Carbohydrate Research, 223 (1992) 221–226
Elsevier Science Publishers B.V., Amsterdam

The structure of mytilan, a bioglycan-immunomodulator isolated from the mussel *Crenomytilus grayanus*

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(Received September 20th, 1990; accepted for publication May 13th, 1991)

ABSTRACT

Mytilan, isolated from the mantle of the mussel *Crenomytilus grayanus* and which possesses high immunomodulating activity, is a non-covalently linked complex of a polysaccharide (95%) and protein (5%) with lectin activities. Chromatography of mytilan on agarose separated the polysaccharide and protein constituents. The protein appeared to have the lectin properties. Methylation analysis, Smith degradation, digestion with alpha-amylase, and ^{13}C-n.m.r. spectroscopy showed the polysaccharide to be a glycogen-like α-D-glucan.

INTRODUCTION

Polysaccharides and glycoconjugates that possess antitumor and immunomodulating activities have been isolated from various marine invertebrates[1–4]. The mussel *Crenomytilus grayanus*, which is widespread in the Sea of Japan, is the source of a bioglycan termed mytilan that has a marked ability to increase the cell-mediated and humoral immune response of animals and humans[5,6]. Mytilan also enhances resistance to infectious diseases caused by bacteria and viruses[7,8].

We now report on the structure of mytilan.

RESULTS AND DISCUSSION

Aqueous extraction of the mantle of the mussel *C. grayanus* afforded the crude bioglycan from which protein was removed by the Sevag procedure[9]. Dialysis and lyophilisation then afforded purified mytilan that contained 95% of carbohydrate and 5% of protein. Attempts to fractionate mytilan by molecular-sieve chromatography on Sephadex G-75, G-100, or G-150 and on Sepharose CL-2B or CL-4B were unsuccessful and mytilan was eluted as a single peak in the void volume. Thus, mytilan appeared to be a polysaccharide–protein complex. By comparison with the behaviour of standard

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0008-6215/92/$ 05.00

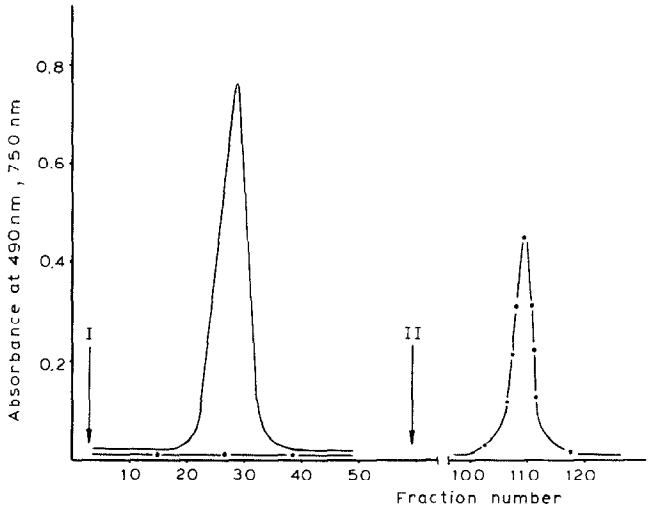


Fig. 1. Affinity chromatography of mytilan on agarose Bio-Gel A-1.5 m: ———, polysaccharide; – · – · –, protein; I, elution with 0.15M sodium chloride; II, elution with 0.1M glycine buffer (pH 2.5).

dextrans (T-250, T-500, T-2000), the weight-average molecular weight of mytilan was estimated to be 3×10^6, a value that was confirmed by analytical ultracentrifugation. A non-covalent bond between the polysaccharide and protein parts of mytilan was proved by affinity chromatography. As shown earlier[10], mytilan possesses lectinic activity, and chromatography of mytilan on agarose (Fig. 1) separated the polysaccharide and protein constituents. The protein components were firmly absorbed, which indicated that they were responsible for the lectin properties. Detailed investigations of these lectins will be published elsewhere.

