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POLYSACCHARIDES AND STEROLS FROM GREEN ALGAE *Caulerpa lentillifera* AND *C. sertularioides*

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Sterols and polysaccharides of green alga Caulerpa lentillifera *grown under laboratory conditions and in mariculture and polysaccharides of green alga* C. sertularioides *grown under natural conditions were studied. The sterol fraction consisted of C_{27}-C_{29} steroidal alcohols with Δ^5-unsaturation in the steroid core regardless of the growth conditions. The dominant (79.9%) steroid component of the sterol fraction was clionasterol. The water-soluble fraction of* C. lentillifera *grown under laboratory conditions was a mixture of 1,4-α- and 1,3-β-D-glucans and protein. The same fraction isolated from* C. lentillifera *grown in mariculture contained only protein. The water-soluble fraction of* C. sertularioides *grown under natural conditions contained 1,3;1,6-β-D-galactan sulfated at C2. The principal components of the base-soluble polysaccharide fractions from all algae samples were 1,4-α-D-glucans.*

Key words: green algae, *Caulerpa lentillifera*, *C. sertularioides*, polysaccharides, sterols, clionasterol.

Marine algae, including representatives of the green algae (Chlorophyta), to which the genus *Caulerpa* belongs, are a rich source of sterols that differ in chemical structure from cholesterol, the main sterol of higher animals. Steroids are important biologically as hormones, vitamins, and structural components of biomembranes.

Polysaccharides are an important component of algae. The heightened interest in them is related to their broad spectrum of biological activity. For example, polysaccharides exhibiting anticoagulant, antitumor, and other activities have been isolated from green algae *Caulerpa racemosa*, *C. brachypus*, *C. okamurai*, *C. scapelliformis*, *Chaetomorpha crassa*, *C. spiralis*, *Codium adhaerens*, *C. fragille*, *C. latum*, *Enteromorpha compressa*, *Monostroma nitidum*, and *Ulva* spp. [1-4].

The goal of our work was to study polysaccharides and sterols from the green algae *Caulerpa lentillifera* and *C. sertularioides*, the composition and structure of which have not been previously studied.

We used samples of *C. lentillifera* grown under laboratory conditions at 25-30°C (natural illumination, collected January 20, 2005, sample 1) and in man-made aquifers in the shade (collected April 15, 2007, Khanh Hoa Province, Ninh Hoa District, Viet Nam, sample 2) and also samples of *C. sertularioides* collected under natural conditions in May 2007 (sample 3).

Steroid components of *C. lentillifera* were isolated from samples 1 and 2 (see Experimental) and identified by mass spectra of their acetates and their GC behavior over a capillary column. Table 1 lists the composition of the sterol fraction of sample 1. The composition and amount of sterols isolated from different samples were identical.

Table 1 shows that the sterol fraction consists of C_{27}-C_{29} steroidal alcohols with Δ^5-unsaturation in the steroidal core. The dominant (79.9%) sterol was clionasterol, for which the absolute configuration at C-24 was determined by comparing PMR spectra with model 24*S* and 24*R* C_{29} sterols [5, 6]. The PMR spectrum contained resonances characteristic of the 24*S* configuration at 0.68 (3H, s, CH_3-18), 1.01 (3H, s, CH_3-19), 0.93 (3H, d, J = 6.8, CH_3-21), 0.83 (3H, d, J = 6.8, CH_3-26), 0.81 (3H, d, J = 6.8, CH_3-27), 0.86 (3H, t, J = 7.0, CH_3-29), 3.5 (m, 1H, H-3), 5.6 (m, 1H, H-6).

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TABLE 1. Sterol Fraction of Alga *Caulerpa lentillifera*

Sterol (acetates)	$[M]^+$ (acetates)	Retention time (relative to cholesterol acetate)	Content, %
Cholesta-5,22*E*-dien-3β-ol	426	0.695	1.4
Cholest-5-en-3β-ol	428	1.000	11.0
24-Methylcholesta-5,22*E*-dien-3β-ol	440	1.098	1.6
24-Methylcholesta-5,24(28)-dien-3β-ol	440	1.240	1.5
24-Methylcholest-5-en-3β-ol	442	1.242	0.6
24-Ethylcholesta-5,22*E*-dien-3β-ol	454	1.333	4.0
24*S*-Ethylcholest-5-en-3β-ol (clionasterol)	456	1.510	79.9

The composition of the sterol fraction was similar to sterols of other algae of the genus *Caulerpa* that were studied previously. The main sterol of this genus was practically always clionasterol [7].

