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Polysaccharides of Diatoms Occurring in Lake Baikal

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Abstract—Polysaccharide composition of neutral, acid- and alkali-soluble fractions of the diatoms *Stephanodiscus meyerii* Genkal et Popovsk and *Aulacoseira baicalensis* (K. Meyer) Simonsen of Lake Baikal has been studied. Neutral polysaccharides were represented by chrysolaminarans (1⟶3; 1⟶6-β-*D*-glucans). The chrysolaminaran from *S. meyerii* consists of the high- and low-molecular-weight fractions (40 and 2–5 kDa, respectively) and contains a large number of β-1⟶6-bound glucose residues. The chrysolaminaran from *A. baicalensis* is a low-molecular-weight 1⟶3; 1⟶6-β-*D*-glucan containing a small number of β-1⟶6-bonds, with mannitol being attached to the reducing unit of its chain. Acid- and alkali-soluble polysaccharide fractions are practically absent in *S. meyerii*. The alkali-soluble fraction from *A. baicalensis* is a low-molecular-weight (2-kDa) glycoprotein, the carbohydrate moiety of which is represented by a heteropolysaccharide.

Diatoms account for approximately 40% of the primary producers on Earth [1]. These unicellular or colonial eukaryotes are widespread in seas, oceans, and freshwater bodies, where they have adapted to the living conditions of plankton and benthos. Polysaccharides account for 10 to 80% of organic matter of diatoms [2, 3] and play a key role in their vital functions. With respect to location, polysaccharides of diatoms can be divided into three groups: reserve, structural, and extracellular [4, 5]. Polysaccharides of each group implement a specific function.

Reserve polysaccharides are the final products of photosynthesis; they represent β-*D*-glucans (chrysolaminarans), which are located in vacuoles [6]. Chrysolaminarans are represented by short chains comprised of 1⟶3-bound β-*D*-glucopyranose residues ($n = 20–60$) branched at C6 and/or C2 [7–9]. In diatoms at the exponential and stationary growth phases, chrysolaminaran accounts for 10–30 and approximately 80% of dry organic matter of cells, respectively. Glucans are accumulated under the conditions of shortage of nutrients, when the synthesis of proteins decreases. Therefore, the protein-to-glucan ratio may be regarded as an index of the trophic properties of water bodies [10, 11].

Structural polysaccharides (heteroglycans) are components of cell walls; together with proteins, they form an organic coat consisting of two layers [12]: the outer envelope and the diatotepum (the inner layer between the cell wall and plasmalemma). The diatotepum consists predominantly of acid polysaccharides and, in some species, sulfated glucuronomannan [13–15]. In addition to the compounds described above, cell walls also contain the structural insoluble 1⟶3-β-glucan callose, strips of which are located at the margin of the hypocingulum [6].

Mucus, or extracellular matrix (extracellular polysaccharides) are secreted by the cell from specialized structures of the carapace. Acid mucopolysaccharides of benthic organisms serve for attaching to and moving on a substrate [16, 17]. It was shown that the secretion of mucus by diatoms enables their movement [18, 19]. In centric organisms, mucous fibers increase their floatability and are involved in the regulation of hovering [20]. In addition to polysaccharides, the extracellular matrix of diatoms also contains protein.

With respect to the strength of binding to the cell, polysaccharides of the extracellular matrix are divided into two groups: weakly bound (soluble) and tightly bound polysaccharides. The weakly bound polysaccharides are retained in supernatant after precipitation of homogenized cells, whereas the tightly bound polysaccharides are extracted from precipitated homogenized cells with water [21].

Study of polysaccharides of diatoms clarifies the function of these organisms and may be important in practice. For example, 1⟶3; 1⟶6-β-*D*-glucans (analogues of chrysolaminarans) are known as biologically active compounds [22].

The goal of this work was to study polysaccharides of the diatoms *S. meyerii* and *A. baicalensis*, the composition and structure of which have not previously been studies.

