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A new procedure for the separation of water-soluble polysaccharides from brown seaweeds

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Abstract

A simple method for the separation and isolation of the water-soluble polysaccharides of brown algae (i.e., laminarans, fucoidans and alginates, respectively) based on hydrophobic chromatography has been developed. In addition, the fractions containing unusually low-sulfated polysaccharides have been isolated by the new procedure.

Keywords: Isolation; Laminaran; Fucoidan; Brown seaweed; Polysaccharide hydrolase

1. Introduction

Fucoidans, laminarans, and alginic acids are the main polysaccharides of brown seaweeds. Fucoidan and laminaran contents vary from 20 to 50% of defatted alga dry weight.

Fucoidans are the family of sulfated homo- and heteropolysaccharides composed mainly of α-(1→2)- and/or α-(1→3)-linked L-fucose residues [1–3]. They can also contain residues of galactose, mannose, xylose and glucuronic acid. Despite a great number of studies on fucoidans, their precise structures remain unclear [1–6]. Fucoidans are nontoxic polyelectrolytes. They possess various pharmacological activities, i.e., antibacterial, antiviral (including anti-HIV), antitumor, immunosuppressive, antipeptic, antilipemic, antigemostatic and anticoagulant [3–5,7–9]. Alginic acids can be used for heavy metal binding, as immunostimulators, etc. [10–12].

The term 'laminaran' describes a group of the reserve, water-soluble (1→3),(1→6)-β-D-glucans with low molecular weight isolated from the seaweeds of Phaeophyta. Laminarans from different sources are well known to vary considerably both in content and structure [1,5,6,13,14]. The study of (1→3),(1→6)-β-D-glucans is of interest taking into account the participation of these substances in animal and plant immunities [15–17].

The abundant supply of algae and the large variety of their species make it possible to exploit the Russian Far-Eastern seaweeds on an industrial scale. The main regions for commercial seaweed harvesting are the coasts of Primorye, Sakhalin and Kuril Islands. During

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seaweed processing, the valuable water-soluble polysaccharides are usually discarded, despite the fact that they may be widely used in the food industry and in medicine. Use of these polysaccharides is restricted for various reasons, including a lack of simple methods for isolating them from extracts. Although several methods for isolation of fucoidans, laminarans and alginic acids are reported, all of them are rather labour intensive, and none allows stepwise separation of the polysaccharides from single source or extract.

The purpose of this study was to investigate the facilities for employment of a hydrophobic sorbent for the separation and isolation of the water-soluble polysaccharides (laminaran, fucoidan, and low-molecular alginic acid). Isolation of these polysaccharides from extracts of the most widely distributed Far-Eastern brown algae (*Laminaria cichorioides*, *Fucus evanescens* and *L. japonica*) was carried out with the procedure reported herein.

2. Results and discussion

To separate nonpolar compounds (pigments, some proteins, polyphenols, etc.) and polysaccharides from *Fucus vesiculosus* extract, hydrophobic chromatography on polystyrene XAD-2 resin was used [4]. This resin did not adsorb polysaccharides.

We have used the hydrophobic resin Polychrome-1 to isolate the water-soluble polysaccharides, the nonpolar and colored compounds from seaweed extracts. This resin was applied some time ago for the isolation of different substances from the marine organism extracts [18].

We discovered that laminaran from extracts of brown algae *L. cichorioides* was adsorbed by Polychrome-1 [19]. In order to study laminaran adsorption to Polychrome-1 and determine the dependence on the structure for binding, various β-D-glucooligo- and polysaccharides obtained from either brown seaweeds or lichen were studied. The species used were 6^3-β-D-glucopyranosyllaminaritriose [20], laminarioligosaccharides (DP 5–7), laminaran from *L. cichorioides* after Smith degradation, laminarans from *L. gurjanovae*, *L. cichorioides* and *F. evanescens* [13,14], translam (a high-molecular-weight and high (1→6)-linked glucose containing (1→3),(1→6)-β-D-glucan produced by enzymatic transformation of the laminarans from *L. cichorioides* [21]) and pustulan from the lichen *Umbellicaria russica* [14,21].

