Carbohydrate Polymers 99 (2014) 101–109

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Structure, enzymatic transformation and anticancer activity of branched high molecular weight laminaran from brown alga *Eisenia bicyclis*

Roza V. Menshova[a,*], Svetlana P. Ermakova[a], Stanislav D. Anastyuk[a], Vladimir V. Isakov[a], Yuliya V. Dubrovskaya[a], Mikhail I. Kusaykin[a], Byung-Hun Um[b], Tatiana N. Zvyagintseva[a]

[a] G.B. Elyakov Pasific Institute of Bioorganic Chemistry, Far Eastern Branch, Russian Academy of Sciences, 159 100-let Vladivostoku Prospect, Vladivostok 690022, Russian Federation

[b] Korea Institute of Science and Technology Gangneung Institute, Ga-17 Block, Gangneung Techno Valley, Daejeon-dong, Gangneung 210-340, Republic of Korea

ARTICLE INFO

Article history:
Received 6 June 2013
Received in revised form 5 August 2013
Accepted 15 August 2013
Available online 21 August 2013

Keywords:
Laminaran
Eisenia
Enzymatic hydrolysis
Structure
Anticancer activity

ABSTRACT

The structure of high molecular weight laminaran from brown alga *Eisenia bicyclis* was investigated by chemical and enzymatic methods, NMR spectroscopy and mass spectrometry. The laminaran from *E. bicyclis* was characterized as 1,3;1,6-β-D-glucan with the high content of 1,6-linked glucose residues (ratio of bonds 1,3:1,6 = 1.5:1), which are both in the branches and in the main chain of the laminaran. The degree of polymerization of fragments, building from 1,3-linked glucose residues with single glucose branches at C-6 or without it, was no more than four glucose residues. The main part of 1,3-linked glucose blocks was builded from disaccharide fragments. 1,6-Linked glucose residues were localized basically on non-reduced ends of molecules. The degree of polymerization of 1,6-linked blocks was not greater than three glucose residues. Laminaran contained laminarioligosaccharides, gentiobiose, gentiotriose and single glucose residues in the branches at the C-6. Laminaran and its products of enzymatic hydrolysis inhibited a colony formation of human melanoma SK-MEL-28 and colon cancer DLD-1 cells. It was shown that decreasing the molecular weight of native laminaran to a determined limit (degree of polymerization 9–23) and increasing the content of 1,6-linked glucose residues increased the anticancer effect. Therefore, they may be perspective antitumor agents.

1. Introduction

Brown algae are a rich and easily renewable source of biologically active polysaccharides, including alginic acids, laminarans and fucoidans. These compounds exhibit a broad spectrum of biological activity and low toxicity in vivo. Laminarans are water-soluble polysaccharides of brown algae, consisting of 1,3- and 1,6-linked β-D-glucose residues. Laminarans from different species of algae are known to vary due to the ratio of 1,3:1,6 bonds and variants of including of these bonds in the molecule of β-D-glucan (Zvyagintseva, Shirokova, & Elyakova, 1994).

The laminarans (1,3;1,6-β-D-glucans) are of interest due to their anticancer, radioprotective and immunomodulatory activities. Laminarans usually have a molecular weight 4–5 kDa. The main chain of most laminarans consist of 1,3-linked β-D-glucose residues with a small amount (≤10%) of branches at C-6 as single β-D-glucose residues. As a rule, these laminarans possess slight biological activity. Branched 1,3;1,6-β-D-glucans with more high molecular weight (8–10 kDa) and 1,6-linked β-D-glucose residues in main chain have the highest immunomodulatory activity (Elyakova et al., 2007; Zvyagintseva, Elyakova, & Isakov, 1995).

Laminaran with an unusual structure was isolated from brown alga *Eisenia bicyclis* (Japan) named as "eisenan". This laminaran was a 1,3;1,6-β-D-glucan, with a high content of 1,6-bonds (ratio of bonds 1,3:1,6 = 1.5:1), a branched structure and a molecular weight close to 6 kDa (Maeda & Nishizawa, 1968; Usui, Toriyama, & Mizuno, 1979). Previously, we isolated laminarans from *E. bicyclis* (Republic of Korea) with a different structure. Laminaran with a ratio of 1,3:1,6 bonds of 1.5:1 and a molecular weight close to 5 kDa was obtained from alga collected in May (Ermakova et al., 2013). The alga collected in July synthesizes two types of laminaran (Menshova, Ermakova, Um, & Zvyagintseva, 2013), one of which has a ratio of 1,3:1,6 bonds of 1.5:1 and a molecular weight of approximately 5 kDa, as the laminaran from *E. bicyclis* purified by Usui. The second fraction of laminaran has the same ratio of 1,3:1,6 bonds (1.5:1), but its molecular weight (19–27 kDa) was higher

* Corresponding author. Tel.: +7 423 2310705; fax: +7 423 2314050.
E-mail address: Menshova-R@yandex.ru (R.V. Menshova).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.08.037

than 4-fold compared usual for laminarans. The high molecular weight laminaran was first isolated by us. The information about the isolation of high molecular weight laminaran from *E. bicyclis* and other brown algae is absent. Therefore, brown algae *E. bicyclis* produces different types of laminarans depending on the habitat and the harvesting time of alga.

The aim of present work was to investigate structural characteristics of high molecular weight laminaran from *E. bicyclis* (Republic of Korea) and to study the anticancer activity of native laminaran and its derivatives to establish the correlation "structure–biological activity".

2. Experimental

2.1. Materials

Organic solvents, inorganic acids and salts, sodium hydroxide were commercial products (Laverna, Russia). Laminarans from brown alga *E. bicyclis* and *Saccharina cichorioides* were isolated by the methods described (Menshova et al., 2013; Zvyagintseva et al., 1999). Endo-1,3-β-D-glucanase from marine mollusk *Pseudocardium sachalinensis* and exo-1,3-β-D-glucanase from marine *Chaetomium indicum* were isolated as described previously (Burtseva, Verigina, Sova, Pivkin, & Zvyagintseva, 2003; Sova, Elyakova, & Vaskovcky, 1970).

Fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin, gentamicin, and the mediums RPMI-1640, DMEM were purchased from Biolot (Russia). CellTiter 96 nonradioactive cell proliferation assay kit was purchased from Promega (USA).

Mass spectrometric (MS) experiments were performed using ultra pure water, produced with Direct-Q 3 equipment (Millipore, USA). DHB (2,5-dihydroxybenzoic acid) matrix for MALDI-TOFMS was purchased from Sigma (USA).

