Polysaccharides of Algae
58.* The polysaccharide composition of the Pacific brown alga *Alaria fistulosa* P. *et* R. (Alariaceae, Laminariales)**

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Quantitative determination of mannitol, fucoidan and alginate in different parts of the thallus (the blade, the midrip, the sporophylls, and the stipe with rhizoids) of the brown alga *Alaria fistulosa*, collected from the coastal waters of Paramushir Island, was carried out. Sporophylls were shown to differ considerably in carbohydrate composition from all other parts of the plant. In particular, the fucoidan content in sporophylls is several times as high as that in the blade. Alginate preparations isolated from sporophylls and from blade have different proportions of monomers (mannuronic acid and guluronic acid residues) and different molecular masses. Fucoidan preparations isolated from these parts of the plant differ slightly in the sulfate content. Their specific feature is a rather high galactose content comparable with the content of fucose. The sporophylls of *A. fistulosa* may be regarded as a rich source for practical isolation of fucoidan.

Key words: *Alaria fistulosa*; brown algae; alginic acid; fucoidan; mannitol; sporophylls.

In our previous communication,[2] we described the polysaccharide composition of a number of the most frequently encountered brown algae of Kamchatka. It was noted that the sporophylls of *Alaria marginata* (a representative of the family Alariaceae, the order Laminariales) contain much more fucoidan than other parts of the plant. Subsequently,*** a similar result was obtained for a sample of the same species gathered at the coast of the Shikotan island. There are rather limited data in the literature (concerning mainly alginic acids) on the differences in the chemical composition of different parts of thalli of the brown algae. It was found[3] that alginates of younger tissues are mainly composed of mannuronic acid residues and therefore they are similar in different species of algae. Conversely, older tissues accumulate alginates with a higher or lower content of guluronic acid, which is responsible for well-known species differences in the composition and properties of alginates.[4] The alginates isolated from stipe and rhizoids often differ from the alginates present in blades in the ratio of mannuronic to guluronic acid residues (the M/G value). Low ratios (corresponding to higher contents of guluronic acid) in the alginates from stipe and rhizoids are attributed to higher strength of the gels formed by these alginates, which ensures higher strength of the corresponding tissues.[5] To our knowledge, no data of this type are available for *Alaria* species. For a representative of the same family *Undaria pinnatifida*, it was found that sporophylls contain less alginates than the blade and midrib; however, these results were not compared with the amounts of fucoidan in the same thallus parts.[6,7] In the present work, we studied the carbohydrate composition of the Pacific alga *Alaria fistulosa* P. *et* R. Representatives of this species are large perennial plants with a high rate of growth, which form substantial accumulations suitable for industrial processing in some areas of the Pacific coast of Russia. This alga is also considered to be promising for sea farming.[8]

Results and Discussion

For analysis we chose a biannual sample of the alga from the typical habitat (the coast of the Paramushir island, Kuril isles). After drying, the thallus was separated into four parts (the blade, the midrib, the sporophylls, and the stipe with rhizoids, Fig. 1). It was found that the blade, the midrib, and the stipe with rhizoids gave approximately equal yields of the dry material, whereas the weight of sporophylls was almost 1.5 times as large as that of all other parts of the plant altogether.

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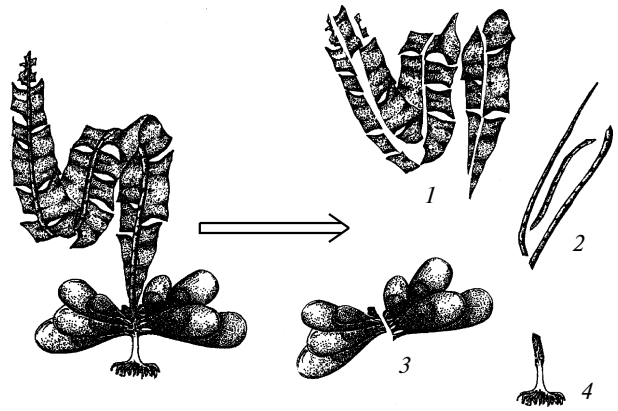

Fig. 1. Separation of the *Alaria fistulosa* thallus for comparative analysis of the carbohydrate composition in parts of the plant: blade (*1*), midrib (*2*), sporophylls (*3*), and stipe with rhizoids (*4*).

To determine the content of mannitol, we subjected the biomass to exhaustive extraction with acidified aqueous ethanol followed by acetylation and quantitative GLC, by analogy with the procedure[9] we used to analyze samples of brown algae from Kamchatka. The content of fucoidans and alginates was determined using the spectrophotometric procedure described in detail in our previous publication.[2] The results are summarized in Table 1.