The capacity of Polychrome-1 toward β-D-glucans was determined with laminarans from *L. cichorioides* and *F. evanescens* and averaged about 10 g/kg of sorbent without dependence on the structure. β-D-Glucans of various structures were completely desorbed by 20% aqueous ethanol. The binding strength of Polychrome-1 was increased up to a certain limit with increase of M_w and amount of β-(1→6)-bound residues of glucose in β-D-glucan. Thus, 6^3-β-D-glucopyranosyllaminaritriose, laminarioligosaccharides with DP 5–7 did not bind to the sorbent. β-D-Glucooligo and polysaccharides with a M_w greater than 1.5 kDa were sorbed to resin. The binding strength was greater for species with M_w of 10 kDa and/or content of (1→6)-bound glucose residues in a molecule up to 25–30% and values beyond that remained practically constant (Fig. 1).

Taking into consideration the binding of β-D-glucans on Polychrome-1, a method for separation and isolation of different water-soluble polysaccharides from brown algae extracts was developed (Fig. 2). Ethanol, acetone and chloroform pretreatment of seaweeds allows one to remove different substances including oligosaccharides with DPs up to 10–12 (about 2 kDa). The laminarans from water extracts of seaweed (including the extracts with low content of laminaran [0.1–0.5 mg of laminaran/mL] were almost completely adsorbed to Polychrome-1 (Tables 1 and 2). This method allowed laminarans to be isolated and separated effectively from fucoidans and water-soluble fragments of alginic acids (Fig. 2, Tables 1 and 2). Fucoidans and alginates passed readily through the Polychrome-1 column. Alginic acids and some fucoidan (the data of ^{13}C NMR) were precipitated from the eluent with glacial HOAc according to Ref. [4], thus leaving the remaining fucoidan in the supernatant, which was separated from the EtOH-soluble sub-

stances by precipitation with EtOH. The laminaran (i.e., material retained on the Polychrome-1 column) was eluted with 15–20% EtOH–water almost completely in second void volume. Hence, the concentration of a laminaran can be achieved from volumes greater than the void volume.

The method was evaluated on extracts from the most widely distributed Far-Eastern brown algae (*L. cichorioides*, *F. evanescens* and *L. japonica*) containing different amounts of laminarans (about 10–12, 3–5 and 0.5–1%, respectively) [5,6,13,14]. The protocols of laminaran isolation with new and traditional methods from the same portion of *L. cichorioides* are given in Ref. [19]. The yields of laminarans and fucoidans obtained using the new method appeared to be comparable with the data of a traditional method (separation of fucoidans and alginates as a complex with cetavlon) for the same seaweeds [5,6,13,14,19] (Table 1).

The structures of polysaccharides obtained in various fractions were investigated by increased sugar-reducing capacity of optical density after incubation with various specific enzymes, followed by determination of mono- and oligosaccharide composition by high-performance liquid chromatography (HPLC). Monosaccharide composition and sulfate content were both determined after acid hydrolysis (Table 2). ^{13}C NMR and IR spectroscopic studies were also undertaken.

The presence of certain signals in the ^{13}C NMR spectra (not shown) enabled the composition of various fractions to be qualitatively established [3,6,7,14,15]. So, for fucoidans the signals of C-1 (96–100 ppm), intensive signals of ring C-atoms (about 68 and 72 ppm, also about 73–78 ppm) and signals of C-6 atoms (17–18.3 ppm) are characteristic. The signals of C-1 (100–102 ppm) and C-6 (175–178 ppm) are characteristic for alginic acids. Signals for the substituted C-1 (around 103.0 ppm), C-3 [β-(1→3)] (85–87 ppm), C-5 [β-(1→6)] (75–76 ppm) and C-6 (62 ppm) regions were observed in the ^{13}C NMR spectra of laminarans.

