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SEASONAL VARIATIONS OF THE COMPOSITION AND STRUCTURAL CHARACTERISTICS OF POLYSACCHARIDES FROM THE BROWN ALGA *Costaria costata*

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Seasonal variations of the polysaccharide composition of the brown alga Costaria costata *were studied. It was found that the alga synthesized in April-May a high-molecular-weight (200–800 kDa) low-sulfated heterofucan; in July, primarily a low-molecular-weight (20–300 kDa) sulfated and acetylated galactofucan. A small amount (<0.01% of total alga mass) of laminaran (1,3;1,6-β-D-glucan) was observed in mature alga.*

Key words: brown algae, polysaccharides, fucoidan, alginates, seasonal variations, monosaccharide composition, molecular weight.

Costaria costata [Turn.] Saund is a widely distributed species of brown alga (Laminariaceae), significant reserves of which are located on the coastal shelf of Primorskii Krai [1].

Three types of polysaccharides occur in brown alga. The contents and structures of these vary during the life cycle [2, 3]. Alginic acids from young tissues consist mainly of mannuronic acid. Therefore, they are similar in various alga species. On the other hand, old tissues accumulate alginic acids with a higher or lower content of guluronic acid. This produces species-specific differences in the composition and properties of the alginic acids [4]. Data on the content and structure of fucoidans in brown algae as a function of development stage of the alga are limited [5, 6]. Very little information on the content and structure of polysaccharides related mainly to alginic acids are available for *C. costata* [7, 8]. A study of fucoidans from brown algae has shown that they exhibit various biological activity such as anticoagulant, antiviral, antitumor, and antioxidant [9–11] that is related to structural features of the polysaccharides, to their degree of sulfating, and/or to their molecular weight. The study of seasonal variations of the polysaccharide composition and structure of individual polysaccharides is of practical significance for determining the optimal times for collecting algae in order to standardize preparations of these polysaccharides for use in medicine and the food industry. Herein results from a study of seasonal variations of the polysaccharide composition and structure of individual polysaccharides of *C. costata* are reported.

C. costata is an annual plant with a short vegetative period. It grows most actively from January through April and reaches its maximum size in the first half of summer. Spore-bearing tissue begins to appear in May-June. Ripening of alga zoospores is completed on the shores of Primor′e in June-July, after which they are dispersed. By August the alga thallus is destroyed [12].

We investigated the content and composition of water-soluble fractions (FLM) consisting of fucoidans (F), laminarans (L), and mannuronans (M) and alkaline-soluble fractions (A) of alginic acids and also structural characteristics of individual polysaccharides as a function of plant life stage including the maximum development of the alga (April, FLM_4 and A_4), preparation of alga to spore genesis (May, FLM_5 and A_5), spore setting (June, FLM_6 and A_6), and start of thallus destruction (July, FLM_7 and A_7).

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TABLE 1. Characteristics of Alginic Acids (Fraction A) Isolated from *C. costata* as Functions of Collection Month

Fraction	M_w, kDa	M_w/M_n	M/G	G	M	MM	MG+GM	GG
				%				
A_4	557	2.2	2.63	0.27	0.73	0.67	0.10	0.23
A_5	349	2.1	2.06	0.32	0.67	n.d.	n.d.	n.d.
A_7	714	2.3	1.87	0.35	0.65	0.62	0.06	0.32

M_W is the average molecular weight; M_W/M_n, the scatter; G, L-guluronic acid; M, D-mannuronic acid; MM, MG, MG, and GM, the corresponding dimeric blocks; n.d., not determined.

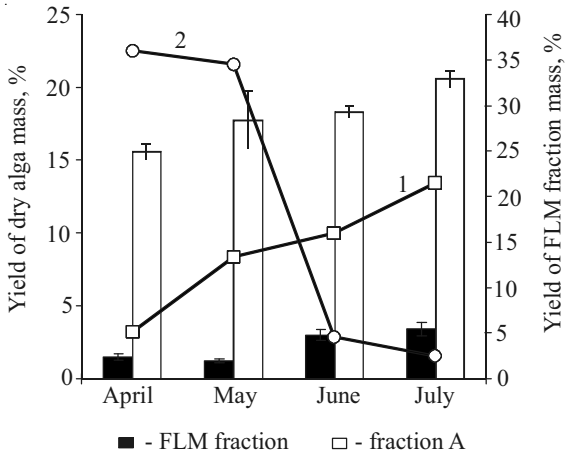

Fig. 1. Yields of fractions A and FLM from *C. costata* (% of dry alga mass); fucoidan (1) and uronan (2) (% of dry FLM fraction); as functions of alga collection month.