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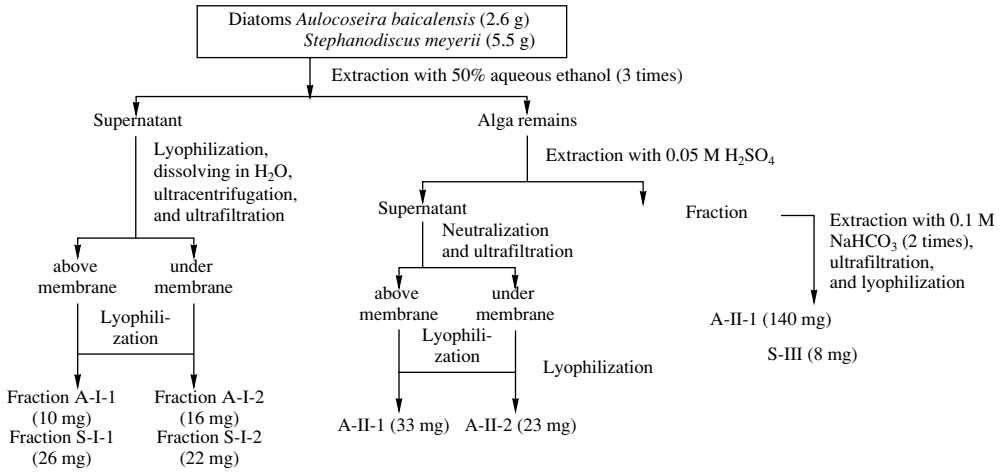

Fig. 1. Scheme of isolation of polysaccharides from diatoms *S. meyerii* and *A. baicalensis.*

MATERIALS AND METHODS

Reagents. Ethanol, acetone, and acetonitrile were from the company Laverna (Russia). Fucose, arabinose, mannose, galactose, xylose, ribose, rhamnose, and glucose were obtained from Merck (Germany). Laminaran from the brown seaweed *Laminaria cichorioides* was obtained as described in [23]. Endo-1⟶3-β-*D*-glucanase (LIV) was isolated from crystalline style of the marine mollusk *Spisula sachalinensis* as described earlier [24].

Diatoms S. meyerii and *A. baicalensis* were collected during blooming in spring 2002 and 2003, respectively.

Extraction of polysaccharides. The diatom *A. baicalensis* (2.6 g) was extracted three times with 50% aqueous ethanol (100 ml). Total extract was dried by lyophilization. Dry matter was dissolved in water (20 ml), centrifuged at 10000 *g* for 10 min, and separated by ultrafiltration using a 1-kDa Millipore membrane (United States). Fractions A-I-1 (10 mg; yield, 0.4%, above membrane) and A-I-2 (16 mg; yield, 0.6%; under membrane) were lyophilized.

After extraction with 0.05 M sulfuric acid, cell remains were extracted twice (at 40°C for 10 h and at 80°C for 2 h) with 0.1 M $NaHCO_3$ (200 ml). Extracts were pooled and dried. This fraction was named A-III (140 mg; yield, 5.4%).

The diatom *S. meyerii* (5.5 g) was treated with 50% ethanol (100 ml). Extract was evaporated until dry. The yield at this stage was 0.7 g (13%). The pellet was dissolved in water (20 ml), centrifuged (10000 *g*, 10 min), and subjected to ultrafiltration through a 1-kDa Millipore membrane. Fractions thus obtained were named S-I-1 (26 mg; yield, 0.5%; above membrane) and S-I-2 (22 mg; yield, 0.4%; under membrane) (Fig. 1). Then, polysaccharides were extracted in the same way as in the case of *A. baicalensis.* The resulting alkali-soluble fraction was named S-III (8 mg; yield, 0.1%).

Determination of saccharides and protein. Neutral saccharides were determined with phenol- and sulfuric acid as described in [25]. Reducing saccharides were determined by the method of Nelson [26]; protein, by the method of Lowry [27].

NMR spectroscopy. ^{13}C NMR spectra were recorded on a Bruker-Physic WM-250 spectrometer (Bruker, Germany) in D_2O and dimethyl sulfoxide at 60°C.