^{13}C NMR spectra of all F-, FL_5-, L_{15}- and A-fractions were obtained excluding L.c.FL_5-1 and L.c.L_{15}-1. As follows from analysis of all these data, F-1 and F-2 fractions contain fucoidans, while A-1 and A-2 fractions contain mixtures of fucoidans and alginic acids in

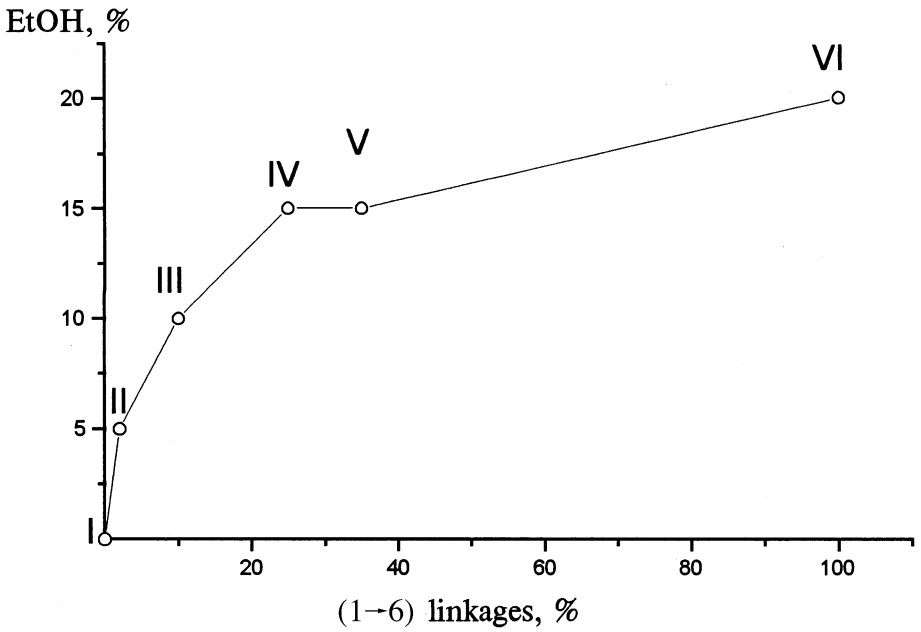

Fig. 1. The dependence of the β-D-glucans sorption to Polychrome-1 on the content of β-(1→6)-linked glucose residues in a molecule. (I) 6^3-β-D-Glucopyranosyllaminaritriose or laminarioligosaccharides (DP 5–7); (II) laminaran from *L. cichorioides* after Smith degradation or laminaran from *L. gurjanovae* (5 kDa); (III) laminaran from *L. cichorioides* (5 kDa); (IV) translam (10 kDa); (V) laminaran from *F. evanescens* (5 kDa); (VI) pustulan [(1→6)-β-D-glucan obtained from the lichen *Umbillicaria russica*, 30 kDa].

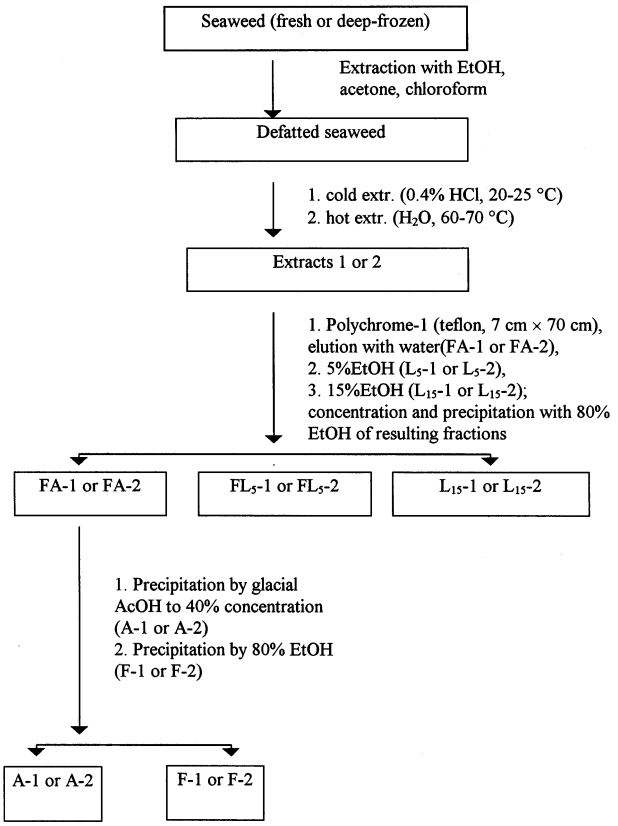

Fig. 2. Scheme for the isolation of water-soluble polysaccharides from brown algae.

contrast to similar fractions obtained by Beress et al. [4] and reported to be alginic acids only. Fucoidans of F-fractions are mainly the high-sulfated ones, but fucoidans from *L. cichorioides* are more high sulfated than the same fractions from *F. evanescens* (Table 2).

