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RHAMNAN SULFATE FROM CELL WALLS OF *MONOSTROMA LATISSIMUM*

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Abstract—Rhamnan sulfate from cell walls of *Monostroma latissimum* was purified through subsequent chromatographic systems. The purified polysaccharide consisted of large amounts of rhamnose residues and appeared to be an entire homopolysaccharide. Antithrombin activity was lower than rhamnan sulfate previously obtained from *M. nitidum* but similar to standard heparin. Studies of the major structural parts of the rhamnan sulfate by periodate oxidation, Smith degradation and permethylation, showed it to consist of 1,3- and 1,2-linked rhamnose residues in a ratio of 3:2. Sulfate was mainly substituted at C-3 or C-4 in the 1,2-linked rhamnose residue. The detection of oligosaccharides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry supports this structure. These results suggested that rhamnan sulfate from the Monostromaceae has quite different structural properties from ulvan from the Ulvaceae. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Sulfated polysaccharides in the plant cell wall are specifically present in seaweeds but not in land plants [1]. Until recently, few structural studies have been done on sulfated polysaccharides from Chlorophyta, except for Ulvales. A water-soluble polysaccharide extracted from *Ulva lactuca* was found to be a sulfated glucurono-xylo-rhamnoglycan, in which the sulfate groups are linked to rhamnose [2]. A similar sulfated polysaccharide was isolated from *U. rigida* [3].

Sulfated polysaccharides show various biological activities, such as antithrombin [4], antitumor [5] and antivirus [6], and these characteristics have been widely investigated in relation to their structures. Surveys and investigations of heparinoid-active polysaccharides showed antithrombin activity *in vitro* for sulfated polysaccharides from Chlorophyta [7]. Antithrombin activity markedly higher than that of standard heparin was found in sulfated polysaccharides extracted from *Monostroma nitidum* using hot water [7]. This active polysaccharide was purified by ion-exchange and gel filtration chromatography, and was found to be a homopolysaccharide-like rhamnan sulfate consisting mainly of *L*-rhamnose. Its antithrombin activity relative to standard heparin was six-times greater and may be correlated with its sulfate ester content and higher ordered structure, given the molecular shape of rhamnan sulfate. To clarify the structure and activity relationship of rhamnan sulfate in the Ulvales, comparative structural studies should be made of rhamnan sulfate for Monostromaceae species. Given that *M. latissimum* is cultivated as an edible alga, analyses of this species are very important in industrial applications. The results of our analyses are presented in this paper.

RESULTS AND DISCUSSION

When algal fronds of *M. latissimum* were successively extracted using cold water (CW) and then hot water (HW), faint coloured materials were obtained, i.e. CW and HW fractions. Their neutral sugar compositions are shown in Table 1.

Antithrombin activity was higher in the HW fraction than in the CW fraction, along with sulfur contents, such as those detected similarly in *M. nitidum* [7]. Further purification was attempted with the HW fraction to obtain higher antithrombin activity with a good yield.

The HW fraction was eluted through a DEAE-Toyopearl 650C anion-exchange column with stepwise alternation of its ionic strength from H_2O to a final 5 M NaCl. Using this procedure, the most abun-

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Table 1. Properties of crude polysaccharides and purified rhamnan sulfate from *M. latissimum*

	CW*	HW*	RS*
Yields (%)	5.0	11.4	4.0
ATA†	0.4	0.7	1.0
Sulfur contents (%)	5.7	5.0	7.2
Protein contents (%)	4.5	4.6	0.0
Uronic acid contents (%)	7.4	7.6	4.0
Neutral Sugar composition (mol%)			
Rhamnose	78.7	67.9	85.4
Arabinose	4.6	4.8	1.4
Xylose	7.6	8.3	5.2
Mannose	6.9	2.4	1.3
Glucose	2.3	11.9	4.7
Galactose	0.0	4.8	2.0
$[\alpha]_D$	-60.0	-42.0	-45.6

* CW and HW, cold water and hot water extracted crude polysaccharide, respectively.

RS, purified rhamnan sulfate.

† Anti-thrombin activity relative to heparin.

dant amounts of polysaccharides with the highest antithrombin activity were obtained in the 1 M NaCl-eluted fraction. In this fraction, rhamnose was also found as a major sugar constituent and the fraction was further purified by Super-Q Toyopearl 650M anion-exchange chromatography. The most frequent fractions eluted in the greatest number were concentrated through re-chromatography under similar elution conditions until eluted as a single, symmetrical peak that appeared to be charged homogeneous materials. Further purification by preparative Toyopearl HW-65F gel filtration chromatography showed the finally obtained single peak to have a homogeneous size distributions. Its partition coefficient (K) was 0.39.