The carbohydrate moiety of mytilan was found to be an α-D-glucan. Thus, the polysaccharide afforded D-glucose only on complete acid hydrolysis and the $[\alpha]_D^{20}$ value of +140° (water) indicated α linkages. The characteristic i.r. band at 820 cm^{-1} and the positive iodine test suggested that mytilan was a glycogen-like polysaccharide. Digestion of mytilan with alpha-amylase gave a residual polysaccharide (AM) together with a mixture of glucose and oligosaccharides that were identified (p.c. and ^{13}C-n.m.r. spectroscopy) as malto-oligosaccharides. Data on mytilan and AM are given in Table I. These results indicated mytilan to have a branched structure.

Treatment of mytilan with ultrasound also gave a mixture of glucose, maltose, and malto-oligosaccharides, together with a non-dialysable fraction (UM) that was resistant to alpha-amylase. The data on alpha-amylase digestion and ultrasonic degradation demonstrated that (1→4)-linked α-D-glucose residues were present in the backbone and side chains of mytilan.

Mytilan, AM, and UM were each subjected to methylation analysis. Methylation was effected using the Hakomori procedure[11] and its modification[12]. Hydrolysis of the permethylated polysaccharides afforded 2,3,4,6-tetra-, 2,3,6-tri- (mainly), and 2,3- and 2,6-di-*O*-methyl-D-glucose. The ratios of methylated sugars from AM and UM were

TABLE I

Molecular characteristics of mytilan and alpha-amylase-treated mytilan (AM)

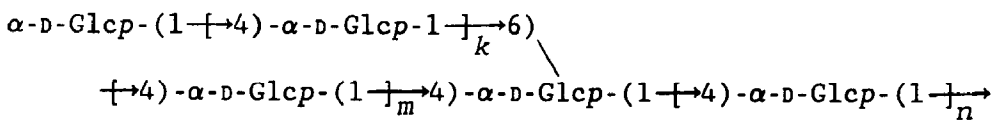
Sample	*Sedimentation coefficient* ($s_{20,w}$, s^{-1})	*Diffusion coefficient* ($D_{20,w}$, sm^2/s)	*Weight-average molecular weight* (M_{ww})
Mytilan	143×10^{-13}	3.0×10^{-7}	3.0×10^{6}
AM	86×10^{-13}	1.9×10^{-7}	0.8×10^{6}

TABLE II

Methylation analysis of mytilan and alpha-amylase-treated mytilan

Methylated sugar	*Mytilan*		*AM*	
	Mol %	*Molar ratio*	*Mol %*	*Molar ratio*
2,3,4,6-Glc	14.7	1.2	16.7	1.2
2,3,6-Glc	64.2	5.4	59.7	4.3
3,4,6-Glc	0.8	0.06	1.1	0.08
2,3-Glc	11.8	1.0	14.1	1.0
2,6-Glc	4.7	0.4	8.4	0.6
3,6-Glc	3.8	0.3	—	—

similar. As may be seen from the data in Table II, the repeating units of mytilan and its derivatives appeared to be similar, as follows:



where $k + m + n = 5$–6 for mytilan and 4–5 for AM and UM.

In addition, each of the three repeating units of mytilan contained two 4-linked α-D-Glc*p* units, one of which was branched at position 3 and the other at position 2, and each of the two repeating units of AM and UM contained one α-D-Glc*p* with branches at position 3.

The results of the methylation studies were confirmed by periodate oxidation data. Smith degradation of mytilan furnished a mixture of glycerol, erythritol, and glucose in the molar ratios 1.7:7.9:1. Glycerol was formed from the terminal residues, the formation of erythritol indicated (1→4)-linked residues as the main constituents of the sugar chains, and the glucose originated from residues that were 2,4- and 3,4-linked.