The IR spectra of fucoidan fractions under study were similar to the spectra published earlier [22]. The absorption patterns of the fucoidan fraction of *L. cichorioides* and *L. japonica* (L.c.F-1, L.c.F-2, L.j.F-1, L.j.F-2) were somewhat different from those of the others for *F. evanescens*, but were similar to each other. Fractions F.e.F-1 and F.e.F-2 of fucoidans from *F. evanescens* gave almost the same spectra. A strong adsorption band of an S=O stretching vibration at 1240 cm^{-1} was found in all polysaccharides, indicating the presence of ester sulfate. In addition, in the spectra of fucoidan fractions F.e.F-1, F.e.F-2, a moderate band at ~ 820 cm^{-1} was observed, which showed that the sulfate groups were in equatorial positions. A weak band at ~ 844 cm^{-1} was also found as a shoulder in the spectra, indicating the presence of a small number of sulfate groups attached in the axial position. In the spectra of fucoidan fractions L.c.F-1, L.c.F-2, L.j.F-1, L.j.F-2, a strong band at 842 cm^{-1} indicated that most sulfate groups were in axial positions and the remainder were in equatorial positions (as a shoulder in the spectra at ~ 820 cm^{-1}).

The lightly bound, low-sulfated polysaccharides of FL_5-fractions contained both glucose and fucose as main components (Table 2) and appeared to be a mixture of (1→3),(1→6)-β-D-glucans and fucoidans (as follows from the data of ^{13}C NMR spectra and enzymatic and acid hydrolysis). It is necessary to note that the sorption of fucoidans (polyelectrolytes) to hydrophobic sorbent is unusual and must be connected with peculiarities of their structures. According to preliminary data for enzymatic hydrolysis, polysaccharides of FL_5-fractions could contain a glucofucan. At the same time the cetavlon-precipitated component of FL_5-2 fraction from *L. cichorioides* contained (1→3),(1→6)-β-D-glucan only (^{13}C NMR data). The fraction is probably distinguished from others reported earlier [3–5,7].

The ^{13}C NMR spectra and action of (1→3)-β-D-glucanases confirmed the presence of (1→3),(1→6)-β-D-glucans in all fractions that were adsorbed on hydrophobic sorbent. Laminaran samples obtained by sorption on the

Table 1
Yield of different fractions [a] of the water-soluble polysaccharides from brown seaweeds obtained by the new procedure

Source	The fractions of the polysaccharides (yield, % of dry weight of the seaweed)							
	F-1	A-1	FL_5-1	L_{15}-1	F-2	A-2	FL_5-2	L_{15}-2
L. cichorioides	7.2	0.5	0.11	3.6	6.5	0.6	2.8	3.7
L. japonica	3.4	n.d.	0	0.2	2.7	n.d.	0	0
F. evanescens	5.3	0.53	2.6	0.6	9.7	1.3	0.5	1.0

[a] The fractions are according to Fig. 2.

Table 2
Some structural characteristics of the polysaccharides isolated from different sources