2.2. Instruments

NMR spectra were obtained using an Avance DPX-500 NMR spectrometer (Bruker, Germany) resonating at 75.5 MHz at 60 °C.

MALDI-TOFMS spectra were recorded using an Ultra Flex III MALDI-TOF/TOF mass spectrometer (Bruker, Germany) with a nitrogen laser (337 nm), reflector and potential LIFT tandem modes of operation.

ESIMS spectra were recorded using an ESI Q-TOF mass spectrometer (Agilent 6510 LC Q-TOF, USA) with a dual electrospray-ionization source.

GLC–MS of alditol acetate derivatives were performed using a Hewlett-Packard 6850 (USA) chromatograph equipped with HP-5MS capillary column (30 m × 0.2 mm) with a temperature gradient of 150 → 230 °C at 3 °C min^{-1}.

2.3. General methods

The content of carbohydrates was determined using the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The content of reduced sugars was carried out using Nelson method (Nelson, 1944).

2.3.1. Smith degradation of laminaran

A sample of the laminaran EbL (40 mg) was dissolved in water (10 mL), added 0.4 M $NaIO_4$ (10 mL) and kept at 4 °C for 24 h. The reaction was stopped by the addition of 1 M ethylene glycol; the mixture was dialyzed against distilled water, and the product obtained was reduced with $NaBH_4$ (120 mg) for 32 h. The excess $NaIO_4$ was neutralized by adding 1 M acetic acid. The solution was dialyzed against distilled water and lyophilized. Acid hydrolysis was carried out with 2 M TFA at 100 °C for 6 h. TFA was neutralized with aqueous NH_3, and the solution was concentrated under a vacuum. The concentrate was then applied onto a Bio-Gel P-2 column (120 × 1 cm, 60 °C, Bio-Rad, USA) and eluted with water. The fractions were concentrated under a vacuum and lyophilized to get the preparations EbLdS1 (5.7 mg), EbLdS2 (4.8 mg) and EbLdS3 (7.4 mg).

2.3.2. Methylation of laminaran

Laminaran was methylated using a modification of the previously described NaOH slurry method (Ciucanu & Kerek, 1984). A sample of the laminaran (2 mg) was solubilized in DMSO (1 mL), and powdered NaOH (100 mg) was added to the solution followed by MeI (0.2 mL). The mixture was stirred for 20 min, and the addition of NaOH and MeI was repeated. The mixture was stirred for 1 h and then cooled on ice. The reaction was stopped by the addition of 1 mL of water. The excess MeI was removed by concentrating under a vacuum, and the resulting solution was passed through a Silica gel 100 C18 column (2.5 × 0.7 cm, Sigma, USA). Methylated laminaran was eluted with 50% MeOH, concentrated under a vacuum, and hydrolyzed with 2 M TFA at 100 °C for 6 h. TFA was neutralized with aqueous NH_3, and the solution was concentrated and lyophilized under a vacuum. Then, the resulting monosaccharides were reduced with $NaBH_4$ and acetylated with Ac_2O in pyridine. Partially methylated alditol acetates were analyzed by GLC–MS as previously described (Bjorndal, Hellerquist, Lindberg, & Svensson, 1970).

2.3.3. Enzymatic hydrolysis

Two samples of the laminaran EbL (3 mg) were dissolved in 0.025 M succinate buffer, pH 5.5 (1 mL). The endo-1,3-β-D-glucanase (LIV) from *Pseudocardium sacchalinensis* (0.1 mL, 10^{-2} units) was added to the first sample of EbL, while the exo-1,3-β-D-glucanase from *Chaetomium indicum* (0.1 mL, 10^{-2} units) was added to the second sample. The incubation was carried out at 37 °C. The same experiment was carried out with the laminaran ScL from *S. cichorioides*. Aliquots (0.05 mL) were taken for registration of the products of enzyme hydrolysis in some period of time. The reaction was stopped by adding 2.5% aqueous NH_3 (0.02 mL).

2.3.4. NMR spectroscopic analysis

^1H, ^{13}C NMR and 2D NMR (COSY, TOCSY, HSQC) spectra for solutions of oligo- and polysaccharides in D_2O with acetone as the internal standard were recorded at 60 °C. The concentration of the samples was 5–20 mg of oligo- or polysaccharide/mL of D_2O.

2.3.5. Mass spectrometric analysis

MALDI-TOFMS. Analyses were carried out in a positive-ion mode using saturated DHB solution in ethanol as a matrix. Sample solution (0.01 mg/mL in water) was prepared from lyophilized samples. "Thin layer" technique was used for sample preparation: 1 μL of matrix solution was applied to a stainless steel plate and allowed to dry. Then, 1 μL of sample solution was applied as the second layer and air dried. Instrument was precalibrated using matrix and angiotensin-II (Sigma, USA) ion signals.

ESIMS. Spectra were acquired in a positive-ion mode with precalibration using a standard "HP-mix". Capillary voltage was set to 4000 V, and the drying gas temperature was 325 °C. Fragmentor voltage was set to 160 V. The isolation window for MS/MS experiments was set to 1.3 mass units for singly-charged ions. Collision energy was optimized between 10 and 45 V to reach abundant intensity of fragment ions. Dried sample was dissolved in 1:1 acetonitrile–water (concentration of the sample was approx. 0.01 mg/mL) and introduced into the mass spectrometer at flow rate of 5 μL/min using a syringe pump (KD Scientific, USA).

2.4. Obtaining of the products of enzymatic hydrolysis

2.4.1. Obtaining of the products of hydrolysis by endo-1,3-β-D-glucanase

Sample of the laminaran EbL (60 mg) was dissolved in 0.025 M succinate buffer, pH 5.5 (20 mL) and the endo-1,3-β-D-glucanase from *P. sacchalinensis* (0.5 mL, 10^{-2} units) was added. The mixture was incubated at 37 °C for 22 h, and the new portion of enzyme (0.1 mL) was then added before the sample was incubated for an additional 2 h. Products of enzymatic hydrolysis were concentrated under a vacuum and lyophilized.

