozzi and Carpita (1991))

	Uronic acids (%)
Heparin	52.2
2-AP–heparin	53.6
ANTS–heparin	61.5
BACH–heparin	64.9
LMW-EPS	24.1
2-AP–LMW-EPS	23.0
ANTS–LMW-EPS	26.5
BACH–LMW-EPS	25.0

and EPS. This lowering of molecular weight was probably due to our experimental conditions (relatively high temperature for 2-AP labelling). Sulphate content (sodium salt) as deduced from sulphur analysis did not change after polysaccharide labelling. Uronic acid content was determined only for heparin and EPS (see Table 2), as fucans possess few uronic acid residues (<10%). The amount of uronic acid was the same in heparin and heparin derivatives, as well as in free EPS and EPS derivatives. Neutral sugars were quantified using TMS derivatisation prior to gas chromatography, (Table 3). In both fucan and EPS, the three labelling methods produced no change in the amount of neutral sugars. Concerning EPS, it should be noted that the sum of analysed sugars represented no more than 54% of the polysaccharidic material and that the colorimetric and chromatographic methods furnished very different values for the uronic acid amount: 24.1 and 8.1%, respectively (see Tables 2 and 3). This is not surprising, because the hydrolysis of uronic acid containing polysaccharides gives often a high percentage of disaccharides (aldobiuronic acids) not quantified by GC, which underestimates the GC determined amounts of both uronic acids and neutral sugars.

3.2. NMR studies

No difference was found between labelled and unlabelled products by the above-mentioned methods, which are rather approximate and not accurate enough to detect minor alterations at the biologically important site. However, in NMR studies, the 1D ^{1}H NMR spectra of the various derivatives showed previously imperceptible modifications relative to the starting materials. The most obvious difference for 2-AP and ANTS derivatives was the appearance of very small signals corresponding to aromatic protons and due to the labelling groups, which consequently were really linked to the oligosaccharide chain. As there is no well resolved proton in the BACH skeleton, its attachment could not be similarly checked.

Concerning heparin, the main resonances of the osidic chain did not appear to change at first and were consistent with previously published data (Larnkjaer, Hansen, & Ostergaard, 1995; Mulloy & Johnson, 1987; Rabenstein, Robert, & Peng, 1995). Some signals were especially informative because they were characteristic of a kind of residue, e.g. the resonances at 5.23 and 3.3 ppm assigned to H1 of 2-*O*-sulphated iduronic acid and H2 of *N*-sulphated glucosamine residues, respectively. There was also a sharp peak at 2.06 ppm corresponding to the chemical shift of CH_3 on an *N*-acetylated glucosamine unit (Glc*N*Ac). Using the area of this last signal as a reference (no deacetylation could occur in the conditions used), it was possible to confirm the absence of any modification by measuring the ratio between the area of a distinctive proton and the area of the 2.06 ppm signal. Thus, it was determined that *N*-sulphated D-glucosamine residues were not *N*-desulphated at all, regardless of the derivative, but that a low percentage of iduronic acid residues was sometimes desulphated at the *O*-2-position.

Concerning fucans, fewer NMR data were available and anomeric protons were poorly resolved, so that quantitative evaluation of modifications was impossible. However, the 5.5 ppm signal present in anticoagulant fucans and assigned to fucose 2,3-*O*-disulphate units (Chevolot et al., 1999) was less intense after derivatisation, which showed some loss of this important residue (see later). For EPS, no obvious difference was apparent. However, as 1D ^{1}H NMR spectra of sulphated derivatives are very complex, some undetectable alterations may have occurred.

Table 3
Sugar composition (% w/w) of labelled and unlabelled low molecular weight polysaccharides (sugar composition was performed by GC analysis using trimethylsilyl derivatives after acidic methanolysis)

Composition	Fuc (%)	Gal (%)	Xyl (%)	Total (%)			
LMW-Fucan	33.0	1.5	1.6	36.1			
2-AP–LMW-fucan	44.7	1.3	1.9	47.9			
ANTS–LMW-fucan	58.2	1.8	3.0	63.0			
BACH–LMW-fucan	43.0	< 1	< 1	43.0			
	Rha (%)	GlcA (%)	GalA (%)	Man (%)	Gal (%)	Glc (%)	Total (%)
LMW-EPS	1.9	3.7	4.4	2.2	12.1	14.3	38.6
2-AP–LMW-EPS	5.0	4.4	5.0	3.8	10.2	13.0	41.4
ANTS–LMW-EPS	2.2	2.5	1.8	1.6	8.8	12.0	27.3
BACH–LMW-EPS	2.8	2.5	2.6	2.6	10.8	13.9	35.25

Table 4
Anticoagulant activity measured by activated partial thromboplastin time performed with the Organon APTT kit (results are expressed as means ± SD ($n = 3$ or 4))