The finally purified active sulfated polysaccharide (Table 1) consisted of a high amount of rhamnose residue with almost negligible amounts of xylose and glucose residues. Since protein was not detectable, sulfur contents originated solely from polysaccharide sulfate esters. The degree of substitution (DS) of sulfate ester was estimated to be 0.4 against an anhydro-sugar residue. The M_r was estimated to be 1.7×10^5 by high-performance gel filtration chromatography. The optical rotation $[\alpha]_D$ was -45.6, (*c* 0.5, H_2O). The IR spectrum showed absorptions at 1250 (S=O), 890 (Type 2b) [8] and 855 (C—O—S) [9] cm^{-1}. Three anomeric signals at δ 5.20, 5.04, and 4.98 ppm in the ^{1}H NMR spectrum of the desulfated material suggest that the material contains different types of rhamnose linkages. The coupling constant ($J_{1,2}$) of rhamnose appeared to be less than 2 Hz, but the spectrum was broadened due to the presence of macromolecules; all anomeric signals were pseudo-singlets. These results suggest that the rhamnose residues of this rhamnan have an α-L configuration. A large peak at δ 1.32 ppm due to CH_3 group also confirmed the existence of rhamnan.

Rhamnan appeared to be the backbone structure in sulfated polysaccharides, so linkage analyses were conducted. After ca 96 h of oxidation, the pectin used as the control consumed 1.01 mol of periodate for the anhydro-sugar residue, which corresponded to the theoretical value of periodate consumption (1 mol) for the anhydro-galacturonic acid residue of pectin. The periodate consumed for the anhydro-sugar residues of desulfated materials was 0.68 mol. Intact rhamnose was detected by gas chromatography after the reaction mixtures were hydrolysed. From these results, ca 30% of periodate-resistant sugar residues were composed of 1,3-linked or branching points. Purified rhamnan sulfate consumed 0.30 mol of periodate per anhydro-sugar residue. The molar differences of periodate consumption between rhamnan sulfate and desulfated material (0.38 mol) agreed well with the DS value (0.4) and ca 40% of sugar residues in the rhamnan were substituted as mono sulfate ester.

Identification of propylene glycol as TMS derivatives from Smith degradation products is evidence for 1,2-linked rhamnose residues. Larger amounts of propylene glycol were found in desulfated materials than rhamnan sulfate. The sulfate ester group was mainly bonded at C-3 or C-4 in the 1,2-linked rhamnose residue.

After successive permethylation of desulfated material using the Hakomori method [20], a completely methylated product was obtained as evidenced by the absence of IR absorption for OH groups. Gas chromatography and mass spectrometric analysis of the partially methylated alditol acetates (Table 2) obtained from the hydrolysate suggested that a major part of the rhamnose residues were linked 1,3-and 1,2 in a ratio of ca 3:2. A minor part of the rhamnose residue was 1,3,4- and 1,2,4-substituted. Although the Monostromaceae and Ulvaceae are closely related, rhamnan sulfates from the former have homogeneous architectures whereas those from the latter are heterogeneous. This structural difference was clearly recognized by the detection of glucuronic acid in oligosaccharides from partial acid hydrolysates of ulvans and the results indicate the heterogeneity of cell wall polysaccharides in *U. lactuca* and *U. rigida*.

Partial acid hydrolysates from *M. latissimum* eluted by gel filtration chromatography revealed seven fractions from higher oligosaccharides (Fraction A) to monosaccharide (Fraction G). Oligosaccharides in each fraction were analyzed using matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOFMS). Measurements for Fractions A and B were poor because of the out of detection range of higher oligosaccharides mixtures eluted by the order of the gel filtration rule. Ion peaks corresponding to rhamnooligosaccharides were observed in Fractions C–F (Table 3). Using the post-source-decay (PSD) fragment analysis on MALDI-TOFMS, fragment ion peaks from the precursor ion

Table 2. Linkage analysis of rhamnan by permethylation analysis

Identification	Origin	*T**	Primary mass fragments (*m/z*)	Molar ratio
3,4-$(Me)_2$-Rham	-2-Rham-1-	0.91	131, 175, 189, 233	20.0
2,4-$(Me)_2$-Rham	-3-Rham-1-	0.98	117, 131, 233	30.1
2-(Me)-Rham	-4,3-Rham-1-	1.14	117	3.5
3-(Me)-Rham	-4,2-Rham-1-	1.38	189, 203	3.0

* Relative retention time to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol

Table 3. Identification of subfractionated rhamnooligosaccharides by MALDI-TOFMS and their relative peak intensities (%)

DP* of oligosaccharides	2	3	4	5	6
Mass number (*m/z*)	333	479	625	771	917
C	—		5	100	8
D	—	10	100	—	—
E	—	100	—	—	—
F	100	—	—	—	—

* DP: Degree of polymerization.