2.4.2. Obtaining of the products of hydrolysis by exo-1,3-β-D-glucanase

Sample of the laminaran EbL (60 mg) was dissolved in 0.025 M succinate buffer, pH 5.5 (20 mL), and the exo-1,3-β-D-glucanase from *C. indicum* (0.5 mL, 10^{-2} units) was added. The mixture was incubated at 37 °C for 3.5 h; then part of the solution (10 mL) was removed to check the products of reaction. Hydrolysis of this sample (EbLexo′) was terminated by heating at 100 °C for 5 min. The incubation of the remaining solution was continuous for an additional 18.5 h, and the new portion of enzyme (0.1 mL) was then added before the sample was incubated for an additional 2 h. Products of enzymatic hydrolysis were concentrated under a vacuum and lyophilized.

2.4.3. Gel-filtration chromatography

Mixtures of the oligosaccharides (EbLendo and EbLexo) obtained after enzymatic hydrolysis were applied to a Bio-Gel P-2 column (120 × 1 cm, 60 °C, "Bio-Rad", USA) and eluted with water. The fractions were concentrated under a vacuum and lyophilized to get the preparations EbLendo1 (22.2 mg), EbLendo2 (8.5 mg), EbLendo3 (10.7 mg), EbLendo4 (3.6 mg) и EbLexo1 (5.6 mg), EbLexo2 (1.9 mg), EbLexo3 (1.4 mg), EbLexo4 (3.1 mg), EbLexo5 (10.4 mg).

2.5. Anticancer activity in vitro

2.5.1. Cell culture

The SK-MEL-28 (ATCC # HTB-72™) human skin melanoma cell line was grown in monolayer in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin in humidified atmosphere containing 5% CO_2. The DLD-1 (ATCC # CCL-221™) human colon cancer cell line was grown in monolayer in RPMI-1640 supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin in humidified atmosphere containing 5% CO_2.

2.5.2. Cell cytotoxicity assay

To estimate cell cytotoxicity, cells (SK-MEL-28 and DLD-1) were seeded (3×10^4) in 96-well plates in 200 μL of DMEM (SK-MEL-28) or RPMI-1640 (DLD-1) supplemented with 10% FBS and incubated at 37 °C in a 5% CO_2 incubator. After 24 h, the medium was removed and replaced by a fresh medium containing the different concentrations (50, 100, 200, and 400 μg/mL) of the oligo- and polysaccharides for additional 24, 48 and 72 h at 37 °C in a 5% CO_2 incubator. After incubation, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS reagent) (10 μL) was added to each well, and cells were then incubated for 4 h at 37 °C and 5% CO_2. Absorbance was measured at 490/630 nm.

2.5.3. Soft agar clonogenic assay

Soft agar assay was performed on human melanoma and colon cancer cells, SK-MEL-28 and DLD-1. In brief, cells (2.4×10^4/mL) were growth in 1 mL of 0.3% basal medium Eagle's agar containing 10% FBS. The culture was maintained at 37 °C in 5% CO_2 incubator for 3 weeks and the cell colonies were scored using a microscope and the ImageJ computer software program as described by Colburn, Wendel, and Abruzzo (1981).

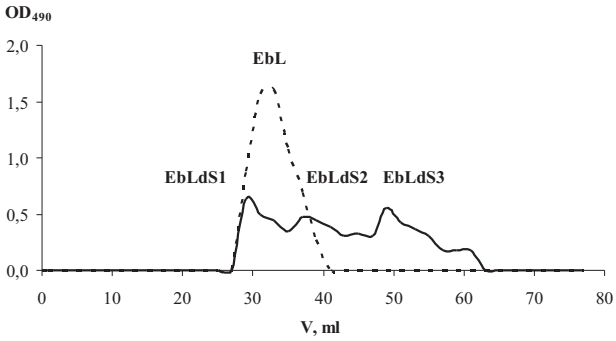

Fig. 1. Gel-filtration of the laminaran EbL and its Smith degradation products on a Bio-Gel P-2 column (120 × 1 cm).

2.5.4. Data analysis

All figures shown in this study are representative of at least three independent experiments with similar results. Statistical differences were evaluated using the Student's *t*-test and considered significant at $p \leq 0.05$.

3. Results and discussion

3.1. Methylation analysis of laminaran

Previously we isolated the laminaran EbL (1,3;1,6-β-D-glucan) with a ratio of bonds as 1,3:1,6 = 1.5:1 and high molecular weight (19–27 kDa) from brown alga *E. bicyclis* (Republic of Korea) (Menshova et al., 2013).

Structural characteristics of the EbL were studied by different methods. Methylation was used for linkage analysis of polysaccharides. 2,3,4,6-Tetra-OMe-Glc; 2,4,6-tri-OMe-Glc; 2,3,4-tri-OMe-Glc and 2,4-di-OMe-Glc, corresponding non-reduced, 1,3-, 1,6- and 1,3,6-linked glucose residues in a ratio of 1:2.8:1.2:0.8, were identified as partially methylated alditol acetates by GLC–MS. The presence of a large number of non-reduced ends and branched points proved a branched structure of polysaccharide. According to the literature data (Usui et al., 1979), 2,3,4,6-tetra-OMe-Glc, 2,4,6-tri-OMe-Glc, 2,3,4-tri-OMe-Glc and 2,4-di-OMe-Glc were obtained as partially methylated alditol acetates in a ratio of 1:3.8:2.0:0.8 from the results of methylation analysis of laminaran from *E. bicyclis* (Japan).

3.2. Smith degradation of laminaran

Depolymerization of the laminaran EbL took place as a result of Smith degradation. Because the products of degradation were a mixture of oligosaccharides, it can be concluded that 1,6-linked glucose residues were included in the main chain of laminaran. Three fractions of oligosaccharides, EbLdS1, EbLdS2 and EbLdS3, were isolated by gel-filtration on Bio-Gel P-2 (Fig. 1).

