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STRUCTURAL CHARACTERISTICS AND ANTITUMOR ACTIVITY OF A NEW CHRYSOLAMINARAN FROM THE DIATOM ALGA *Synedra acus*

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The 1→3;1→6-β-D-glucan chrysolaminaran with molecular weight 8.5 kDa and 3.6:1 ratio of 1→3:1→6 bonds was isolated from cultivated diatom alga Synedra acus *and characterized. It was shown that the isolated chrysolaminaran inhibited growth and colony formation of human colon tumor cells.*

Keywords: diatom alga, chrysolaminaran, 1→3;1→6-β-D-glucan, antitumor activity.

Diatom algae are unicellular or colonial eukaryotic organisms that inhabit seas, oceans, and freshwaters and have adapted to habitats of plankton and benthos. Diatom algae and terrestrial plants are responsible for about 40% of the primary production on Earth [1]. Polysaccharides make up from 10 to 80% of the organic mass of diatoms [2, 3] and play an important role in their metabolism. Polysaccharides of diatom algae are divided into three groups depending on their location in the cell: storage, structural, and extracellular [4, 5]. Each of these fulfills its own specific function.

Storage polysaccharides are localized in vacuoles and represent the final photosynthesis products [6]. They are β-D-glucans, are called chrysolaminarans, and consist of short chains of 1→3-bonded β-D-glucopyranoses (n = 20–60) with branching at C6 and/or C2 [7–9].

The study of polysaccharides from diatom algae is important for both an understanding of the functional processes of these organisms and their practical use. Analogs of chrysolaminarans, 1→3;1→6-β-D-glucans, isolated from yeast, fungi (lentinan), and culture filtrates of *Schizophyllum commune* (schizophyllan) have been used in cancer immunotherapy [10, 11], act as immunomodulators, and increase the resistance to infections [12]. This makes them an attractive alternative to cytotoxic drugs. In contrast with other natural biopolymers, purified β-1,3-glucans retain their biological activity over time so that the mechanism of their action on the cellular and molecular level can be studied. It is currently known that the biological action of β-1,3-glucans is mediated through two membrane receptors, CR3 [13] and Dectin-1 [14].

The biological activity of algae polysaccharides (laminarans and chrysolaminarans) has been studied less than that of glucans from yeast and fungi.

Our goal was to isolate chrysolaminaran from cultivated diatom alga *Synedra acus* and to study its structure and antitumor activity.

A chrysolaminaran preparation consisting of a polysaccharide–protein complex with a 1:8 protein:carbohydrate ratio was isolated from *S. acus* cultivated under laboratory conditions by extraction with hot water. According to the literature, the protein:carbohydrate ratio in similar complexes can vary from 0.33 to 6.5 depending on the physiological condition of the cell [15]. Removal of protein from the *S. acus* complex by the Sevage method [16] did not completely free the polysaccharide from the protein. A sample of the chrysolaminaran without protein could be obtained using hydrophobic chromatography over Polykhrom-1. The chrysolaminaran yield was 0.35% of the moist alga weight.

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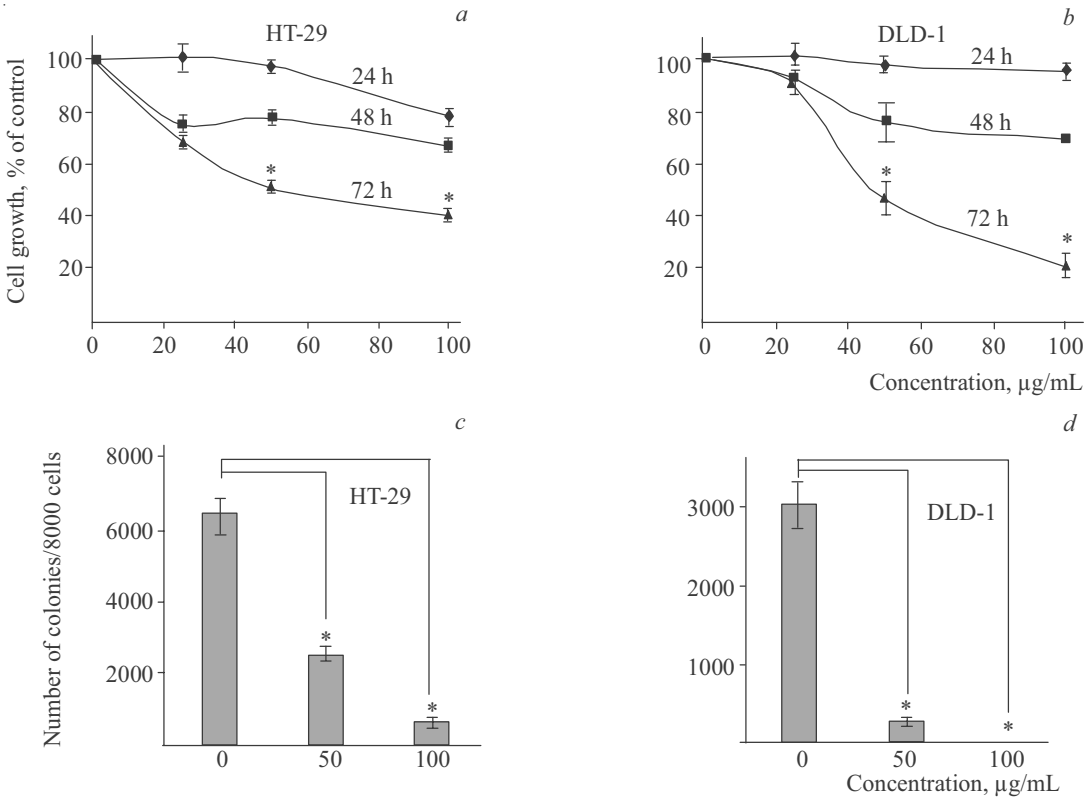