at *m/z* 771 $[M]^+$ in the Fraction C were observed at *m/z* 625, 479 and 333, corresponding to rhamnopentaose. Each of them showed a regular mass interval of 146 amu corresponding to the loss of rhamnosyl residue [R]. Fragment ion peaks at *m/z* 625, 479 and 333 corresponded to the chemical species of $[M-R_1]^+$, $[M-R_2]^+$ and $[M-R_3]^+$. No ion peak was found at *m/z* 363, but only rhamnobiose in Fraction F corresponding to the disaccharide fraction, signifying that aldonobiuronic acid was not in the rhamnan from *M. latissimum*. Rhamno-oligosaccharides higher than rhamnohexaose found in Fraction C were expected in Fractions A and B, so rhamnan sulfate from *M. latissimum* consisted of a series of homogeneous rhamno-oligosaccharides.

Antithrombin activity of the rhamnan sulfate from *M. latissimum* was similar to that of heparin, but lower than that of rhamnan sulfate previously obtained from *M. nitidum*, which was found to be ca 6 times greater than that of heparin. These are structurally similar to rhamnan sulfate derived from *M. nitidum* and *M. latissimum*, in that both were composed of 1,3- and 1,2-linked rhamnose residues; the content and substituted positions of ester sulfate from *M. nitidum*, estimated at DS 0.7, were mainly substituted at C-2 of the 1,3-linked rhamnose residue. The partially desulfated materials of this polysaccharide, although showing DS values similar to *M. latissimum*, still showed higher antithrombin activity than that from *M. latissimum*. The detailed mechanisms of antithrombin activity of seaweed sulfated polysaccharides are unknown. It is very important, however, to clarify the contents and sites of sulfate esters in sugar residues and linkage modes between the sugar residues. The polarity and stable conformation of the macromolecule are determined by these factors. Further spectroscopic studies in comparative conformational analysis, including linkage analysis for the different species of rhamnan sulfate, are required. Antiviral properties will be reported elsewhere.

EXPERIMENTAL

Material

Monostroma latissimum, was supplied by the Mie Fisheries Cooperative Association, Tsu, Mie Prefecture. It was harvested in April 1994 and air-dried.

General

All evapns were conducted below 40° under red. pres. Total sugars were estimated by the PhOH–H_2SO_4 method [10]. Uronic acids were determined by the carbazole–H_2SO_4 method [11]. Protein content was determined by Lowry method [12]. IR of free sugars was measured in a KBr disk, or for methylated polysaccharides in $CHCl_3$ liq. film. Optical rotations were determined at 20° using a 10 cm light path length cell as a wave length of 589 nm. The ^1H NMR spectrum was recorded in a D_2O soln with a α-500 NMR spectrometer at 50°; TMS was used as the ext. standard.

Assay of antithrombin activity

The method is described elsewhere [7].

Extraction of crude polysaccharides

Dried algae (100 g) were dipped into 1 l of H_2O and kept at room temp. for 1 h. The well-expanded algae were then homogenized and kept at room temp. for a further h. After filtration, the supernatants were dialyzed against running tap H_2O for 3 days and H_2O for 2 days and then lyophilized. The product obtained is referred to as cold H_2O extracted crude polysaccharide (CW). H_2O (700 ml) was added to the residual algae and heated under reflux for 2 h at 80°. After filtration, the product, hot H_2O-extracted crude polysaccharide (HW), was obtained from the extract after dialysis against running tap H_2O for 3 days and H_2O for 2 days, and lyophilization as described above.

Fractionation and purification of active polysaccharide

When antithrombin activities were compared for the two extracts, stronger activity was shown for HW than CW. The active component was fractionated from HW by DEAE-Toyopearl 650C anion-exchange column chromatography (50 × 4.4 cm i.d.). After an appropriate amount of carbohydrates in the HW soln was determined by PhOH–H_2SO_4 methods and loaded on the column, it was eluted with 4 bed vols of H_2O until no more PhOH–H_2SO_4 positive material appeared. Further elutions were continued in the same way by stepwise addition of 0.5, 1.0, 3.0, and 5.0 M NaCl. The column was then washed with a 0.2 M NaOH soln. Each eluted fr. was extensively dialyzed against H_2O and then lyophilized. Since the stepwise fractionated sample at a 1 M NaCl revealed the highest activity, it was further purified on a Super-Q Toyopearl 650M column (30 × 2.2 cm i.d.). The column was eluted with a linear gradient NaCl from 0.5 to 2.0 M NaCl. Frs (9 ml) were collected at a flow rate of 1.8 ml min^{-1}. The fr. obtained was extensively dialysed against H_2O and then lyophilized.