These fractions were studied by MALDI-TOFMS and NMR spectroscopy (spectra not shown). According to the mass spectrometry data, oligosaccharides from the fractions EbLdS1 and EbLdS2 have intervals of degree of polymerization 7–18 and 5–9, respectively. The ^{1}H NMR spectra of EbLdS1 and EbLdS2 contained not only signals characteristic of H-1 of 1,3-linked β-D-glucose residues (4.7–4.8 ppm), but also low intensive peaks 4.55 and 4.52 ppm, corresponding to H-1 and H-6 of 1,6-linked glucose. The ratio of bonds 1,3:1,6 of oligosaccharides from the fractions EbLdS1

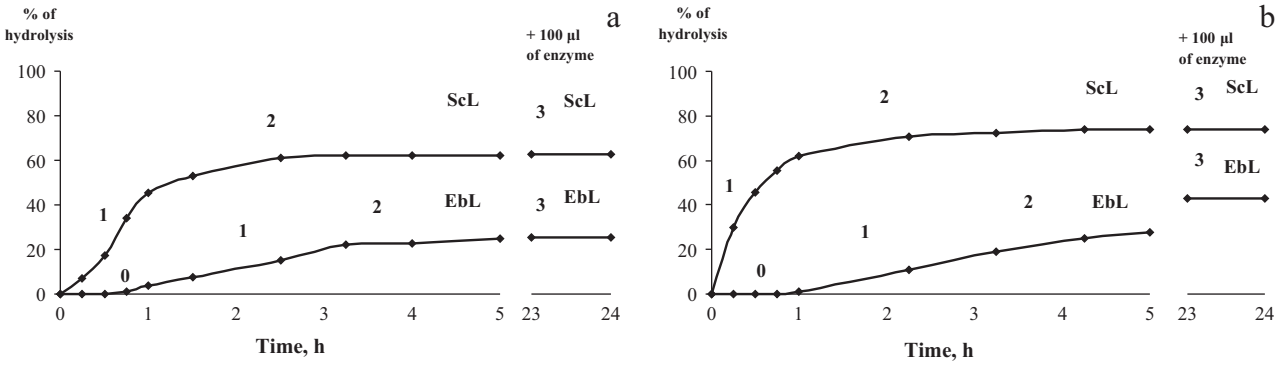

Fig. 2. The dynamics of accumulation of products of the laminarans ScL and EbL hydrolysis catalyzed by endo- (a) and exo-1,3-β-D-glucanases (b) based on the Nelson method.

and EbLdS2 was about 4:1. It was suggested that some glucose residues in the laminaran were glycosylated at C-6 of residues of laminarioligosaccharides. Thus, the construction of these fragments was stabilized under conditions of Smith degradation. ^{1}H NMR spectrum of low molecular weight the fraction EbLdS3 contained only peaks that were characteristic of 1,3-linked β-D-glucose residues (4.7–4.8 ppm). Mass spectrum of EbLdS3 included signals m/z $[M+\text{glycerol}]^+$ in interval 439.3–763.5, corresponding to a degree of polymerization of oligosaccharides 2–4. Based on these data, we suggested that fragments built from 1,3-linked β-D-glucose residues, either with or without a single glucose residue in branches at C-6, contained no more than 4 glucose residues.

3.3. Enzymatic hydrolysis of laminaran

The laminaran EbL was investigated using enzymes with a known type of action. We studied the dynamics of the accumulation of hydrolysis products of the laminarans from *S. cichorioides* (ScL) and *E. bicyclis* (EbL) by enzymes differing in their type of action: endo-1,3-β-D-glucanase LIV from *P. sacchalinensis* and exo-1,3-β-D-glucanase from *C. indicum*. The laminaran ScL from *S. cichorioides* with known structural characteristics (1,3;1,6-β-D-glucan, ratio of bonds 1,3:1,6 = 9:1) (Zvyagintseva et al., 1994) was used in the enzymatic processes for comparison with the laminaran EbL (Fig. 2).

The accumulation of reduced sugars during enzymatic hydrolysis must have a linear dependence from time until the rate of reaction is slowed down due to the disappearance of the substrate. For the reaction of endo- and exo-1,3-β-D-glucanases with the laminaran ScL, the curves of accumulation of hydrolysis products consist of three stages. The first stage (1) showed linear dependence of the accumulation of hydrolysis products with reaction time. In the second stage (2), the accumulation of hydrolysis products slowly decreased due to the consumption of the substrate, and then the stage with the plateau began (3). In contrast to the laminaran ScL, the curves of the hydrolysis of the laminaran EbL by action endo- and exo-1,3-β-D-glucanases contained lag-periods (0). Their presence indicated that laminaran EbL consisted of fragments containing a large amounts of 1,6-linked glucose residues. Additionally, the lag-period for reaction with exo-1,3-β-D-glucanase may also be due to the presence of significant amounts of 1,6-linked glucose residues on the non-reduced ends of polysaccharide molecules. Degrees of exhaustive hydrolysis of the laminarans EbL and ScL by endo-1,3-β-D-glucanases were 25.2% and 62.2%, respectively, and by exo-1,3-β-D-glucanases were 42.8% and 74.1%, respectively.

The accumulation of the laminaran EbL and ScL enzymatic hydrolysis products with a degree of polymerization of 2–5 based on ESIMS kinetics was showed in Fig. 3. A sensitivity of ESIMS in relation to glucose in comparison with other products, unfortunately, is very low and cannot be correctly determined by the method (Bezukladnikov, Elyakova, Zvyagintseva, & Mirgorodskaya, 1989; Bezukladnikov, 1990). The disaccharide was the main product of the laminaran ScL hydrolysis by endo-1,3-β-D-glucanase using ESIMS detection (Fig. 3a). Tri- and tetrasaccharides were accumulated less than disaccharide. Pentasaccharide was accumulated at the beginning of the reaction and then used with enzyme as substrate. The characteristic of the laminaran EbL hydrolysis by endo-1,3-β-D-glucanase was different from the laminaran ScL hydrolysis (Fig. 3b). Trisaccharide was the main product of enzymatic reaction, while the formation of di- and tetrasaccharides was weak. It was suggested that the rate of hydrolysis of pentasaccharide was similar to the rate of its appearance.

Dependence of the accumulation of the laminaran ScL hydrolysis products by action of exo-1,3-β-D-glucanase from time was shown in Fig. 3c. Oligosaccharides were accumulated consecutively: initially di-, then tri-, tetra- and, finally, pentasaccharide. In contrast to the hydrolysis of the laminaran ScL by endo-1,3-β-D-glucanase, in the case of reaction with exo-1,3-β-D-glucanase, the formation of oligosaccharides was not immediately observed. Apparently, in the beginning of the reaction only glucose was produced, and then oligosaccharides were formed from fragments of the laminaran, which were unavailable for enzyme attack. The dependence of the accumulation of the laminaran EbL hydrolysis products by exo-1,3-β-D-glucanase with time was similar to the laminaran ScL (Fig. 3d). However, the amount of tetrasaccharide was higher than trisaccharides in hydrolysis products of the laminaran EbL. This was due to peculiarities of substrate structure, and the presence of a large amount of 1,6-linked glucose residues. In addition, quantities of hydrolysis products from the laminaran ScL by endo- and exo-1,3-β-D-glucanases were higher compared with the laminaran EbL. The main product of the enzymatic hydrolysis of ScL was disaccharide. Products of EbL hydrolysis included large amounts of di-, tri-, tetra- and pentasaccharides.