Three polysaccharide fractions, P1, water-soluble, and P2 and P3, base-soluble, were isolated and characterized from each of the three samples of algae.

Results for compounds of water- (P1-1) and base-soluble (P2-1 and P3-1) fractions from sample 1 are listed below. The protein content in fraction P1-1 was rather high, about 20%. The amino-acid composition of the protein components of fraction P1-1 (amino-acid content expressed in mole percent) was as follows: Asp (13.3), Thr (6.7), Ser (7.2), Glu (18.2), Gly (16.0), Ala (9.1), glucosamine (1.2), Val (5.2), Ile (3.2), Leu (4.3), Tyr (0.9), Phe (1.5), His (0.5), Lys (3.7), Arg (1.6), citrulline (3.5), Pro (3.9). The protein was dominated by Glu, Asp, Gly, and Ala. Proline (3.9) and its precursor citrulline (3.5) are constant components of plant cells [8] and were also present in appreciable amounts.

Fractions were purified of protein by the Sevag method. The ranges of molecular weights of fraction P1-1 and P2-1 polysaccharides that were determined by gel chromatography were 20-60 and 20-40 kDa, respectively. Fraction P3-1 consisted of higher molecular weight polysaccharides with MW >70 kDa. A study of the monosaccharide composition showed that all fractions of sample 1 consisted of Glc, Gal, Man, and Xyl in different proportions (Table 2). Glucose dominated the monosaccharide composition of all these fractions.

The ^{13}C NMR spectrum of fraction P1-1 contained resonances characteristic of 1,4-α-D-glucan (amylose) with C1 (101.21 ppm), C2 (72.96), C3 (74.91), C4 (78.99), C5 (72.96), C6 (62.29), and 1,3-β-D-glucan (laminaran) with C1 (104.08 ppm), C2 (74.9), C3 (86.1), C4 (69.85), C5 (77.3), C6 (62.47) [9]. 1,3-β-D-Glucan has been observed previously only in *Caulerpa simpliciuscula* [10]. Resonances characteristic of 1,4-α-D-glucan dominated the ^{13}C NMR spectra of fractions P2-1 and P3-1.

IR spectra of water- and base-soluble polysaccharide fractions isolated from *C. lentillifera* (sample 1) lacked absorbances at 1230-1255 cm^{-1} and 815-850, which are typical of sulfate groups (spectra are not shown). For example, polysaccharides containing sulfates have been isolated from *Ulva rigida*, *Spongomorpha indica*, *C. racemosa*, and *C. dwarkense* [1, 11, 12].

Water-soluble fraction P1-2 was isolated from *C. lentillifera* grown in mariculture (sample 2, yield about 2% of the defatted alga weight) and consisted of practically only protein. The sugar content according to the phenol—H_2SO_4 method was less than 0.1% of the fraction weight. Monosaccharides also were not found in this fraction after hydrolysis. Base-soluble fractions P2-2 and P3-2, like sample 1, contained amylose according to ^{13}C NMR spectroscopy.

Three fractions P1-3, P2-3, and P3-3 were also isolated and characterized from wild green alga *C. sertularioides* (sample 3). Analysis of the monosaccharide composition of fraction P1-3 showed predominantly galactose and to a lesser extent glucose (Table 2). The ^{13}C NMR spectrum of fraction P1-3 showed strong resonances with chemical shifts 102.6, 79.3, 77.1, 74.6, 70.4, and 68.7 ppm. Furthermore, weaker resonances corresponding to 1,4-α-D-glucan were also present. The ^{13}C NMR spectra suggested that fraction P1-3 contained acetyls (21.71 ppm CH_3; 175.92 ppm C=O).