Fig. 1. Effect of chrysolaminaran on growth of human colon cancer cells (*a*, *b*) and on growth of human colon cancer cell colonies (*c*, *d*).

Analysis of the monosaccharide composition showed that the sample consisted of only glucose. The ^{13}C NMR spectrum of the isolated polysaccharide contained resonances at 103.5, 74.2, 84.8, 69.0, 76.8, and 61.4 ppm that were characteristic of C-1, C-2, C-3, C-4, C-5, and C-6 of glucoses bonded through β-1→3-bonds. Furthermore, the spectrum showed resonances at 103.8 (C-1) and 70.2 (C-6) that were characteristic of β-1→6-bonded glucoses. The PMR spectrum of the isolated polysaccharide contained resonances corresponding to anomeric protons (5.2 and 4.73 ppm) and protons of β-1→3- (4.8–4.9) and β-1→6-bonded (4.2) glucoses. These results led to the conclusion that the isolated polysaccharide was a 1→3;1→6-β-D-glucan and a typical chrysolaminaran.

The ratio of 1→3:1→6 bonds in the polysaccharide was 3.6:1 according to PMR spectroscopy.

The molecular weight of the isolated chrysolaminaran was 8.5 kDa according to size-exclusion chromatography. The characteristics agreed well with those published for other chrysolaminarans [17].

We studied the antitumor activity of the isolated chrysolaminaran using HCT-116 and DLD-1 human colon cancer cells. The chrysolaminaran did not exhibit toxicity to HCT-116 and DLD-1 cells at concentrations up to 200 mg/mL. This is an important property for practical application because most drugs with antitumor activity are highly toxic so that their use is limited. We used the MTS method to study the effect of the chrysolaminaran on tumor cell growth. Cells were treated with various doses of chrysolaminaran (25, 50, and 100 μg/mL) for 72 h. Cell proliferation was determined as described above. Inhibition of cancer cell proliferation had a distinct concentration dependence (*, $p < 0.5$). The IC_{50}/72h values were 54.5 and 47.7 μg/mL for HCT-116 and DLD-1, respectively (Fig. 1).

The effect of the chrysolaminaran on growth of HCT-116 and DLD-1 colonies in soft agar was studied using concentrations of 50 and 100 μg/mL. The results showed that treatment of the cells with the chrysolaminaran at 50 μg/mL decreased the number of colonies compared with the control. Treatment of DLD-1 cells with the chrysolaminaran at 100 μg/mL practically completely inhibited formation and growth of colonies (Fig. 1). It is known that laminarans (1→3;1→6-β-D-glucans), analogs of chrysolaminarans that are isolated from brown algae, usually exhibit weak or moderate biological activity. It was shown that the biological activity of 1→3;1→6-β-D-glucans increases with increasing molecular weight and also depends on the presence and position of β-1→6-bonded glucoses [18].

Thus, the chrysolaminaran isolated from diatom alga *S. acus* was nontoxic, inhibited proliferation of cancer cells and colony formation, and could become the principal drug for treating human colon cancer.

EXPERIMENTAL

Reagents. We used ethanol, acetone, and acetonitrile (Lavern, Russia) and fucose (Fuc), arabinose (Ara), mannose (Man), galactose (Gal), xylose (Xyl), ribose (Rib), rhamnose (Rha), and glucose (Glu) (Merck, Germany). Laminaran from brown alga *L. cichorioides* was obtained as before [19]. Nutrient medium MEM, BME, phosphate buffer, penicillin, streptomycin, L-glutamine, trypsin, EDTA, fetal bovine serum (FBS), and sodium bicarbonate were purchased commercially from Biolot (Russia).