Thus, the kinetics of accumulation of the laminaran hydrolysis products by endo- and exo-1,3-β-D-glucanases were shown to be significantly different according to peculiarities of the structures of the substrates.

3.4. NMR analysis of laminaran and its enzymatic hydrolysis products

The laminaran EbL was investigated by 1D and 2D NMR spectroscopy (^{1}H, ^{13}C, COSY, TOCSY, HSQC). Results of the investigation showed that the ^{1}H NMR spectrum of EbL contains 4 groups of signals in anomeric region, corresponding the following fragments of structure: →3)-β-D-Glc*p*-(1→3)- and/or →3,6)-β-D-Glc*p*-(1→3)- (4.77 ppm); →6)-β-D-Glc*p*-(1→3)- and/or β-D-Glc*p*-(1→3)- (4.72 ppm); →3)-β-D-Glc*p*-(1→6)- and/or

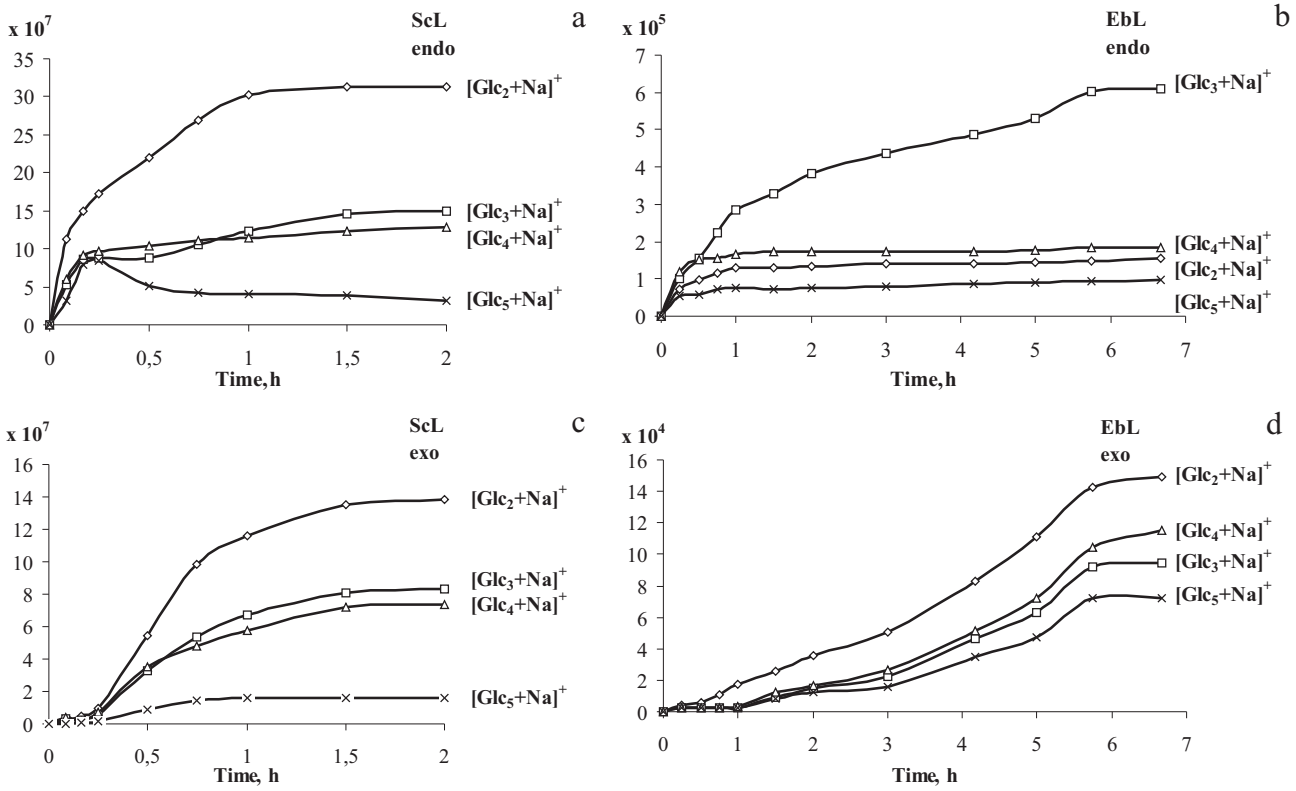

Fig. 3. The kinetics of the accumulation of the laminarans ScL and EbL hydrolysis products by endo- (a, b) and exo-1,3-β-D-glucanases (c, d) based on ESIMS experiments. (A) H1 →3)-β-D-Glc*p*-(1→3)- and/or →3,→6)-β-D-Glc*p*-(1→3)-; (B) H1 →6)-β-D-Glc*p*-(1→3)- and/or β-D-Glc*p*-(1→3)-; (C) H1 →3)-β-D-Glc*p*-(1→6)- and/or →3,→6)-β-D-Glc*p*-(1→6)-; (D) H1 →6)-β-D-Glc*p*-(1→6)- and/or β-D-Glc*p*-(1→6)-; (E) H1 →)-α-D-Glc*p* and/or α-D-Glc*p*; and (F) H1 →)-β-D-Glc*p* and/or β-D-Glc*p*.

→3,6)-β-D-Glc*p*-(1→6)- (4.55 ppm); →6)-β-D-Glc*p*-(1→6)- and/or β-D-Glc*p*-(1→6)- (4.52 ppm) (Fig. 4a).

In order to obtain the enzyme hydrolysis products with the aim of investigating its structural characteristics and anticancer activity, the laminaran EbL was incubated with endo- (using a mixture of oligosaccharides EbLendo) and exo-1,3-β-D-glucanases (EbLexo′ and EbLexo). The fractions EbLendo, EbLexo′ and EbLexo were obtained at laminaran degrees of hydrolysis of 25.2%, 18.7% and 42.8%, respectively.