TABLE 2. Properties of Polysaccharides from Fractions P1, P2, and P3 from Green Algae *Caulerpa lentillifera* and *C. sertularioides*

Fraction	Yield, %*	MW, kDa	Monosaccharide composition, mole fractions			
			Glc	Gal	Man	Xyl
P1-1	2.7	20-60	1	0.2	0.2	0.1
P2-1	4.1	20-40	1	0.1	0.07	0
P3-1	3.5	>70	1	0.3	0.2	0.2
P1-3	1.0	-	0.3	1	0.4	0.2
P2-3	0.8	-	1	0.5	0.2	0.3
P3-3	2.3	-	1	0.2	0	0

*Yield determined as percent of defatted alga dry weight.

Deacetylation, despite the loss of information about the position of the acetyls, is often used to make NMR spectra of polysaccharides simpler and easier to interpret [13]. Deacetylation of fraction P1-3 followed by dialysis on a centrifugal membrane separated a low-molecular-weight 1,4-α-D-glucan and produced fraction P1(1)-3 in 44% yield that contained a polysaccharide consisting mainly of galactose. The IR spectrum of fraction P1(1)-3 exhibited absorption bands at 1252 cm^{-1} (S=O stretch) and at 818 (C–O–S bending) that are characteristic of sulfates that could be located on either C2 or C6 of galactose (spectra not shown) [14]. Resonances in the ^{13}C NMR spectrum of this polysaccharide were assigned using COSY and HSQC 2D correlation spectra as 102.6 ppm (C1), 79.3 (C2), 77.1 (C3), 68.7 (C4), 74.6 (C5), and 70.4 (C6) [9]. The anomeric proton H_a bound to C1 resonated at 4.63 ppm with SSCC J = 7.3 Hz; H_b, at δ 4.38 ppm. This was indicative of the β-configuration of the glucoside bond with C2 substituted by SO_3. The chemical shift of C6 of 70.6 ppm was consistent with glycosylation of the CH_2OH of the β-glycoside bond. This was confirmed by ^{13}C NMR data reported for sulfated 1,3;1,6-β-D-galactans [15]. Thus, the studied polysaccharide was a 1,3;1,6-β-D-galactan sulfated at the galactose C2 atom.

Resonances typical of a 1,4-α-D-glucan dominated ^{13}C NMR spectra of fractions P2-3 and P3-3. Fraction P2-3 contained a small impurity of 1,3;1,6-β-D-galactan sulfated at the galactose C2, which was the main component of fraction P1-3.

Thus, the sterol composition of *C. lentillifera*, regardless of the cultivation conditions, consisted mainly of clionasterol. According to the literature, this is typical for algae of the genus *Caulerpa*. The polysaccharide composition of the studied species grown in mariculture lacked sulfated polysaccharides. Sulfated 1,3;1,6-β-D-galactan was observed only in wild *C. sertularioides*. A small amount of 1,3-β-D-glucan, the main reserve polysaccharide of brown and diatomaceous algae [16], was observed in *C. lentillifera* grown under laboratory conditions. *C. lentillifera* is rich in water-soluble proteins, the amino-acid composition of which is rather variable.

EXPERIMENTAL

Isolation of Sterol Fraction. Moist alga (3.0 g dried alga) was extracted with ethanol at room temperature (3 × 200 mL). The ethanol extract was evaporated to dryness in vacuo (500 mg), dissolved in water, and extracted with butanol (3 × 50 mL). The butanol extracts were combined, evaporated to dryness (100 mg), and chromatographed twice over a silica gel column using $CHCl_3$ to produce the sterol fraction (9 mg, 0.3% of dry alga weight) that was pure by TLC.

Polysaccharide Extraction [14]. Green alga *C. lentillifera* that was defatted with ethanol and ground (samples 1 and 2, 3.2 and 12.8 g, respectively) and *C. sertularioides* (sample 3, 12.0 g) were extracted with water (200, 900, and 800 mL, respectively) at 80°C for 30 min. The procedure was repeated three times. The total extract was lyophilized after evaporation, dialyzed, and precipitated with ethanol (1:4) to afford water-soluble fractions (P1) from the three alga samples P1-1 (87 mg), P1-2 (38 mg), and P1-3 (120 mg), respectively.