The glycogen-like structure of mytilan followed from the ^{13}C-n.m.r. data. The ^{13}C-n.m.r. spectra of mytilan and glycogen (Fig. 2) showed a substantial similarity[13], but there were differences. The chemical shifts of the ^{13}C resonances of mytilan compared

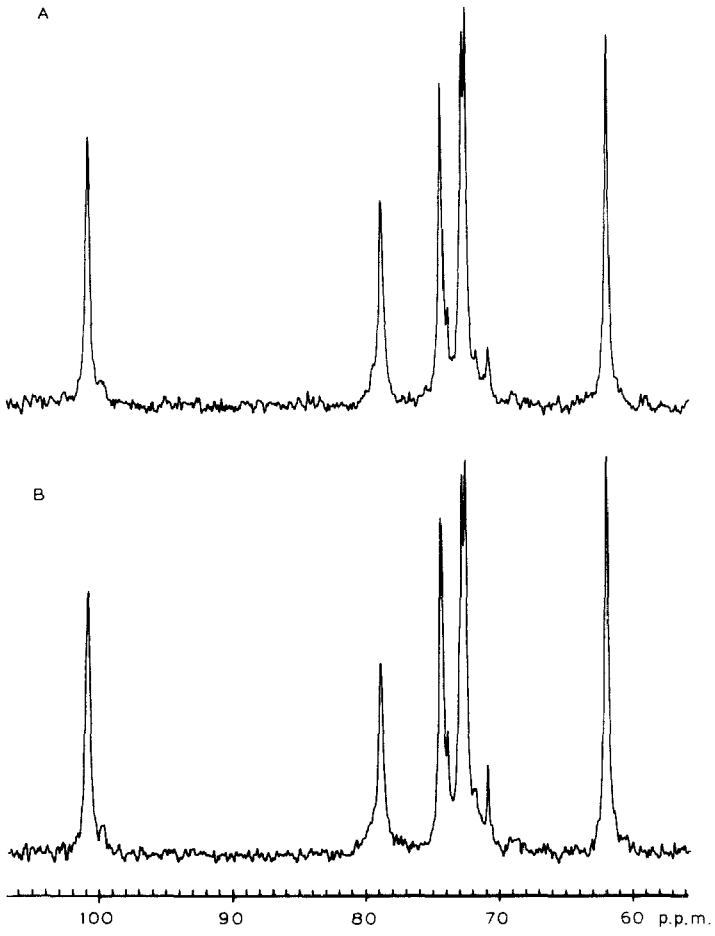


Fig. 2. ^{13}C-N.m.r. spectra (62.9 MHz) at 80° of solutions in D_2O of A, glycogen; and B, mytilan.

with reference data[14] indicated that mytilan consisted mainly of (1→4)-linked α-D-glucose residues with 4,6-linked residues present as minor components. The signals for 2,4- and 2,3-linked D-glucose residues were not detected, probably because of their low intensities. The intensity of the signal at 70.7 p.p.m. for C-4 of terminal Glc and C-4,5 of 6-linked Glc of mytilan was twice that of the corresponding signal of glycogen. Thus, mytilan appeared to be more highly branched than glycogen.

EXPERIMENTAL

General. — The mussel *C. grayanus* was collected in the sublittoral of the Sea of Japan. The alpha-amylase was a commercial sample (Reanal, Budapest). Solvents were evaporated under reduced pressure at $<40°$.

Total carbohydrate was determined by the phenol–sulfuric acid method[15] and protein by the Lowry method[16].

P.c. was performed on Filtrak FN-3, FN-12, and FN-15 paper with 6:4:3 1-butanol–pyridine–water and detection with aniline hydrogen phthalate or alkaline silver nitrate. For g.l.c., sugars and their methylated derivatives were converted into the alditol acetates, acetylated methyl glycosides, or acetylated aldononitriles. A Pye–Unicam 104 gas chromatograph, equipped with a flame-ionisation detector and a glass column (0.4 × 150 cm) packed with 3% of QF-1 or 3% of ECNSS-M on Gas Chrom Q, was used. G.l.c.–m.s. was performed with an LKB-9000S instrument, using a column of 3% of QF-1 on Gas Chrom Q.

Molecular-sieve chromatography was carried out on columns of Sephadex G-75, G-100, or G-150, and Sepharose CL-2B or CL-4B, using 0.15M sodium chloride as eluent. Affinity chromatography was performed on agarose Bio-Gel A-1.5 M (Bio-Rad), using 0.15M sodium chloride and 0.1M glycine buffer (pH 2.5) as eluents.