Monosaccharide composition. Polysaccharide samples (5 mg) were hydrolyzed with 4 N CF_3COOH at 100°C for 2 h. Monosaccharide composition of the products of acid hydrolysis was determined in the form of polyol acetates by GLC on an HP-5MS column (0.2 mm × 30 m) (Agilent 6850, Germany). Chromatography was performed at temperatures programmed from 150 to 230°C (rate, 3°C/min) using helium as a carrier gas. Mass spectrometry of polyol acetates was performed on an HP GC System 6890 spectrometer equipped with an HP-5 ms column (30 m × 250 mm × 0.25 μm nominal) with 5% phenylmethylsiloxane (MSD 5973, Hewlett Packard, United States). Helium was used as a carrier gas; temperature was programmed from 150 to 280°C (rate, 5°C/min).

Paper chromatography was performed on Whatman-1 paper (Germany) in the system *n*-butanol–pyridine–water (6 : 4 : 3). Saccharides were visualized with silver nitrate.

Deproteinization by the method of Sevage. Aqueous solution of fraction A-III (50 mg/ml) was mixed with chloroform (1 : 0.2) and then with *n*-butanol (chloroform–*n*-butanol, 1 : 0.2). The mixture was shaken for 30 min and then centrifuged at 10000 *g* for 10 min. Thereafter, the polysaccharide was precipitated with 80% ethanol and dried with acetone. This procedure

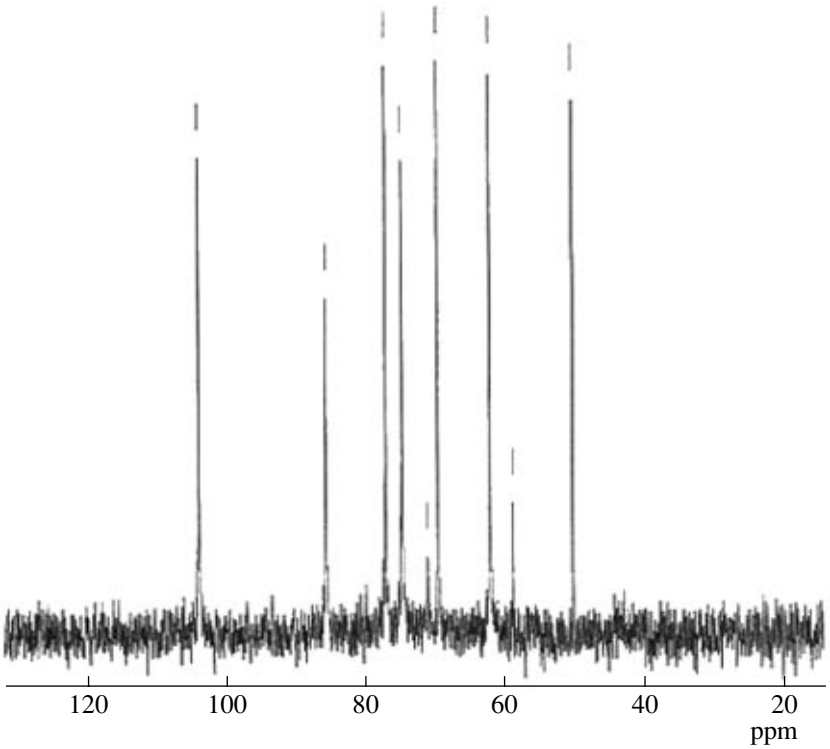

Fig. 2. ^{13}C NMR spectrum of chrysolaminaran S-I.

was repeated several times. The yield of the polysaccharide was 32 mg (64%).

Molecular weight of the compounds obtained was determined by gel filtration on a column (1.0 × 30 cm) packed with Sephadex 75-HR 10/30 (Amersham Pharmacia Biotech AB, United States), which was equilibrated with phosphate buffer (pH 7.2) supplemented with 0.15 M NaCl. Compounds were eluted with the same buffer at a rate of 0.4 ml/min. Dextrans with molecular weights of 10, 20, 40, and 80 kDa and inulin (5 kDa) were used as standards.