Figure 1 shows the change of yield of isolated fractions as a function of alga collection month. The *C. costata* polysaccharides consisted mainly of alginates (fractions A_4–A_7). From April to July, the content of sodium alginate calculated per alga dry weight increased from 15.6 to 20.6%. ^{13}C NMR spectra of these polysaccharides contained strong resonances with chemical shifts 101.2 ppm (C-1), 70.6 (C-2), 72.6 (C-3), 79.2 (C-4), 77.3 (C-5), and 176.1 (C-6) that corresponded with 1,4-bonded β-D-mannuronic acid units and weaker resonances with chemical shifts 66.0 (C-2) and 80.7 (C-4) that were characteristic of 1,4-bonded α-L-guluronic acid units [13]. The season of alga collection had a great effect on the monomer composition of the alginates. PMR spectroscopy showed that alginates from alga collected in April consisted mainly of D-mannuronic acid (M) units. As the alga matured, the fraction of L-guluronic acid (G) increased. The ratio of manuronic and guluronic acids (M/G) varied from 2.63 in the April specimen to 1.87 in the July one (Table 1).

An alginate with M/G 1.55 was previously isolated from *C. costata* collected in August [7]. A decrease of M/G ratio in alginates as a function of age was observed also for other representatives of the family Laminariaceae. The M/G ratios in alginates of young and mature thalli of *Laminaria saccharina* were 1.84 and 0.8, respectively [14]. The ratio of these monosaccharides and the distribution of monomeric units in the polymer chain (MM-, GG- and MG-, GM-blocks) is a very important characteristic of alginic acids that largely determines their physicochemical properties and biological activity. It has been found that alginates constructed primarily of polymannuronic blocks exhibit an antitumor effect [15, 16]. Alginates containing primarily polyguluronic blocks exhibit biosorptive properties [17].

Alginates with a low M/G ratio form a more solid gel; with a high ratio, more elastic [18, 19]. A gel does not form if the content of L-guluronic acid in the polysaccharide is <20–25%. The strength of the gel from alginates with similar M/G ratios increases linearly with increasing molecular weight of the polysaccharide [20]. The molecular weights of alginates extracted by us from alga collected in April through July were in the range 349–714 kDa (Table 1). Alginates with molecular weights from 185 to 300 kDa were isolated previously from Primor′e *C. costata* [3]. The difference in the molecular weights might be explained by differences in the analytical methods or the different times of alga collection. Alginates with molecular weights of 544 and 949 kDa, which were similar to the values found by us, were isolated from the algae *Laminaria digitata* and *L. hyperborea* [21].

TABLE 2. Content and Characteristics of Fucoidans (F) from Fractions of Water-Soluble Polysaccharides (FLM) Isolated from *C. costata* as Functions of Collection Month

Fraction	Fucoidan, %*	M_w, kDa	SO_3Na, %*	Neutral monosaccharides, mol%					
				Fuc	Gal	Man	Rha	Xyl	Glc
FLM_4**	5.0	n.d.	2.3	47.5	13.0	18.2	9.2	10.1	2.0
FLM_5	13.4	200–800	5.3	47.4	13.1	18.2	13.2	6.4	2.2
FLM_6	16.0	n.d.	9.0	53.6	16.6	9.1	5.4	9.3	6.0
FLM_{6fert}	28.3	n.d.	17.2	65.2	17.9	3.8	2.4	2.2	8.5
FLM_7	21.5	20–300	12.3	61.7	17.9	4.8	3.7	4.4	7.4

*, % of FLM fraction weight; **, alga collection month; n.d., not determined.

Thus, mature *C. costata* synthesizes in July significant quantities of high-molecular-weight alginate capable of forming gel. On the other hand, alginates giving less viscous solutions enriched in MM-blocks can be isolated in April and May from the alga. Alginates isolated from *C. costata*, in contrast with most brown algae, gave colorless solutions. This in addition to the high yield made *C. costata* alginate more preferred for use in medicine and the food industry.