The content of mannitol in the midrib (see Table 1) is 2—3 times as high as in other parts of the plant. Previously,[9] a similar result has been obtained for another species of the same genus *A. marginata*. In all probability, the midrib serves for the transport of this readily soluble reserve metabolite, formed in the blade as a primary product of photosynthesis, along the thallus.

Spectrophotometric determination of fucoidan in acidic extracts of particular parts of the alga has shown that the blade, the midrib, and the stipe contain relatively small and approximately equal amounts of this polysaccharide, whereas its content in sporophylls is about an order of magnitude higher. Acid hydrolysis of particular parts of the thallus followed by GLC determination of neutral monosaccharides as alditol acetates also attested to the predominance of fucoidan in sporophylls. However, as regards the content of laminaran, which can be estimated from the yield of glucose upon acid hydrolysis, the parts of thallus barely differed from one another. Spectrophotometric determination of alginates in alkaline extracts of the biomass has shown high content of this polysaccharide in the blade and the midrib, somewhat lower content in sporophylls (apparently, due to the increased content of fucoidan in this part of the plant), and even lower content in the stipe, which can be attributed to the presence of acid-resistant polysaccharides nondetectable by the analytical methods employed.*

It follows from the data of Table 1 that the sporophylls differ appreciably from other parts of the plant mainly in the presence of a large amount of fucoidan. To analyze this difference more comprehensively, we isolated polysaccharides by fractional extraction, separately from the blade and the sporophylls. The extraction procedure included treatment of the biomass with a dilute aqueous solution of calcium chloride with moderate heating to solubilize readily soluble polysaccharides (fucoidan and laminaran), whereas alginic acids are retained under these conditions in the biomass as insoluble calcium salts. Subsequently the biomass was treated with dilute hydrochloric acid to remove calcium cations and the alginic acids were extracted as sodium salts with an aqueous solution of sodium carbonate. The highly colored sodium carbonate extracts were decolorized by treatment with bromine,[2] and alginic acids were precipitated by acidification. This protocol gave five polysaccharide preparations A—E from each biomass sample (see Experimental); the yields and the compositions of the preparations are listed in Table 2. Of most interest are preparations A and D containing fucoidan and alginates.

* It is worth noting that GLC analysis of neutral monosaccharides in acid hydrolyzates of the biomass as alditol acetates does not imply separate determination for mannose and mannitol. The mannose + mannitol sums given in the corresponding column of Table 1 do not differ much from the results of direct determination of mannitol in the midrib or the sporophylls but do differ considerably from those found in the blade and the stipe. In the latter case, the content of the substance that gives mannose upon hydrolysis is especially high, which accounts for the lower content of the alginate in the stipe compared to other thallus parts found by spectrophotometry.

Table 1. Carbohydrate compositions of parts of the thallus of the brown alga *Alaria fistulosa*

Part of the plant	Yield	Mannitol	Fucoidan	Alginate	Neutral monosaccharides in the acid hydrolyzate, % of the biomass weight				
	%				Fucose	Xylose	Mannose + mannitol	Glucose	Galactose
Blade	13.2	4.6	0.7	27.5	2.1	0.2	6.9	1.0	1.1
Midrib	16.5	11.9	0.6	29.5	1.0	0.1	11.0	1.0	0.5
Sporophylls	58.7	6.4	7.8	21.4	4.2	0.1	6.2	1.0	3.0
Stipe	11.6	5.6	0.5	14.1	1.0	0.2	18.1	1.0	0.4

Table 2. Yields and composition (content of neutral monosaccharides in % of the total weight) of the polysaccharide preparations obtained on successive extraction of the *A. fistulosa* blade and sporophylls

Preparation	Yield (%)	Fucose	Xylose	Mannose	Glucose	Galactose
			Blade			
A	2.2	14.7	1.5	3.8	2.9	7.2
B	1.7	0.6	—	4.9	0.8	—
C	1.4	5.1	0.8	10.3	1.8	1.5
D	18.4	0.3	—	8.2	1.2	—
E	4.2	7.2	1.2	6.3	2.1	3.9
			Sporophylls			
A	14.5	20.6	0.3	1.5	1.6	19.0
B	2.9	0.8	—	11.1	1.6	0.6
C	0.9	2.5	0.2	3.1	2.2	2.2
D	6.1	0.4	—	5.2	0.7	0.5
E	3.7	8.3	0.5	5.6	1.8	4.7

As regards the sum of water-soluble polysaccharides (see Table 2), the blade and the sporophylls almost do not differ from each other, whereas the yields of some polysaccharide preparations are markedly different. As was to be expected in view of tentative data, the yield of fucoidan from the blade was relatively low, sodium alginate being the major polysaccharide. Conversely, a fucoidan preparation was obtained as the main polysaccharide material upon extraction of sporophylls, whereas the preparative yield of the alginate is rather low in this case. The other three preparations (B, C, and E) obtained from each biomass sample in minor yields contain, judging by their monosaccharide composition, some fucoidan mixed with polysaccharides of different nature. These were not studied here in more detail.