Obtaining products of exhaustive enzymatic hydrolysis. Endoglucanase LIV (2×10^{-2} U/ml) was added to the laminaran from *L. cichorioides* (2 mg/ml) and the chrysolaminarans S-I-2 and A-I-2 in 0.05 M succinate buffer (pH 5.2), and the mixture was incubated at 37°C for 1 day.

The products of enzymatic hydrolysis of β-*D*-glucans were analyzed by HPLC on an Agilent chromatograph equipped with an Asahipak NH_2P-50 4A column (Shoko Co., LTD, Japan) in the system acetonitrile–water (60 : 40).

RESULTS AND DISCUSSION

Polysaccharides of the diatom S. meyerii. Aqueous–alcohol extract from the diatom *S. meyerii* (5.5 g) was used to isolate and purify the polysaccharide fraction S-I, the yield of which was approximately 1% (58 mg) of the weight of wet cells (Fig. 1).

The ^{13}C NMR spectrum of fraction S-I contained signals with chemical shifts at 104.2, 85.8, 77.3, 69.8, and 62.4 ppm, which are characteristic of 1⟶3-β-*D*-glucans. These data provide evidence that fraction S-I was representative of chrysolaminaran (Fig. 2).

The distribution of molecular weights of the total polysaccharide fraction S-I was studied using gel-penetrating chromatography on Superdex 75-HR 10/30 (Fig. 3). It can be seen that the chrysolaminaran from the diatom *S. meyerii* consists of the high-molecular-weight fraction S-I-1 (~40 kDa) and low-molecular-weight fraction S-I-2 (~2–5 kDa).

The low-molecular-weight fraction S-I-2 was isolated from the total chrysolaminaran preparation (S-I) by ultrafiltration. Using ^{13}NMR spectroscopy, it was shown that fraction S-I-1 represented a low-molecular-weight 1⟶3; 1⟶6-β-*D*-glucan enriched in β-1⟶6-bound glucose residues, compared to the total preparation (104.5 ppm) (Figs. 2 and 4). The acid-soluble fraction was virtually absent in this alga. The yield of the alkali-soluble fraction S-I-3 was approximately 0.1% (8 mg) of the weight of wet cells. Fraction S-I-3 consisted predominantly of uronic acids, as determined by paper chromatography.

Polysaccharides of the diatom A. baicalensis. Polysaccharide fractions A-I-1, A-I-2, A-II-1, A-II-2, and A-III were isolated from the diatom *A. baicalensis*

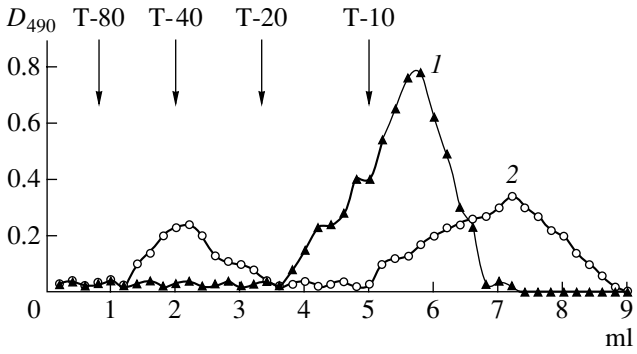

Fig. 3. Chromatogram obtained by gel filtration of (*1*) laminaran and (*2*) chrysolaminaran S-I on Superdex 75-HR 10/30.

(Fig. 1). Fractions A-I-1 and A-I-2 contained predominantly protein. It is known that, in addition to polysaccharides, the extracellular matrix and cell walls of diatoms contain protein [28]. The content of the latter depends on the species and stage of growth of the alga. For example, the content of protein in *Skeletonema costatum* accounted for 7% of the weight of substances secreted [28]; in *Chaetoceros affinis*, <1.5% [29].