The yields of water-soluble polysaccharide fractions (FLM_4–FLM_7) from *C. costata* were low and increased during April through July by 1.5–2 times (Fig. 1). The fraction of uronan in these fractions decreased from 36.0 to 2.5%; that of fucoidan increased from 5.0 to 21.5%. The ^{13}C NMR spectrum of FLM_4 from young alga was dominated by resonances with chemical shifts 101.2 ppm (C-1), 71.3 (C-2), 72.7 (C-3), 79.3 (C-4), 77.2 (C-5), and 176.4 (C-6), which were characteristic of polymannuronic acid [22]. A resonance with chemical shift 17.08 ppm was typical of α-L-fucans and was insignificant. The molecular weight of FLM_4 was in the range 430–465 kDa. Thus, water-soluble polysaccharides of the alga in April were practically pure polymannuronic acid. This agreed well with the literature [22]. Fraction FLM_5 also contained significant quantities of polymannuronic acid. Mannuronan (fraction M_5) with molecular weight 165–220 kDa and a ^{13}C NMR spectrum characterstic of this class of polysaccharides precipitated from a solution of FLM_5 at pH 2.0 [22]. Fraction FLM_5 in addition to water-soluble mannuronan contained significant amounts of fucoidan (Fig. 1).

The fucoidan content in the alga increased by five times as it matured (Fig. 1). Substantial changes in the molecular-weight distribution, the monosaccharide composition, and fucoidan content occurred simultaneously. Vegetative plants synthesized in April and May small amounts of low-sulfated fucoidan with a heterogeneous monosaccharide composition (Table 2). Fucose dominated the monosaccharides (about 50% of the neutral sugars); galactose, mannose, and rhamnose were present in the fucoidan in approximately equal amounts. Their total content was comparable with that of fucose (Fuc:Gal:Man:Rha ratio 1:0.27:0.38:0.19). Xylose and glucose were observed in trace quantities. The fucoidan had molecular weight in the range 200–800 kDa.

The fraction of fucose and galactose (total from 60 to 80 mol%) increased in the polysaccharide from April through July; that of mannose and rhamnose decreased significantly (Table 2). A fucoidan containing these monomers in mole ratio 1:0.29:0.08:0.06 that was actually a galactofucan was isolated from generative plants in June-July. The ratio between fucose and galactose in the fucoidan was practically constant as the alga grew. The fraction of sulfates and polysaccharides in summer specimens increased by more than five times compared with the spring specimens (from 2.3 to 12.3% of the FLM fraction weight). The molecular weight of the fucoidan in July specimens decreased noticeably and fell in the range 20–300 kDa. Thus, mature alga synthesized in July a low-molecular-weight fucoidan that was more sulfated and had a less heterogeneous monosaccharide composition than that of spring specimens.

The observed variations in the composition and structure of polysaccharides extracted from *C. costata* could have been related to the start of spore setting in the alga. As noted earlier [23, 2], the physiological and chemical status of algae changes significantly during spore setting. In order to check the hypothesis about the role of spore genesis in the change of composition and structure of water-soluble polysaccharides, we took from June collections of *C. costata* including a mixture of fertile and sterile specimens thalli with mature sporangiae and isolated from them a fraction of water-soluble polysaccharides (fraction FLM_{6fert}). Comparative analysis of FLM_{6fert} and FLM isolated from algae of other collections (Table 2) showed that the maximum accumulation of fucoidan occurred during formation of reproductive organs. Our observations agreed well with

those obtained previously for *Alaria fistulosa* [24] in which the highest amount of fucoidan accumulated in reproductive organs, sporophyllae. An increase of fucoidan level during maturation of sporangiae was also observed in *L. japonica* [5]. Comparison of the monosaccharide composition of the fucoidans of FLM_5 and FLM_{6fert} (Table 2) showed that the dominant monosaccharides in the fucoidan became fucose and galactose with the constant mole ratio 1:0.27–0.29 as the plants transitioned to spore setting. The content of sulfates in the fucoidan increased. The change of monosaccharide composition, increased sulfate content, and decreased fucoidan molecular weight as the soruses matured (Table 2) suggested that not only the content but also the structure of this polysaccharide were related to its physiological role in the development of the reproductive organs and the spore yield.