Sources	Fractions	M_w Distribution (kDa)	Fuc:SO_4 (mol/mol)	IR spectra (λ, cm^{-1})	Neutral monosaccharide composition [a], normalized mol % Fuc/Glc/Gal/Man/Xyl/Rha	Content of sugars [b] (%)	Action of enzymes, sugars (%) [c]
L. cichorioides	L.c.F-1	19–38	1:1.6	842	72/5/8/8/7/0	25	3 [d]
	L.c.FL5-1	n.d. [e]	1:0.1		48/35/0/8.5/0/0	65	40 [f]
	L.c.L_{15}-1	4–5			0/100/0/0/0/0	98	65 [f]
	L.c.F-2	19–28	1:1.8	842	81/3/4/2/2/8	38	5 [d]
	L.c.FL_5-2	17.7–31.6	1:0.1	n.d. [e]	31/67/0/2/0/0	80	65 [f]
	L.c.L_{15}-2	5–6			0/100/0/0/0/0	99.5	70 [f]
L. japonica	L.j.F-1	n.d. [e]	n.d. [e]	842	72/3.5/20/2/0/2.5	20	10 [d]
	L.j.L_{15}-1	4–5	n.d. [e]		10/82/8/0/0/0	96	65 [f]
	L.j.F-2	n.d.	n.d. [e]	842	94/0/3.5/1/1.5/0	30	15 [d]
F. evanescens	F.e.F-1	150–500	1:0.8	820	90/6/1.2/0/2.8/0	22	15 [d]
	F.e.FL_5-1	15–28			53/32/3.5/0/3.1/0	48	30 [f]
	F.e.L_{15}-1	4–5			5/95/0/0/0/0	92	43 [f]
	F.e.F-2	200–500	1:1.2	820	90.5/1.5/0/0/7/0	32	20 [d]
	F.e.FL_5-2	17.7–40	1:0.2	n.d. [e]	38/58/0/2/2/0	60	35 [f]
	F.e.L_{15}-2	5–6			13/81/2/0/4/0	96	50 [d]

[a] Determined by acid hydrolysis, followed by HPLC.
[b] Determined by the phenol–sulfuric acid method.
[c] The action of corresponding enzymes determined by Nelson's method as % liberated sugars from theoretically possible.
[d] Fucoidan hydrolase from *Littorina* sp.
[e] n.d., not determined.
[f] *Endo*-(1→3)-β-D-glucanase from *S. sachalinensis*.

Polychrome-1 (L_{15}-fractions) contained from 80% (*F. evanescens*) up to 98–99.5% (*L. cichorioides*) glucose. Ash was almost completely absent (less than 0.1–0.2%) (Table 2).

It should be noted that there are marked differences in susceptibility of similar fractions to the action of responding enzymes. Thus, the A-fractions from *L. cichorioides* were degraded by alginate lyase from *Littorina* sp. far better than the same fractions from *F. evanescens*. Fucoidan hydrolase from *Littorina* sp. essentially did not act on fucoidans from *L. cichorioides*. Laminarans from *L. cichorioides* were hydrolyzed by *endo*-(1→3)-β-D-glucanase from *Spisula sachalinensis* better that the same fractions from *F. evanescens*. These differences are certainly connected with the peculiarities of substrate structure (Table 2).

We believe that the new procedure is simpler than previously reported methods and it allows laminarans to be separated from fucoidans and alginic acids present in brown seaweed water extracts. Studies of the biological activity, structure and properties toward specific enzymes of the polysaccharides thus obtained are in progress.

3. Experimental

General methods and analytical techniques.—The following colorimetric assays were used: phenol–sulfuric acid reaction for total carbohydrate [23] and Nelson's method for reducing sugars [24]. IR spectra of polysaccharides were recorded for a KBr pellet of a test sample with a Carl Zeiss IR-75 spectrometer. ^{13}C NMR spectra (10-mm tubes, internal methanol, δ 50.1) were obtained at 60 °C on a Bruker WM-250 NMR spectrometer (62.9 MHg). Observation pulses of 17 μs were employed with relaxation delay of 0.8 s, acquisition time 0.5 s, number of transients collected ~40,000–60,000. Samples of polysaccharides (about 60 mg) were dissolved in D_2O, and the fractions containing alginic acid were solubilized by adjusting the pH to 10–11 with NaOH solution.

Enzymatic methods.—The specific enzymes [*exo*-(1→3)-β-D-glucanase from *Eulota maakii* [25] and *endo*-(1→3)-β-D-glucanase from *Spisula sachalinensis* [26], fucoidan hydrolase and alginate lyase from *Littorina* sp. [12]) were used for identification of laminarans, fucoidans and alginic acid, respectively. The enzymes (0.1 mL; $1–2 \times 10^{-2}$ U/mL) were added to the solution of polysaccharides (1–5 mg/mL of 0.1 M acetate buffer, pH 5.5, 0.5–2 h at 25 °C) twice to reach exhaustive hydrolysis. The enzymatic reaction products were detected by Nelson's method for fucoidan hydrolase and (1→3)-β-D-glucanases and by optical density measurement at λ 235 nm for alginate lyase.