Acid hydrolysate of fraction A-I-2 contained glucose and xylose (ratio, 4 : 1), which were identified in the form of polyol acetates using GLC–MS and paper chromatography. An analysis of the ^{13}C NMR spectrum of this fraction showed that the latter was represented predominantly by 1⟶3; 1⟶6-β-*D*-glucan, the terminal reducing unit of which was linked with mannitol (64 ppm) (Fig. 5). Note that mannitol has not been detected earlier in the polysaccharide fraction of diatoms. According to the results of acid hydrolysis, fraction A-II-2 (3–5 kDa) consisted only of glucose residues. The treatment of this fraction with 1⟶3-β-*D*-glucanase yielded reducing saccharides. The degree or enzymatic hydrolysis of fraction A-II-2 was approximately 40% of the total amount of saccharides in it. Based on these data, fraction A-II-2 was also classified with chrysolaminarans.

Fraction A-III, which was extracted with 0.1 M sodium bicarbonate, contained the protein and carbohydrate components, as indicated by the ^{13}C NMR spectroscopic data. The determination of protein content by the method of Lowry showed that protein in this fraction accounted for approximately 70%. We assumed that the polysaccharide isolated from this fraction represented a component of cell walls of the diatom, which formed the organic envelope covering the silica carapace of cells, because high protein content is characteristic of its components [12]. According to the published data, the protein-to-carbohydrate ratio may vary from 0.3 to 6.5, depending on the physiological state of the cell [14]. In fraction A-III, this ratio was 2.3.

Deproteinization of this fraction by the method of Sevage [30] did not allow the protein component to be removed. The protein and carbohydrate components of this fraction were not separated as well by gel-penetrating chromatography on Superdex 75-HR 10/30. Possible, this fraction represented a carbohydrate–protein complex (glycoprotein) with a low molecular weight (approximately 2 kDa, as evaluated by the results of gel-penetrating chromatography). The carbohydrate component of fraction A-III consisted of the residues of mannuronic acid and neutral polysaccharides (specifically, mannose, galactose, glucose, xylose, raffinose, and ribose), as indicated by the results of paper chromatography of the products of acid hydrolysis of this fraction. The composition and content of neutral polysaccharides were determined in the form of polyol acetates by GLC–MS. The ratio between neutral polysaccharides (in molar percent) in this fraction was as follows: 1% mannose, 0.5% galactose, 0.4% xylose, 0.3%

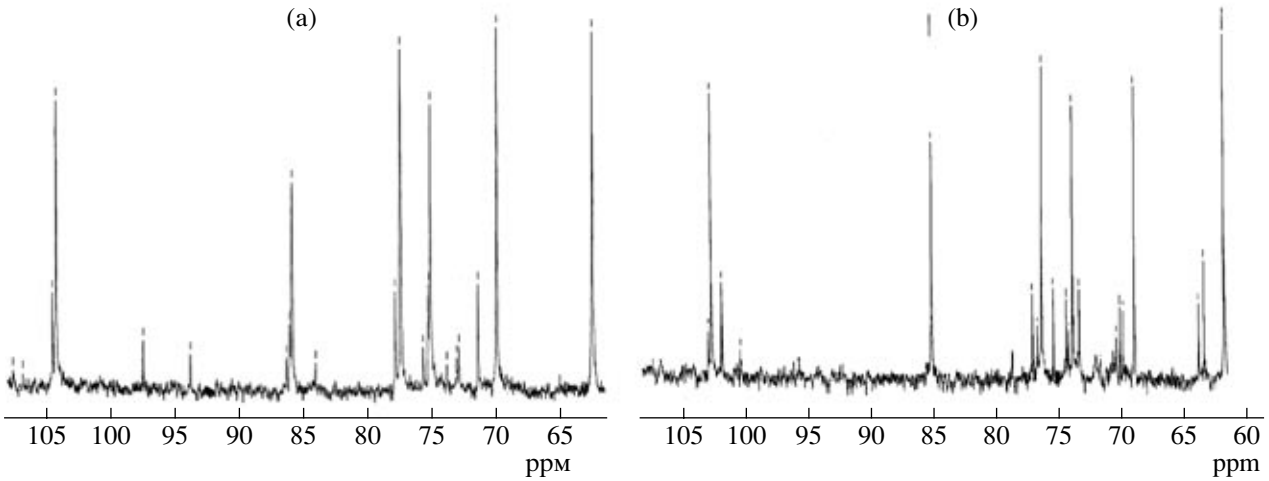

Fig. 4. ^{13}C NMR spectrum of low-molecular-weight fractions of chrysolaminarans S-I-2 (a) and A-I-2 (b).