Methods. Neutral sugars were determined by the phenol:H_2SO_4 method [20]. Reducing sugars were detected by the Nelson method [21]. Protein content was estimated by the Lowry method [22].

Cultivation of Diatom Alga *S. acus*. A laboratory culture of *S. acus* that was obtained from material collected from phytoplankton of Lake Baikal was used as the starting material for the cultivation. The optimal growth conditions for *S. acus* were 12°C, 250–300 $\mu E \cdot m^{-2} \cdot s^{-1}$ illumination intensity, and culture medium consisting of (mg/L) $Ca(NO_3)_2 \cdot 4H_2O$ (20), KH_2PO_4 (2), $MgSO_4$ (12), $NaHCO_3$ (30), Na_2EDTA (2.2), H_3BO_3 (2.4), $MnCl_2 \cdot 4H_2O$ (1.3), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (1), $Na_2SiO_3 \cdot 9H_2O$ (25), $FeCl_3$ (1.6), cyanocobalamine (vitamin B_{12}) (0.04), thiamine (vitamin B_1) (0.04), and biotin (0.04) [23].

The innoculate was cultivated in 1000-mL glass flasks at room temperature with periodic stirring under natural day–night lighting conditions.

Extraction. Chlorophyll and lipids were removed by extraction (5×) with aqueous ethanol (80%) at room temperature.

Extraction of Polysaccharides. Defatted diatom alga (10 g) was soaked with hot distilled water (70°C, 100 mL) and sonicated using a homogenizer (Sonopuls HD 2070, Bandenlin, Germany). The procedure was repeated three times. The homogenate was centrifuged for 20 min at 10,000 rpm. The total extract was concentrated in a rotary evaporator and dried by lyophilization.

Hydrophobic Chromatography. A sample (160 mg) was dissolved in water (10 mL), placed on a Polikhrom-1 column (2 × 15 cm), and eluted with water (150 mL) and aqueous ethanol (10%, 150 mL). Fractions were analyzed using phenol:H_2SO_4. Fractions containing carbohydrates were combined, concentrated in a rotary evaporator to 2 mL, and dried by lyophilization. The yield of carbohydrates was 35 mg.

Protein Removal by the Sevage Method. An aqueous solution of polysaccharide (20 mg/mL) was treated with $CHCl_3$ (1:0.2, v/v) and then *n*-BuOH ($CHCl_3$:*n*-BuOH 1:0.2, v/v). The mixture was shaken for 30 min and centrifuged. The aqueous phase was removed. Polysaccharide was precipitated by ethanol (80%) and dried with acetone. The procedure was repeated several times. The polysaccharide yield was 31%.

Monosaccharide Composition. A sample of polysaccharide (5 mg) was hydrolyzed by HCl (2 N) at 100°C (4 h). The monosaccharide composition of the acid-hydrolysis products was determined by HPLC on a carbohydrate analyzer (Biotronik, ISA-07/S2504 column, 0.4 × 25 cm, Shimadzu) with detection by the bicinchoninate method [24].

Molecular weight of chrysolaminaran was estimated by size-exclusion chromatography over a Superdex 75-HR 10/30 column (1.0 cm × 30 cm) (Amersham Pharmacia Biotech AB) equilibrated with phosphate buffer (pH 7.2) containing NaCl (0.15 M) and eluted with the same buffer at flow rate 0.4 mL/min. The standards were dextrans of molecular weight 10, 20, 40, and 80 kDa and laminaran of molecular weight 4.5 kDa.

NMR spectroscopy. NMR spectra were recorded in D_2O at 60°C on a DRX-500 instrument (Bruker, Germany).

Human colon cancer cells HT-29 and DLD-1 were cultivated in McCoy and RPMI-1640 media, respectively, with added FBS (10%), L-glutamine (2 mM), penicillin (50 U/L), and streptomycin (50 μg/L) in an incubator at 37°C and 5% CO_2.

Determination of Effect of Chrysolaminaran on Cell Proliferation. HT-29 and DLD-1 cells (3×10^4/mL) were innoculated into 96-well planchets and cultivated in medium (200 μL) at 37°C for 24 h, treated with chrysolaminaran (25, 50, and 100 μg/mL), incubated for 24, 48, and 72 h, treated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS reagent, 15 μL), and placed in an incubator (37°C) for 4 h. The amount of formazan released by the cells was determined using optical density at 492 nm/690 nm (A492/A690) (Bio-Tek Instruments spectrophotometer, USA). Growth of cells (%) treated with chrysolaminaran was calculated relative to untreated control cells. Two independent experiments with five samples for each concentration were performed for each substance.