The ^{1}H NMR spectrum of fraction of hydrolysis products EbLendo was demonstrated in Fig. 4. Signals that were characteristic of structural fragments →3)-β-D-Glc*p*-(1→3)- and/or

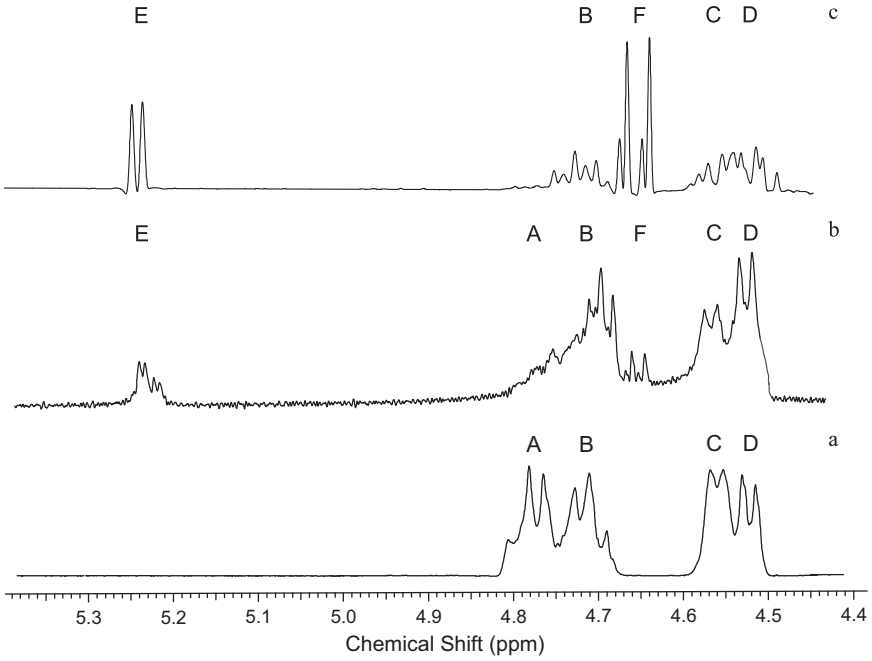

Fig. 4. ^{1}H NMR spectra of the laminaran EbL (a) and its enzyme hydrolysis products EbLendo (b) and EbLexo (c).

→3,6)-β-D-Glc*p*-(1→3)- almost completely disappeared. The intensity of peaks corresponding to fragments →6)-β-D-Glc*p*-(1→3)- and/or β-D-Glc*p*-(1→3)-; →6)-β-D-Glc*p*-(1→6)- and/or β-D-Glc*p*-(1→6)- in comparison with fragments →3)-β-D-Glc*p*-(1→6)- and/or →3,6)-β-D-Glc*p*-(1→6)- was found to increase. This was due to increasing of amounts of non-reduced ends β-D-Glc*p*-(1→3)- and β-D-Glc*p*-(1→6)- in the process of enzyme hydrolysis. New signals 5.25 ppm and 4.67 ppm, corresponding to H-1 of α- and β-D-glucose residues, respectively, appeared. The ratio of bonds 1,3:1,6 decreased from 1.5:1 to 1:1, thus, about third of 1,3-linkages was hydrolyzed by endo-1,3-β-D-glucanase.

^{1}H NMR spectrum of fraction of hydrolysis products EbLexo was demonstrated in Fig. 4. Peaks, corresponding to structural fragments →3)-β-D-Glc*p*-(1→3)- and/or 3,6)-β-D-Glc*p*-(1→3)-, were absent. The intensity of peaks corresponding to fragments →6)-β-D-Glc*p*-(1→3)- and/or Glc*p*-(1→3)- decreased significantly. New signals of 5.25 ppm and 4.67 ppm, corresponding to H-1 of α- and β-D-glucose residues, respectively, appeared. The ratio of bonds 1,3:1,6 changed from 1.5:1 to 0.6:1; thus, about two-thirds of the 1,3-linkages were hydrolyzed by exo-1,3-β-D-glucanase.

3.5. Isolation and structural characteristics of oligosaccharides

Products of the enzymatic hydrolysis EbLendo and EbLexo were separated on Bio-Gel column (Fig. 5). As a result, the preparations of the glucooligosaccharides EbLendo1, EbLendo2, EbLendo3, EbLendo4 и EbLexo1, EbLexo2, EbLexo3, EbLexo4, and EbLexo5 were obtained, which differed in degrees of polymerization and the ratios of bonds 1,3:1,6. EbLexo′1 was the highest molecular weight fraction isolated from EbLexo′ by gel-filtration on Bio-Gel P-2. Characteristics of laminarioligosaccharides are presented in Table 1.

Previously, Pang et al. studied the hydrolysis of laminaran from *E. bicyclis* (Japan) by endo-1,3-β-D-glucanase from *Arthrobacter* sp. (Pang et al., 2005). They isolated three main fractions with a degree of polymerization of 4–5 (8.4%), 6–7 (8.8%) and more than 8 (26.0%). The reaction products contained glucose and trisaccharide however, in contrast to our results, disaccharide was absent.

The fraction EbLendo3 obtained by gel-filtration on Bio-Gel (yield 17.8%), consisting of mainly trisaccharide and traces of disaccharide, was investigated by 1D and 2D NMR spectroscopy (^{1}H, ^{13}C, COSY, TOCSY, HSQC). ^{1}H and ^{13}C NMR spectra of EbLendo3 contained signals with chemical shifts, which are characteristic for both 1,3- and 1,6-linked glucose residues. 2D-spectroscopy analysis showed that trisaccharide mainly has the following structure: β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→3)-D-Glc (chemical shifts are shown in Table 2).

The yield of fraction EbLendo4, containing glucose, was 6.1% from the native laminaran EbL. It is possible to measure the amount of glucose in 1,3-linked blocks without branches at C-6 with a degree of polymerization of more than 2. Thus, based on the degree of laminaran hydrolysis by endo-1,3-β-D-glucanase (25.2%), the main part of 1,3-linked blocks that were available for enzyme attack were disaccharide fragments. This conclusion was shown by the small amounts of disaccharide from reaction products and the results of Smith degradation of laminaran.

MALDI-TOFMS analysis of the low molecular fractions EbLexo5, EbLexo4, EbLexo3 and EbLexo2, which were obtained by gel-filtration on Bio-Gel P-2 column, showed those fractions to contain mono, di-, tri- and tetrasaccharides, respectively (Table 1). Exhaustive enzymatic hydrolysis of *E. bicyclis* (Japan) laminaran by exo-1,3-β-D-glucanase from *Basidiomycetes* sp. QM806 was described (Nanjo, Usui, & Suzuki, 1984). It was found that laminaran degraded to glucose, gentiobiose, gentiotriose and gentiotetraose.