Two alginate preparations, those from the blade and from sporophylls, differed not only in the yield upon extraction, but also in the properties. The alginate from the blade forms colorless viscous solution, which indicates a high molecular mass of the polysaccharide. Conversely, in the case of alginate from sporophylls, the decoloring procedure used was inefficient and the solution viscosity (and correspondingly the molecular mass of the polysaccharide) was much lower than that of the blade alginate. The weight-average molecular masses calculated from viscometric data by a known method[10] were 35000 and 20000 for alginates from the blade and sporophylls, respectively. The ^{13}C NMR spectra of both preparations were typical of brown algal alginates.[11] It follows from these spectra that the substances differ substantially in the ratio of β-D-mannuronic (M) and α-L-guluronic (G) acid residues. The M/G values found from the NMR spectra are 1.89 and 0.76 for the blade and sporophyll alginates, respectively.

The fucoidan preparations obtained from the blade and from the sporophylls had similar compositions despite different yields. To characterize these preparations in more detail, we studied their behavior in anion exchange chromatography. This method has been repeatedly used previously to compare fucoidans obtained from different brown algal species,[12] different anion exchangers being used for the separation. By analogy with our previous works,[1,13] we carried out fractionation on a DEAE-Sephacel column using stepwise elution with sodium chloride solutions with increasing concentrations. The yields and the composition of the resulting fractions are listed in Table 3.

The degree of sulfation of molecules is the principal factor determining the behavior of fucoidans in the anion exchange chromatography. Both preparations to be separated are nonuniform as regards this feature, giving three main fractions eluted with 0.5, 1.0, and 1.5 *M* NaCl solutions, respectively. The ratio of these fractions indicates that the average degree of sulfation of the preparation obtained from sporophylls is somewhat higher than that of the preparation from the blade. The content of the

Table 3. Characteristics of fractions obtained by anion exchange chromatography of preparations A containing fucoidan and isolated from the blade and the sporophylls of *A. fistulosa*

Fraction	Elution (*M* NaCl)	Yield (%)	Neutral monosaccharides in the acid hydrolyzate, mol per mole of fucose					Uronic acids	SO_3Na
			Fucose	Xylose	Mannose	Glucose	Galactose	%	
					Blade				
1	0.5	33.3	1.00	0.29	0.35	0.20	0.06	11.6	7.8
2	1.0	45.6	1.00	0.13	0.16	0.11	0.37	10.4	16.6
3	1.5	21.1	1.00	0.06	0.05	0.08	0.84	3.9	23.0
					Sporophylls				
1	0.5	9.3	1.00	0.22	0.38	0.37	0.30	7.2	14.5
2	1.0	46.7	1.00	0.06	0.07	0.05	0.67	4.0	19.7
3	1.5	40.0	1.00	0.03	—	0.02	0.89	2.0	24.6
4	2.0	4.0	1.00	0.19	0.03	0.05	0.86	1.5	19.6

minor monosaccharides (xylose, mannose, glucose) and uronic acids decreases on passing from fractions *1* to fractions *3*, whereas the contents of the sulfate and galactose increases. Thus, fractions *3* can be considered to be the best purified fucoidan samples. The compositions of these fractions obtained from the two preparations are fairly similar. Their characteristic feature is a high content of galactose, comparable with the fucose content. It is noteworthy that fucoidan with a similar composition containing approximately equal amounts of fucose and galactose and exhibiting an antiviral activity has been isolated recently[14] from the sporophylls of *Undaria pinnatifida*. Fucoidans from other brown algae often contain galactose but, as a rule, as a minor component. The position of this monosaccharide in the fucoidan molecules is usually unknown. In rare cases, it is possible to separate fucoidans into two fractions, one consisting almost entirely of fucose and sulfate, whereas the other concentrating, in addition to fucose, other monosaccharides typical of fucoidans such as galactose and glucuronic acid.[15,16] In only one case, thorough fractionation of fucoidan resulted in the isolation of a sulfated galactan with a unique structure, however, in a very low yield.[17] Xylose, mannose, glucose, and uronic acids are often detected in minor amounts in fucoidans isolated from other brown algae.