Carbohydrate analysis.—Acid hydrolysis of polysaccharides was carried out with 4 N HCl at 100 °C for 2 h. Mono- and oligosaccharide compositions of enzymatic and acid hydrolysis products of polysaccharide were determined with a Biotronik carbohydrate analyzer (Durrum- × 4–20; 0.63 × 30 cm; 60 °C; bicinchoninate method [27]; Shimadzu C-R2AX). Monosaccharides (Rha, Rib, Man, Fuc, Gal, Xyl, Glc) and laminaribiose were used as intrinsic standards for HPLC.

Determination of molecular weight.—To determine the molecular-weight distribution, the polysaccharides were subjected to size-exclusion chromatography on the columns with Sephadex G-50 (1 × 100 cm, water, flow rate 15 mL/h) and Sepharose CL-4B (1 × 100 cm, water, flow rate 15 mL/h). Dextrans of 10, 20, 40, 80 and 500 kDa molecular weight were used as calibrants.

Sulfate content.—For sulfate the appropriate fractions were quantitatively assayed by turbidity measurement after hydrolysis with 4 N HCl and addition of gelatin–$BaCl_2$ [28].

L. cichorioides was collected in Troitsa Bay, Sea of Japan in August 1997. *F. evanescens* was collected near Iturup Island (August 1997), Sea of Okhotsk during a cruise of the research vessel 'Academik Oparin'. *L. japonica* was collected in Rifovaya Bay, Sea of Japan (August 1996).

Isolation and separation of water-soluble polysaccharides.—The process was carried out according to the scheme in Fig. 2. Fresh or deep-frozen seaweeds (3 kg) were pretreated with EtOH, acetone and $CHCl_3$. Samples of defatted, deionized, dry and powdered alga fronds (about 200 g) were successively ex-

tracted with cold 0.4% HCl (4–5 L, 20–25 °C) and hot (4–5 L, 60–70 °C) water. The resultant cold (1) and hot (2) extracts were subjected to hydrophobic chromatography on Polychrome-1 [Teflon (Russia), 2 kg; column 7×70 cm, void volume about 2 L]. The polysaccharide fractions were eluted with water (FA-fractions), 5% aq EtOH (FL_5-fraction) and 15% aq EtOH (L_{15}-fraction) successively until the eluates were free from carbohydrates with phenol–sulfuric acid reagents. Following the procedure in Ref. [4], glacial HOAc was slowly added to the water-eluted fractions (approximately to 40%) to precipitate the fractions of water-soluble fragments of alginic acid (A-fractions). The fucoidans (F-fractions) and laminarans (L-fractions) were precipitated from the supernatant with aq EtOH (80%). The yields of A-, F-, FL- and L-fractions from extracts of different brown algae are given in Table 1. The yields of polysaccharides were determined from the dry weight of alga. The binding capacity of Polychrome-1 was determined with the laminarans from *L. cichorioides* and *F. evanescens* by passing through the column an excess of the laminaran sample (30 g); about 20 g were sorbed on the Polychrom-1 column and 9–10 g were eluted (nonabsorbed) from the sorbent by water.

6^3-β-D-Glucopyranosyllaminaritriose, laminarioligosaccharides (DP 5–7), laminaran after Smith degradation, laminarans from *L. gurjanovae*, *L. cichorioides* and *F. evanescens* [(1→3),(1→6)-β-D-glucans with contents of β-(1→6)-bound Glc of 2–3, 10–12, 30–35%, respectively; M_w about 5–6 kDa] obtained by methods [13,14,20], translam [(1→3),(1→6)-β-D-glucan; M_w about 10–12 kDa and content of β-(1→6)-bound Glc about 20–25%] obtained by enzymatic transformation of laminaran according to Ref. [21] and pustulan from *Umbellicaria russica* [(1→6)-β-D-glucan; M_w about 30–35 kDa] were used as standards [14,19].

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