Both ^{1}H and ^{13}C NMR spectra (not shown) of the fraction EbLexo4 corresponded to gentiobiose spectra (Nanjo et al., 1984). Signals indicating the presence of other linkages were absent. ^{1}H and ^{13}C NMR spectra (not shown) of the fraction EbLexo3 contain peaks, corresponding to gentiotriose and trisaccharide with the structure β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→6)-D-Glc in a ratio of 0.3:0.7. The fraction EbLexo2 was a mixture of tetrasaccharides based on NMR spectroscopic analysis. Tandem ESIMS of the $[Glc_4 + Na]^+$-ion at *m*/*z* 689.17 from the fraction EbLexo2 contained series of signals of type $^{0,2}X/^{0,2}A$, corresponding to glucose residues from tetrasaccharide (Fig. 6). Since the mechanism of formation of the above-mentioned fragment ions that required a free proton at a hydroxyl, located at C-3 (Tissot, Salpin, Martinez, Gaigeot, & Daniel, 2006), it was suggested that tetrasaccharide consisted of 1,4- or 1,6-linked glucose residues. Based on MS/MS and NMR data, gentiotetraose was identified as one of the tetrasaccharides from the EbLexo2 fraction.

Based on difference in structures of oligosaccharides, obtained by exo-1,3-β-D-glucanase hydrolysis, we suggested, that these oligosaccharides were derived from structurally different fragments of the native laminaran. We suggested that products of the laminaran EbL hydrolysis by exo-1,3-β-D-glucanase from *C. indicum*, consisting of only 1,6-linked glucose residues (gentiobiose, gentiotriose and gentiotetraose) were formed from fragments of branches of native laminaran. Recently, it was also shown that gentiobiose was obtained after enzymatic hydrolysis of the laminaran from *S. cichorioides* (the main chain consisted 1,3-linked glucose with a single glucose residues branches at C-6) (Shirokova & Elyakova, 1983). However, products containing 1,3-linked glucose on a non-reduced end, such as β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→6)-D-Glc and β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→6)-D-Glc, were obtained from fragments of the main laminaran chain.

3.6. Anticancer activity of laminaran and its enzyme hydrolysis products

As a result of enzymatic hydrolysis of the laminaran, we obtained fractions of oligosaccharides and studied their structural characteristics (degree polymerization intervals and ratio of bonds 1,3:1,6). The highest molecular weight fractions EbLendo1, EbLendo2, EbLexo′1 and EbLexo1 with known structural characteristics and the native laminaran EbL were selected to examine the effect on the cytotoxicity of SK-MEL-28 human melanoma and DLD-1 human colon cancer cells with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and inner salt (MTS assay). Some correlations were determined: “structural characteristics–anticancer effect”. These samples did not show any significant cytotoxicity after treatment for 24 h, 48 h, and 72 h at concentrations from 1 to 200 μg/mL. These results indicated that EbL, EbLendo1, EbLendo2, EbLexo′1 and EbLexo1 were less cytotoxic to SK-MEL-28 and DLD-1 human melanoma and colon cancer cells.

SK-MEL-28 and DLD-1 human melanoma and colon cancer cells were treated with 100 μg/mL of the laminaran and laminarioligosaccharides in a soft agar matrix and incubated at 37 °C in a 5% CO_2 incubator for 15 days for SK-MEL-28 cells, and 20 days for DLD-1 cells. We determined whether all samples could have an inhibitory effect on the transformation of SK-MEL-28 and DLD-1 cells.

The potency of inhibition of DLD-1 cell transformation by EbL, EbLendo1, EbLendo2, EbLexo′1 and EbLexo1 was higher than SK-MEL-28 cells. The main correlations were observed for both cell lines (Fig. 7).

The high molecular weight laminaran EbL inhibited cell transformation of SK-MEL-28 and DLD-1 cells by 15% and 38%, respectively. The fraction EbLendo1 and EbLexo′1 with interval of

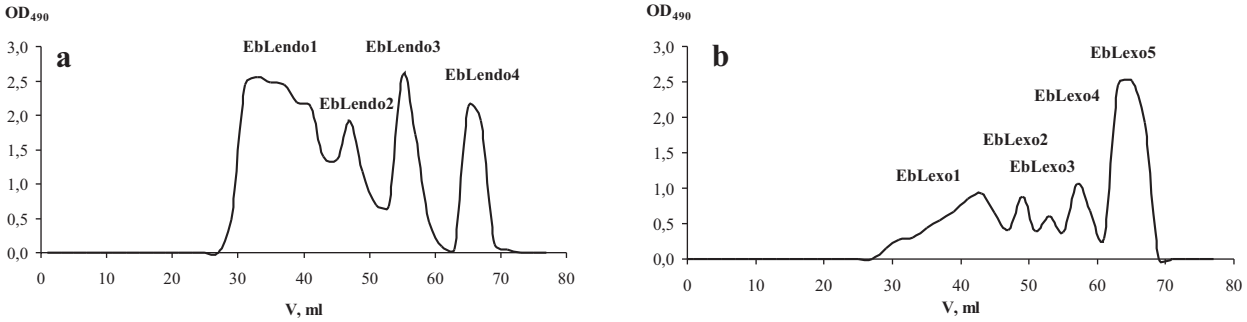

Fig. 5. Gel-filtration of enzyme hydrolysis products of the laminaran EbL – EbLendo (a) and EbLexo (b) on a Bio-Gel P-2 column (120 × 1 cm).

Table 1
Characteristics of enzymatic hydrolysis of laminaran products EbL by endo- and exo-1,3-β-D-glucanase.

Fraction	Yield (%)*	Degree of polymerization**	Ratio of bonds 1,3:1,6***
Endo-1,3-β-D-glucanase			
EbLendo1	37.0	Glc_9–Glc_{23}	1:1
EbLendo2	14.2	Glc_4–Glc_{12}	0.9:1
EbLendo3	17.8	Glc_2–Glc_3	1:1
EbLendo4	6.1	Glc	–
Exo-1,3-β-D-glucanase			
EbLexo′1	–	Glc_9–Glc_{23}	1.4:1
EbLexo1	18.7	Glc_4–Glc_{12}	0.6:1
EbLexo2	6.3	Glc_4	0.5:1
EbLexo3	4.7	Glc_3	0.6:1
EbLexo4	10.3	Glc_2	0:1
EbLexo5	34.7	Glc	–

* % of laminaran sample weight.
** Obtained by MALDI-TOFMS.
*** Obtained by ^{1}H NMR spectroscopy.