The remaining alga was extracted with KOH solution (1 M) containing $NaBH_4$ (20 mM) at 37°C (4 h) and then at 4°C for 17 h. The resulting extract was neutralized with AcOH (until the pH was 6.0), dialyzed by ultrafiltration using Millipore-1 kDa membranes (Millipore, USA). Base-soluble fractions (P2) from the three alga samples, P2-1 (130 mg), P2-2 (78 mg), and P2-3 (99 mg) were lyophilized.

The remaining alga was treated with KOH solution (4 M) containing $NaBH_4$ (20 mM) and extracted analogously. The yields of base-soluble fractions (P3) from the three alga samples were P3-1 (113 mg), P3-2 (315 mg), and P3-3 (272 mg), respectively.

Purification from protein of fraction P1-1 (87 mg in 2 mL) was carried out by the Sevag method [17].

Deacetylation of polysaccharides of fraction P1-3 (100 mg) was carried out by treatment with aqueous ammonia solution (12%) for 14 h at 37°C. The resulting precipitate was removed by centrifugation. The supernatant was dialyzed by ultrafiltration using Millipore-10 kDa centrifugal membranes (Millipore, USA) to afford two fractions, fraction P1(1)-3 above the membrane (44 mg) and fraction P1(2)-3 under the membrane (21 mg).

Molecular weights of polysaccharides were estimated by gel-filtration over a Superdex 75-HR 10/30 column (1.0 cm × 30 cm) (Amersham Pharmacia Biotech AB, USA) equilibrated with phosphate buffer (pH 7.2) containing NaCl (0.15 M) and eluted by the same buffer at 0.4 mL/min. The standards were dextrans with molecular weights 10, 20, 40, and 80 kDa.

Neutral sugars were determined by the phenol-H_2SO_4 method [18].

Protein content was determined by the Bradford method [19].

Amino-acid composition of proteins in water-soluble fraction P1-1 were determined after hydrolysis of the fraction by HCl (6 M) at 100°C (24 h) in a Biochrom-30 analyzer (200 × 4.6, Ultropac-8 μm [Li^+]). The ratio of amino acids was determined by comparing peak areas of the samples with those of a standard mixture of amino acids (Sigma, USA).

Monosaccharide Composition of Polysaccharides. Fractions of polysaccharides (5 mg each) were hydrolyzed by TFA (2 M) at 100°C (4 h). The monosaccharide compositions of the fractions were determined on an IC-5000 Biotronik carbohydrate analyzer (Germany, Shim-pack ISA-07/S2504 column, 0.4 × 25 cm) using potassium borate buffer at elution rate 0.6 mL/min. Sugars were detected using the bicinchonate method with a Shimadzu C-R2 AX integration system. Monosaccharides (Rha, Rib, Man, Fuc, Gal, Xyl, Glc) were used as standards.

IR spectra of polysaccharides in films were recorded on a Vector 22 Fourier transform FTIR spectrophotometer (Bruker, Germany) at 4 cm^{-1} resolution.

GC of sterol fraction was performed in an Agilent 6850 chromatograph with an HP-5MS capillary column (30 m × 0.25 mm, Agilent, USA) at 290°C.

GC—MS Analysis. Sterol fractions were acetylated by Ac_2O—Py (1:1) for 16 h at room temperature and analyzed in an Hewlett—Packard HP 6890 mass spectrometer (ionization energy 70 eV, He carrier gas) with an HP-5MS capillary column (30 m × 0.25 mm, Agilent, USA) at 270°C.

Preparative column chromatography of sterol fractions was carried out over KSK silica gel (50-160 μm, Sorbpolimer, Russia).

TLC of sterol fractions was performed on Sorbfil plates with a fixed layer of STKh-1A silica gel (5-17 μm, Sorbpolimer).

^{13}C NMR spectra of polysaccharides were recorded on a Bruker Avance DPX-300 spectrometer (D_2O, operating frequency 75.5 MHz, methanol internal standard, δ_C 50.15 ppm at 60°C).

PMR spectra of sterols were recorded on a Bruker DRX 500 spectrometer ($CDCl_3$, operating frequency 123.8 MHz, Me_4Si internal standard).

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