ribose, 0.3% glucose, 0.3% raffinose, and 0.2% unidentified polysaccharide.

Note that the yield of fraction A-III was 5.4% (140 mg) of the weight of wet alga cells, which was approximately 50 times greater than the yield of fraction S-III. This difference may be related to the thickness of cell wall of the diatoms studied, as we used sufficiently mild conditions of extraction in order to isolate native biopolymers.

The characteristics of all fractions isolated are summarized in the table. It can be seen that the composition of easily soluble biopolymers considerably varied. Note that weakly bound protein fractions were absent in polysaccharide fractions of *S. meyerii.*

A comparative analysis of structures of the chrysolaminarns isolated was performed usinig 1⟶3-β-*D*-glucanase from *S. sacchalinensis.* The laminaran from the brown seaweed *L. cichorioides*, the fine structure of which was determined earlier [31], was used for comparison.

1⟶3-β-*D*-glucanase from *S. sachalinensis*, the specificity and mechanism of action of which were studied earlier at the Pacific Institute of Bioorganic Chemistry (Far East Division, RAS) [9], catalyzed the hydrolysis of both laminaran (1⟶3; 1⟶6-β-*D*-glucan) from *L. cichorioides* and chrysolaminaran fractions S-I-2 and A-I-2 at similar rates. The degree of hydrolysis was also similar (50, 44, and 42%, respectively).

The composition of reaction products determined by HPLC was different for all polysaccharide samples studies (Fig. 5). We plotted the logarithms of retention time of oligosaccharides versus their degree of polymerization. In the case of homologous oligomers, this dependence should represent a straight line. An analysis of dependencies obtained showed that the majority of products of enzymatic hydrolysis of polysaccharides represented laminarioligosaccharides; the latter were identified using laminaribiose and laminaritriose. As seen from Fig. 5, there was another line situated above the line corresponding to laminarioligosaccharides, which was apparently formed by glucoligosaccharides of varying structure containing both 1⟶3- and 1⟶6-bonds. It is also seen from Fig. 5 that 1⟶3; 1⟶6-glucoligosaccharides of most variable structures are formed from the chrysolaminaran of the diatom *S. meyerii*. It should be noted that the chrysolaminaran of *S. meyerii* yields 1⟶3; 1⟶6-β-*D*-glucoligosaccharides with a greater degree of polymerization (n = 6–9) than the laminaran of the brown seaweed *L. cichorioides* (n = 3–7). Production of mixed 1⟶3; 1⟶6-tri- and 1⟶3-β-tetrasaccharides from the laminaran of *L. cichorioides* was reported earlier; the structure of these oligosaccharides was determined using the methylation technique [31]. In addition, the laminaran from *L. cichorioides* is characterized by a regular distribution of 1⟶6-bound glucose residues alongside its molecule [32]. A comparison of the reaction products allowed us to conclude that 1⟶3; 1⟶6-β-*D*-glucans of the diatoms *S. meyerii* and *A. baicalensis* and the brown seaweed *L. cichorioides* have different structures. Apparently, chrysolaminaran molecules have sites where β-1⟶6-bound glucose residues are located. Possibly, the chrysolaminaran from *A. baicalensis* also has a site with an increased number of β-1⟶6-bonds, which is located in the vicinity of the reducing end of the molecule.