Analytical gel filtration chromatography

Gel filtration chromatography was performed on a Toyopearl HW-65F column (100 × 1.1 cm i.d.) and the sample was eluted with 0.2 M NaCl at a flow rate of 1 ml min^{-1}. The elute was determined by the PhOH–H_2SO_4 method and an RI detector. The partition coefficient was estimated from K = (Ve-Vo)/(Vt-Vo). Vo is the void vol. determined using Blue Dextran 2000, Vt is the total bed vol. by L-rhamnose used as the standard, and Ve is the elution vol. of the sample. M_r was estimated via M_r markers using an analytical gel filtration chromatography (TSK-GEL GMPW$_{XL}$ column, 300 × 7.8 mm i.d.). The eluted sample with 0.2 M NaCl at a flow rate of 0.8 ml min^{-1} was detected by RI.

Acid hydrolysis and gas chromatography [13]

Aldoses in complete hydrolysates with 2 N H_2SO_4 were converted TFA alditol derivatives. They were analyzed by GC using an OV 1701 fused silica capillary column at an oven temp. of 170°. N_2 was used as the carrier gas.

Desulfation of purified rhamnan sulfate

Solvolytic desulfation was conducted with DMSO and MeOH mixts [14]. Decreased amounts of sulfate ester were calculated from sulfur analysis.

Microdetermination of sulfur

Sulfur was determined by flask combustion in O_2 gas in the presence of H_2O_2 [15]. After the sample in the combustion flask was ignited in an O_2 atmosphere with an aliquot H_2O_2, the inner soln was titrated against a standard soln of 50 mM $BaCl_2$, using a 0.1% dimethylsulfonazo III as indicator. A standard soln of $BaCl_2$ (1 ml) corresponded to 0.16 mg of sulfur. DS was calculated as the molar ratio of sulfur to the anhydro-sugar residue.

Periodate oxidation

Samples (10 mg) were added to the mixed soln of 0.3 M $NaClO_4$ (3 ml) and 0.1 M $NaIO_4$ (2 ml) in a 0.1 M sodium acetate buffer (pH 5.0), and was prepared with a final periodate conc of 40 mM. Commercially prepared pectin (α-1,4-D-galacturonan) was oxidized under the same reaction conditions as controls. Oxidation was continued at 4° in the dark with continuous stirring and portions (20 μl) of the reaction mixt. were removed after 3, 6, 9, 12, 24, 48, 72 and 96 h. After dilution to 10 ml with H_2O, the amount of consumed periodate per anhydro-sugar was calculated by the A of the resulting soln at 223 nm [16].

Smith degradation [17]

After periodate consumption reached a constant value for the pectin soln used as the control, excess amounts of ethylene glycol (1 ml) were added, and the mixture was dialysed against H_2O for 48 h. $NaBH_4$ (5 mg) was added to the nondialysable soln and reduced for 24 h at room temp. After excess $NaBH_4$ was destroyed by the addition of aq. OHAc soln, the reaction mixt. was dialysed again for 24 h against H_2O. By addition of 3% HCl in MeOH (0.5 ml) to the nondialysable material, the oxidized polysaccharide was then methanolysed at 90° for 24 h. After neutralization with solid Ag_2CO_3, the pp. was filtered off and rinsed thoroughly with MeOH. Combined filtrates and washings were evapd under vacuum until dry at −15° [18]. This dried material was trimethylsilylated in pyridine [19] and analyzed by GC on a packed glass column (100 × 0.2 cm i.d.) containing 2% SE-30 on Chromosorb W. Each peak was identified from R_t values compared with those of authentic samples.

Permethylation analysis

Acid hydrolysates of permethylated samples were converted to partially methylated alditol acetates with $NaBH_4$ and acetylated. They were analyzed by GC using a DB-225 fused silica capillary column (25 m × 0.32 mm i.d.) and GC-MS using a HP-5 (30 m × 0.32 mm i.d.) fused silica capillary column with an ionizing potential of 70 eV, respectively. The peaks on the chromatogram were identified from comparison with reported *RRt* values [21] and previous GC-MS data [22].

Partial acid hydrolysis of rhamnan sulfate

The dissolved rhamnan sulfate in 0.2 N H_2SO_4 was heated at 100° for 75 min and partial acid hydrolysates obtained fractionated by Toyopearl HW-40S gel filtration chromatography (100 × 2.2 cm i.d.), monitoring using the PhOH-H_2SO_4 method.

MALDI-TOFMS

A sample (0.5 μl) and cobalt powder matrix were well mixed with glycerol on the sample plate. A Kompact Maldi IV apparatus was used in linear flight pathway mode with positive polarity of the detector; the acceleration energy of ions was 20 kV [23]. The laser power was adjusted slightly above the threshold, in which case the required ion appears constantly from shot to shot. All detected oligosaccharide ions were observed as pseudomolecular ions $[M+Na]^+$) [24].

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