I.r. spectra were obtained with a UR-20 spectrophotometer. ^{13}C-N.m.r. spectra (62.9 MHz) were obtained with a Bruker HX-250 or Jeol FX 90Q spectrometer on solutions in D_2O at 80° with methanol (δ 50.15) as the internal standard. Chemical shifts are reported in p.p.m. relative to that of Me_4Si.

Ultracentrifugation was carried out on an analytical ultracentrifuge MOM 3170 under the conditions of high-speed sedimentation and approximation to equilibria at 20°, using solutions of 0.001–0.005 g/mL in 0.15M sodium chloride.

Weight average molecular weights of the glucans were estimated, using a column (1.2 × 35 cm) of Sepharose CL-4B with 0.05M sodium chloride as the carrier at 12.0 mL/h. Purified dextran fractions (T-250, T-500, and T-2000) were used as standards.

The ultrasonic treatment of mytilan involved an ultrasonic disintegrator USDM-2T at 22 kHz in a current of argon at 0°, to afford the degraded mytilan (UM), $[\alpha]_D^{20}$ +150° (*c* 0.1, water).

Optical rotations were measured at 20° on solutions in water, with a Perkin–Elmer Model 141 polarimeter.

Isolation of mytilan. — An aqueous extract[2] of the mantle of *C. grayanus* was centrifuged, and the supernatant solution was treated by the Sevag procedure[9], then dialysed, and lyophilised to give mytilan as a white powder (4–5% of the parent material), $[\alpha]_D^{20}$ +140° (*c* 0.1, water). The sample was stored at +4°. Sugar and protein contents were estimated to be 95.0 ±1.0 and 5.0 ±1.0%, respectively.

Hydrolysis of mytilan. — (*a*) *With acid.* Mytilan (5 mg) was heated with M sulphuric acid (0.5 mL) in a sealed tube for 6 h at 100°. P.c. of the hydrolysate and g.l.c. of the derived alditol acetate revealed glucose.

(*b*) *With alpha-amylase.* To a solution of mytilan (300 mg) in distilled water (60 mL) was added alpha-amylase (3 ml), and the solution was incubated for 3 h at 37°. The degree of hydrolysis was estimated on the basis of the release of reducing sugar (expressed as glucose). The mixture was heated for 5 min at 100° to inactivate the enzyme, concentrated to a small volume, and diluted with ethanol (4 vol.). The supernatant solution was concentrated to yield a mixture of glucose, maltose, and malto-oligosaccharides that were fractionated by preparative p.c. The total yield of oligosaccharides was 135 mg. A solution of the insoluble, degraded glucan in water was lyophilised to yield amylase-treated mytilan (AM, 155 mg), $[\alpha]_D^{20}$ +150° (*c* 0.1, water).

Methylation analysis. — Separate solutions of mytilan (50 mg), AM (20 mg), and UM (20 mg) in methyl sulfoxide (20 mL) were each methylated by the method of Hakomori[11] or its modification[12]. The completeness of methylation was tested by i.r. spectroscopy.

Each fully methylated polysaccharide (5 mg) was heated with dry methanolic 0.5M HCl (5 mL) for 7 h at 100° in a sealed tube. The acid was removed by repeated evaporation with methanol *in vacuo* at 37°. The methyl glycosides of the methylated sugars were then acetylated.

Also, each fully methylated polysaccharide (5 mg) was hydrolysed with 90% aq. formic acid (1 mL) in a sealed tube for 2 h at 100°, and then (after removal of the formic acid) in 0.25M sulphuric acid (1 mL) for 10 h at 100°. The partially methylated sugars in the hydrolysate were reduced with $NaBH_4$ and then acetylated to yield the alditol acetate derivatives.

Smith degradation. — Mytilan (50 mg) was stirred with 0.015M sodium periodate (50 mL) at 4° in the dark for 10 days. The resulting polyaldehyde was treated with $NaBH_4$ (50 mg) at room temperature for 24 h, the $NaBH_4$ was decomposed by the addition of acetic acid, and the solution was dialysed against distilled water, then lyophilised to yield the polyalcohol (45 mg). The polyalcohol (10 mg) was hydrolysed with 0.5M sulphuric acid for 4 h at 100°, and the products were acetylated with acetic anhydride (0.2 mL) in pyridine.

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