To summarize the observations, one can conclude that sporophylls and other parts of the thallus of alga *A. fistulosa* differ appreciably in the polysaccharide composition. A specific feature of sporophylls is accumulation of large amounts of fucoidan. This fact can be considered together with the previously reported increase in the fucoidan content in *Laminaria japonica* upon the formation of sporangia.[18] It is quite probable that high amounts of fucoidan are needed for normal functioning of sporogenous tissues. From the practical standpoint, it is expedient to utilize the *A. fistulosa* biomass using separately sporophylls to obtain fucoidan and other parts of the plant to isolate alginate. Brown algal fucoidans are well known[19] to be of interest as biopolymers endowed with diverse biological activities, although no reliable correlations between the polysaccharide structure and particular biological features have been elucidated so far. Since the unusually high content of galactose residues is a characteristic feature of fucoidan from *A. fistulosa*, it is expedient to use this polysaccharide for elucidating the biological role of this monosaccharide residues in fucoidan molecules. The structural analysis of fucoidan from *A. fistulosa* and study of its biological activity will be discussed in our subsequent publications.

Experimental

GLC analysis was carried out on a Hewlett—Packard 5890A chromatograph equipped with a flame ionization detector and a 3393A integrator using a HP Ultra-2 capillary column, in a flow of nitrogen, and at a temperature rise from 175 to 290 °C at a rate of 10 °C min^{-1}. For acid hydrolysis, 2 *M* trifluoroacetic acid (1 mL) containing *myo*-inositol (0.9 mg mL^{-1}) was added to biomass (20—30 mg) or polysaccharide specimen (10—12 mg) samples, the mixture was heated for 8 h at 100 °C, and the acid was co-evaporated *in vacuo* with ethanol. The transformation of the liberated neutral monosaccharides into alditol acetates and quantitative GLC analysis were carried out by a known procedure.[20] The content of mannitol in the biomass was also determined by GLC after extraction with acidified ethanol and acetylation.[9]

The spectrophotometric analysis of fucoidan and alginate was carried out by a procedure described previously.[2] The sulfate content in polysaccharide was determined by turbidimetry[21] after hydrolysis with 2 *M* trifluoroacetic acid (100 °C, 8 h). The content of uronic acids was found by spectrophotometry upon the reaction with 3,5-dimethylphenol and sulfuric acid[22] using the calibration plot for an authentic sodium alginate (BDH, UK). Quantitative measurements were carried out using a Ultrospec 4050 spectrophotometer (LKB Biochrom). The ^{13}C NMR spectra of alginates were recorded on a Bruker AM-300 instrument, the monomer ratio was calculated as described previously.[11] The viscosities of alginate solutions of different concentrations in 0.1 *M* NaCl were determined in a capillary viscometer and the molecular weights were calculated by a known procedure.[10]

Collection of the alga. A 2-year old alga sample collected in July, 2002 at the coast of the Paramushir island (North Kuril isles) was first dried in air and then *in vacuo* over P_2O_5 to a constant weight and crushed to obtain particles not larger than 0.25 mm.

Isolation of polysaccharides. A suspension of the biomass (10 g) in 2% aqueous calcium chloride (100 mL) was stirred for 6 h at 75 °C. The extract was separated by centrifugation and the precipitate was treated twice under the same conditions. The combined extracts were concentrated, dialyzed and freeze-dried to give preparation A. The alga residue was stirred for 6 h with 0.2 *M* HCl (3×100 mL, once at room temperature and twice at 50 °C), and the acid extracts were concentrated and dialyzed. The precipitate formed upon dialysis was separated by centrifugation, washed with ethanol and acetone, and dried *in vacuo* to give preparation B. The supernatant was freeze-dried to give preparation C. The alga residue was stirred for 6 h at 50 °C with a 3% aqueous solution of Na_2CO_3 (3×200 mL). Bromine (0.85 mL) was added dropwise with stirring to the combined dark-colored extracts and the reaction mixture was left for ~14 h and acidified by concentrated HCl. The precipitated alginic acid was separated by centrifugation, washed with water, and suspended in water (100 mL). For dissolution, solid NaOH was added in portions to obtain a clearly alkaline pH and the solution was dialyzed and freeze-dried to give preparation D. After separating the alginic acid precipitate, the acidic solution was dialyzed and freeze-dried to give preparation E. The yields and the monosaccharide composition of preparations A—E obtained upon the extraction of the blade and sporophylls are listed in Table 2.

Anion exchange chromatography of fucoidans. Preparation A (300 mg) obtained from the blade or from sporophylls was dissolved in 0.1 *M* NaCl (20 mL), the minor insoluble residue was separated by centrifugation and rejected, and the solution was applied onto a DEAE-Sephacel column (Cl^- form, 25×2 cm) equilibrated with 0.1 *M* NaCl. The column was washed succes-

sively with 0.1, 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 *M* NaCl, every time until the phenol—sulfuric acid assay[23] showed the absence of carbohydrates in the eluate. The resulting solutions were dialyzed and freeze-dried. The yields and the compositions of the fucoidan fractions are given in Table 3.

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