The laminaran content increased in *C. costata* as it grew. This could be estimated from the yield of glucose upon acid hydrolysis of the water-soluble polysaccharides (FLM). From May to July the laminaran content increased by 3.5 times (Table 2) but did not exceed 1% of the whole fraction (0.01% of the dry alga weight). The most laminaran was observed in July in mature alga.

Thus, the content of water-soluble polysaccharides in *C. costata* increased from April to July, reaching a maximum during spore setting of the alga. Consequently, this period is optimal for collecting alga in order to isolate these polysaccharides. Therefore, we selected extracts obtained from July specimens for further study. Fraction FLM_7 was fractionated over a column of hydrophobic carrier in order to isolate larminaran. Water eluted fucoidan (FM_7) that made up 94% of the initial fraction. The ^{13}C NMR spectrum of the fucoidan exhibited a resonance at 16.9 ppm that was characteristic of the CH_3 group of fucopyranose. Resonances at 21.66 and 173.9 ppm indicated that the polysaccharide contained *O*-acetyl groups. A resonance at 62.56 ppm was consistent with hexapyranoses with unsubstituted CH_2OH groups; at 176.06, a carbonyl group. This suggested that the sample contained uronic acids. The IR spectrum of FM_7 showed an absorbance at 1264 cm^{-1} that was characteristic of an S=O bond [25]. Absortion bands at 823 cm^{-1} suggested that sulfates were present in the fucoidan mainly in the equatorial position on C-2 or C-3 of the hexoses. A shoulder at 846 cm^{-1} indicated that a small quantity of sulfates were located in the axial position on C-4. Thus, the fucoidan from *C. costata* that was isolated from July alga was sulfated primarily on C-2 and C-3 and was an acetylated galactofucan.

Fraction FL_7 was eluted by 15% aqueous alcohol and contained approximately equal amounts of laminaran and fucoidan. The laminaran (L_7) was separated from the fucoidan on a membrane with retention limit 10 kDa. A study of the structure of L_7 showed that it was a typical representative of 1,3;1,6-β-D-glucans [26]. The ^{13}C NMR spectrum of this fraction showed resonances with chemical shifts 103.9 (C-1), 74.6 (C-2), 85.6 (C-3), 69.5 (C-4), 77.3 (C-5), and 62.1 (C-6) ppm that were characteristic of 1,3-bonded β-D-glucopyranose and weaker resonances with chemical shifts 70.1 (C-6), 75.9 (C-5), and 71.0 (C-4) that corresponded to 1→3,6- and 1,6-bonded glucoses, respectively [26]. The ratio of the 1,3- and 1,6-bonded β-D-glucopyranoses was 5:1. The spectrum also contained a minor resonance with chemical shift 64.5 ppm that was characteristic of mannite. The molecular weight of laminaran from *C. costata* was 4.4 kDa. Laminarans are considered low-molecular-weight polysaccharides. Their molecular weights are usually less than 5–6 kDa. A high-molecular-weight laminaran was observed in algae *F. evanescens* and *L. cichorioides* found under sterile conditions. However, fertile algae of these same species contained laminarans with the usual molecular weights [22].

Thus, our investigations found that the highest amounts of laminaran, fucoidan, and alginate occurred in mature algae collected in June-July. However, algae can be collected at a different time of year in order to obtain fucoidans and alginates with a different structure and, therefore, with different biological properties. Thus, *C. costata* should be collected in May in order to isolate an alginate enriched in mannuronic acid; in the summer months, a low-molecular-weight sulfated and partially acetylated galactofucan and a low-molecular-weight alginate enriched in guluronic acid.

EXPERIMENTAL

We used fresh alga *C. costata* collected from April to July in 2007 and 2008 in Peter the Great Bay, Sea of Japan.

The content of carbohydrates was determined using the phenol–H_2SO_4 method [27]; of sulfates in the polysaccharides, a turbidimetric method [28]; of uronic acids, spectrophotometrically (Cecil-2021 spectrophotometer, England) by reaction with 3,4-dimethylphenol and H_2SO_4 [29] using D-glucurono-3,6-lactone (Sigma, USA) as a standard; of fucoidan, spectrophotometrically by the literature method [30].