	Activated partial thromboplastin time (seconds)		
	0 μg/ml	1.5 μg/ml	3 μg/ml
Heparin	36.8 ± 1.0	85.0 ± 2	178.0 ± 5
2-AP–heparin	39.0 ± 1.6	75.0 ± 1.5	112.3 ± 4.1
ANTS–heparin	38.7 ± 1.1	86.9 ± 2.0	148.0 ± 4.2
BACH–heparin	38.9 ± 1.1	85.0 ± 2.4	137.2 ± 3.2
	0 μg/ml	20 μg/ml	40 μg/ml
Fucan	40.1 ± 0.5	78.4 ± 5.8	117.0 ± 4
2-AP–fucan	38.9 ± 1.1	56.6 ± 1.5	103.0 ± 1.3
ANTS–fucan	38.7 ± 1.0	54.9 ± 3.2	92.3 ± 4.0
BACH–fucan	38.9 ± 1.1	47.5 ± 0.5	110.1 ± 0.6
	0 μg/ml	10 μg/ml	20 μg/ml
LMW-EPS	39.0 ± 1.6	85.0 ± 2	121.1 ± 2
2-AP–LMW-EPS	37.6 ± 0.9	72.2 ± 1.4	107.2 ± 2.6
ANTS–LMW-EPS	38.7 ± 1.1	87.0 ± 1.4	110.1 ± 4.4
BACH–LMW-EPS	39.0 ± 1.6	83.9 ± 0.6	143.7 ± 1.6

3.3. Yield

The efficiency of polysaccharide labelling was determined by quantifying the remaining reducing ends by tetrazolium blue assay. After dialysis, no residual free 2-AP or ANTS was detected by gel electrophoresis or HPLC, with a detection limit of 10^{-8} and 10^{-12} M, respectively (data not shown). The reducing ends labelled with 2-AP or ANTS were estimated to be 90–100% by this method. Measurement was impossible by NMR because the anomeric protons of reducing ends were overlapped by other protons. Concerning BACH derivatives, the amounts of reagent linked to polysaccharides were determined according to Vynios, Faraos, Spyracopoulou, Aletras, and Tsiganos (1999), which indicated that 50–60% of the chains were biotinylated.

3.4. Anticoagulant activity

The in vitro anticoagulant activity of labelled and free polysaccharides was evaluated by APTT (Table 4). In the case of heparin, the same anticoagulant effect was obtained for tagged and free heparin: the concentration required to double control time was approximately 1.5 μg/ml for each product. A very weak activity loss was noted for 2-AP–heparin, probably because of the greater chain shortening observed for this derivative (heparins usually show a lower effect in APTT assay when molecular weight decreases). It was impossible to check the integrity of the antithrombin-binding pentasaccharidic unit by NMR because of signal overlapping. Nevertheless, these results were consistent with NMR data because sulphates borne by glucosamine residues (those important for anticoagulant activity) were not modified (see above). Anticoagulant activities seemed lower with all tagged fucans at both concentrations tested, but particularly at 20 μg/ml, although the decrease of molecular mass was no greater than with heparin. This was probably due to alterations of the anticoagulant sequence, as the presence of a few fucose 2,3-*O*-disulphate residues (as shown by NMR, see above) is important for the anticoagulant activity of fucan (Chevolot et al., 1999). The anticoagulant activity of EPS was roughly not modified by labelling. Otherwise, the concentration doubling control time was approximately 10 μg/ml for free and tagged EPS.

4. Conclusion

In conclusion, labelling the reducing ends of naturally or artificially sulphated polysaccharides with tags (2-AP, ANTS and BACH) had no marked effect on the chemical composition and biological activity of the three polysaccharides. However, molecular size reduction and low desulphation, detectable only by careful examination of NMR spectra, were sometimes observed. These alterations were slightly greater with 2-AP derivatisation and almost totally lacking with BACH, however, the labelling yield was relatively low with the latter reagent. Among the three reagents tested, ANTS provided the best compromise for retaining structural integrity and biological activity. Very recently, Ramsay, Freeman, Grace, Redmond, and MacLeod (2001) proposed novel hydroxylamines as mild tagging reagents. This possibility seems quite promising, but the compounds are not commercially available. Moreover, only oxime was quantitatively formed with a sulphated model compound (Glc*N*SO3). This is a limitation to the method, because oximes are hydrolysable and form a mixture of various isomers that complicate chromatographic separation. At the present time, there is no 'perfect' reagent available, and it is necessary in any case to test and select the most convenient reagent for each kind of oligosaccharide. The present study indicates that ANTS as a labelling reagent induces few alterations. In addition, this reagent provides three negative charges, thereby facilitating the study of neutral oligosaccharides by electrophoresis or electrospray mass spectrometry. Thus, this sensitive technique could be a very useful tool for the discovery and characterisation of new bioactive sulphated polysaccharides.

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