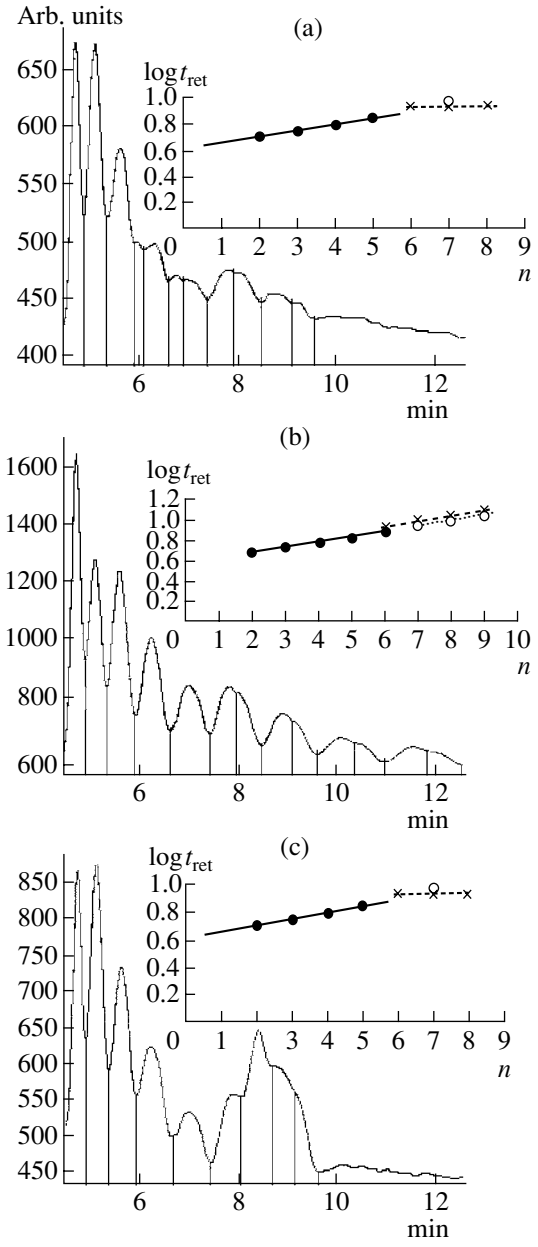

Fig. 5. Chromatogram obtained by gel filtration of the products of exhaustive enzymatic hydrolysis of (a) laminaran from *L. cichorioides* and chrysolaminarans from (b) *S. meyerii* and (c) *A. baicalensis* catalyzed by 1⟶3-β-*D*-glucanase from *S. sachalinensis*.

Thus, the plankton diatoms *S. meyerii* and *A. baicalensis*—endemic species of Lake Baikal [33]—

Characteristics of biopolymers isolated from diatom algae of Lake Baikal

Alga	Fraction	Extraction	Characteristics				
			yield, %	composition	ratio* β-1 ⟶ 3; β-1 ⟶ 6	molecular weight, kDa	Note
A. baicalensis	A-I-1	Aqueous ethanol	0.4	Protein		ND**	Protein
	A-I-2	Aqueous ethanol	0.6	Polysaccharide (glucose, xylose, mannitol)	90 : 10	ND	Chrysolaminaran
	A-II-1	H_2SO_4	1.3	Protein		ND	Protein
	A-II-2	H_2SO_4	0.9	Glucan	ND	3–5	Chrysolaminaran
	A-III	$NaHCO_3$	5.4	Protein + polysaccharide		2	Polysaccharide–protein complex
S. meyerii	S-I-1	Aqueous ethanol	0.5	Glucan	95 : 5	40	Chrysolaminaran
	S-I-2	Aqueous ethanol	0.4	Glucan	80 : 20	2–6	Chrysolaminaran
	S-III	$NaHCO_3$	0.1	Acid polysaccharide	ND	ND	Acid polysaccharide

* Determined from ^{13}C NMR spectra.

** Not determined.

differ both in the composition of biopolymers and in the structure of polysaccharides. Apparently, these species-specific features are related to biological characteristics of species and ensure different mechanisms of their adaptation to life in a deep water body.

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