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

PMR spectra were recorded on the same instrument (acetone internal standard, δ_H 2.233 ppm at 70°C). The ratio of integrated intensities of resonances of anomeric protons were calculated as before [31]. IR spectra were recorded on a Vector 22 Fourier-transform FTIR spectrometer (Bruker, Germany) in KBr disks with 4 cm^{-1} resolution.

Extraction of Polysaccharides. Thalli of algae were extracted twice with ethanol for 3 h at 40°C (alga:ethanol 1:0.8 w/w) to remove low-molecular-weight compounds. Then algae were dried in air and in vacuo, ground to particle size <1 mm, and extracted for 3 h twice with HCl (pH 2.0–2.3) at 60°C (ratio 1:20). The extracts were combined, concentrated to one fifth the volume, neutralized with aqueous $NaHCO_3$ solution (3%) until the pH was 5.7–6.1, dialyzed against 10 volumes of distilled water, and lyophilized to obtain FLM preparations. The solid alga was again extracted successively with 3 and 1.5% aqueous Na_2CO_3 solutions (1 L) for 8 h at 60°C and rinsed with five volumes of water. The extracts and rinsings were combined, concentrated, and dialyzed in an ultrafiltration apparatus using a membrane with retention limit 100 kDa (Biotest, Russia). The concentrate was evaporated in a rotary evaporator to 3L. Polysaccharide was precipitated by one volume of ethanol. The solid was washed twice with ethanol and dried in vacuo. The resulting polysaccharide was dissolved in water (500 mL). The pH was adjusted to 2 using HCl (12%). The resulting precipitate of alginic acid was separated by centrifugation for 10 min at 10,000 rpm and 15°C. The solid was dissolved in a small amount of water with careful addition of solid NaOH until the pH was 8.6. The solution was dialyzed and lyophilized to afford preparation A.

Total acid hydrolysis of the polysaccharides (5 mg) was carried out using trifluoroacetic acid (2 M, 0.5 mL, 8 h, 100°C). The acid was distilled under vacuum with ethanol. Partial hydrolysis of the alginate samples for PMR analysis was performed as before [32].

The monosaccharide composition of the polysaccharides was determined in an Biotronik IC-5000 carbohydrate analyzer (Germany) using a Shim-pack ISA-07/S2504 column (0.4 × 25 cm, potassium borate buffer, flow rate 0.6 mL/min). Monosaccharides were detected using a bicinchoninate method. The standards were monosaccharides (Rha, Rib, Man, Fuc, Gal, Xyl, Glc).

Hydrophobic Chromatography. A weighed portion (1.34 g) of FLM_7 was dissolved in water (100 mL) and placed on a column of Polikhrom-1 (15 × 200 mm). The column was eluted successively with distilled water (70 mL) and aqueous ethanol (15%, 55 ml). Fractions (7 mL) were collected. Fractions containing polysaccharides were concentrated in a vacuum evaporator and lyophilized to afford polysaccharides (1.26 g) eluted by water (fraction FM_7, 94% of total polysaccharides) and eluted by aqueous ethanol (63 mg, fraction FL_7, 4.7%). Laminaran (L_7) was separated from a fucoidan impurity by ultrafiltration on an NMWL 10000 membrane (Millipore, USA).

Molecular weights of polysaccharides were determined by HPLC on a Shimadzu LC-20A instrument (Japan) with an RID-10A refractometric detector. Alginate samples were dissolved in $NaNO_3$ (0.2 M); fucoidans, in doubly distilled water and filtered through a Kurabo 25A membrane filter (Japan) of pore size 0.45 μm. Alginates were separated over a Shodex Asahipak GS-520 HQ column (7.5 mm × 300 mm) (Showa Denko, Japan) at 40°C with elution by $NaNO_3$ solution (0.2 M, 0.8 mL/min); fucoidans, over successively connected Shodex Asahipak GS-520 HQ and Shodex Asahipak GS-620 HQ (7.5 mm × 300 mm) (Showa Denko, Japan) columns at 50°C with elution by H_2O (0.8 mL/min). The columns were calibrated using a set of Shodex Standard P-82 pullulan standards (Showa Denko, Japan) with molecular weights 667 kDa and 180 Da (Polymer Laboratories, USA) and dextran blue (Amersham, Sweden).

Statistical processing of data used the Excel program.

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