Soft agar Method. The anticancer effect of the chrysolaminarans was estimated by the published soft agar method [25] with certain modifications. HT-29 and DLD-1 (8×10^3 cells/mL) human colon cancer cells were treated with different (50 and 100 μg/mL) doses of chrysolaminaran in BME agar (1 mL, 0.33%) containing FBS (10%) over BME agar (3.5 mL, 0.5%) with FBS (10%) and the indicated chrysolaminaran concentrations. Cells were cultivated at 37°C and 5% CO_2 for

30 d. Colonies were counted using a Motic inverted microscope and the Image-Pro Plus program. Two independent experiments with three samples for each concentration were performed for each substance.

Statistical treatment of data was carried out using the Student t-criterion with a fixed confidence probability of 95% (SigmaPlot 2000 program, version 6, SPSS Inc., USA).

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REFERENCES

1. D. M. Nelson, P. Tregueer, M. A. Brzezinski, A. Leyneart, and B. Queguiner, *Global Biogeochem. Cycles*, **9**, 359 (1995).
2. S. Myklestad, *J. Exp. Mar. Biol. Ecol.*, **15**, 261 (1974).
3. E. A. Romankevich, *Geochemistry of Organic Matter in the Ocean*, Springer, Berlin, 1984.
4. E. Granum and S. M. Myklestad, *J. Phycol.*, **37**, 772 (2001).
5. E. Granum and S. M. Myklestad, *Hydrobiologia*, **477**, 155 (2002).
6. L. Waterkeyn and A. Bienfait, *La Cellule*, **74**, 198 (1987).
7. A. Beattrie, E. L. Hirst, and E. Percival, *Biochem. J.*, **79**, 531 (1961).
8. B. S. Paulsen and S. Myklestad, *Carbohydr. Res.*, **62**, 386 (1978).
9. M. J. McConville, A. Bacic, and A. E. Clarke, *Carbohydr. Res.*, **153**, 330 (1986).
10. J. Yan, V. Vetvicka, Y. Xia, A. Coxon, M. C. Carroll, T. N. Mayadas, and G. D. Ross, *J. Immunol.*, **163**, 3045 (1999).
11. T. J. Yoon, T. J. Kim, H. Lee, K. S. Shin, Y. P. Yun, W. K. Moon, D. W. Kim, and K. H. Lee, *Int. Immunopharmacol.*, **8**, 36 (2008).
12. K. H. Kim, Y. W. Kim, H. B. Kim, B. J. Lee, and D. S. Lee, *Biotechnol. Lett.*, **28**, 439 (2006).
13. G. D. Ross, J. A. Cain, B. L. Myones, S. L. Newman, and P. J. Lachmann, *Complement*, **4**, 61 (1987).
14. G. D. Brown and S. Gordon, *Nature*, **413**, 36 (2001).
15. B. E. Volcani, in: *Cell Wall Formation in Diatoms: Morphogenesis and Biochemistry*, T. L. Simpson and B. E. Volcani (eds.), Springer-Verlag, New York, 1981, p. 157.
16. M. G. Sevage, *Biochem. Z.*, **273**, 419 (1934).
17. S. A. Alekseeva, N. M. Shevchenko, M. I. Kusaikin, L. P. Ponomorenko, V. V. Isakov, T. N. Zvyagintseva, and E. V. Likhoshvai, *Prikl. Biokhim. Mikrobiol.*, **41**, 213 (2005).
18. T. N. Zvyagintseva, N. N. Besednova, and L. A. Elyakova, *Structure and Immunotropic Action of 1→3;1→6-β-D-Glucans*, Dal′nauka, Vladivostok, 2002.
19. T. N. Zvyagintseva, N. M. Shevchenko, I. B. Popivnich, B. B. Isakov, A. S. Scobun, L. B. Sundukova, and L. A. Elyakova, *Carbohydr. Res.*, **322**, 32 (1999).
20. M. Dubois, K. A. Gilles, J. Hamilton, P. A. Robers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
21. N. J. Nelson, *Chemistry*, **153**, 375 (1944).
22. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
23. A. L. Vereshchagin, O. Yu. Glyzina, T. N. Basharina, T. A. Safonova, N. A. Latyshev, S. A. Lyubochko, E. S. Korneva, D. P. Petrova, V. V. Annenkov, E. A. Danilovtseva, E. P. Chebykin, N. A. Volokitina, and M. A. Grachev, *Biotekhnologiya*, **4**, 55 (2008).
24. A. E. Manzi, L. D. Powell, and A. Varki, *Curr. Protoc. Mol. Biol.*, 17 (2001).
25. N. H. Colburn, E. J. Wendel, and G. Abruzzo, *Proc. Natl. Acad. Sci. USA*, **78**, 6912 (1981).