Table 2
NMR data for fraction EbLendo3.

Residue	13C chemical shifts (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*-(1→6)-	103.7	74.2	76.4	70.6	76.8	61.7
→6)-β-D-Glc*p*-(1→3)-	103.9	74.3	76.4	70.5	75.7	69.8
→3)-β-D-Glc*p*	96.7	74.5	86.4	69.2	76.6	61.7
→3)-α-D-Glc*p*	92.9	71.7	84.2	70.5	72.1	61.6
Residue	^{1}H chemical shifts (ppm)					
	H-1	H-2	H-3	H-4	H-5	H-6
β-D-Glc*p*-(1→6)-	4.52	3.33	3.53	3.43	3.47	3.75; 3.92
→6)-β-D-Glc*p*-(1→3)-	4.69	3.42	3.57	3.54	3.64	3.89; 4.22
→3)-β-D-Glc*p*	4.67	3.45	3.74	3.55	3.44	3.77; 3.94
→3)-α-D-Glc*p*	5.25	3.75	3.90	3.53	3.88	3.76; 3.84

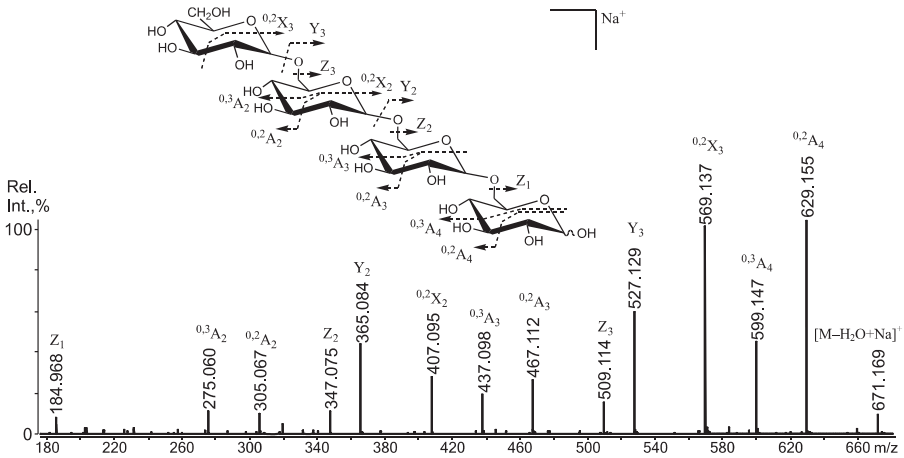

Fig. 6. Tandem ESIMS of the $[Glc_4 + Na]^+$-ion at *m*/*z* 689.17 from the fraction EbLexo2.

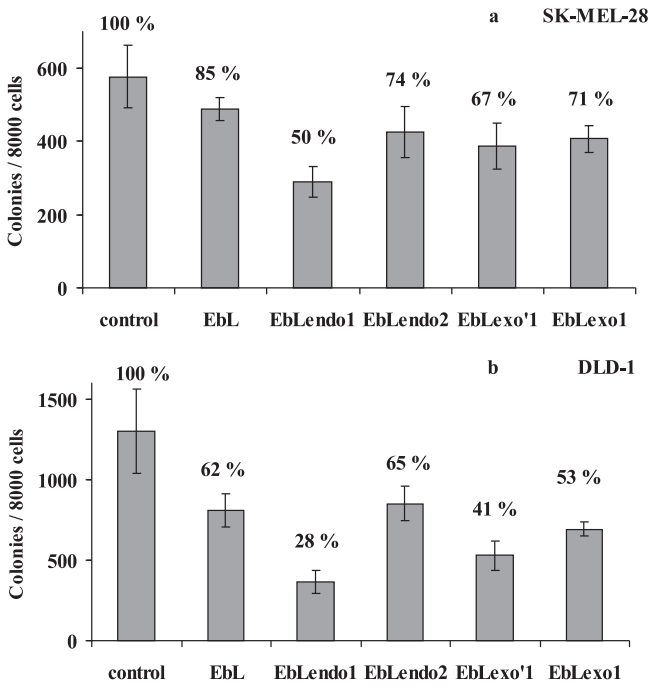

Fig. 7. The inhibitory effects of the fractions EbL, EbLendo1, EbLendo2, EbLexo′1 и EbLexo1 on colony formation in human skin melanoma cell line SK-MEL-28 (a) and human colon cancer cells DLD-1 (b) comparing untreated control cells.

oligosaccharides degree of polymerization 9–23 showed the highest effect on both cell lines in comparison with EbL. Decreasing molecular weight to 4–13 glucose residues (the fraction EbLendo2 and EbLexo1) led to a decrease in activity. Comparison of the anticancer effect of EbLendo1 with EbLexo′1, and EbLendo2 with EbLexo1, characteristics the same interval of molecular weight and different ratios of bonds 1,3:1,6 (Table 1), showed an increase of anticancer activity with increasing contents of 1,6-linked glucose residues.

According the literature data, the effect of laminaran from *E. bicyclis* (Japan) and its enzyme hydrolysis products on human monocytes to inhibit the proliferation of U937 cells was investigated. The native laminaran with molecular weight about 6 kDa and ratio of bonds 1,3:1,6 = 1.5:1 did not show an anticancer effect. Oligosaccharides with a degree of polymerization of 4–5 and 6–7 were less active. Only the fraction with an average degree of oligosaccharide polymerization of 13 and ratio of bonds 1,3:1,6 = 1.5:1 showed a significant effect (Pang et al., 2005; Usui et al., 1979). The literature data are in close agreement with our investigation: the inhibition of colony formation of cancer cells was increased with higher levels of 1,6-linked glucose residues in the laminaran and the enzymatic hydrolysis products.

4. Conclusions

As a result, the structure of the laminaran EbL can be represented as follows: the laminaran EbL was found to be a branched high molecular weight 1,3;1,6-β-D-glucan, building of the structural fragments →3)-β-D-Glc*p*-(1→3)- and/or →3,6)-β-D-Glc*p*-(1→3)-; →6)-β-D-Glc*p*-(1→3)- and/or β-D-Glc*p*-(1→3)-; →3)-β-D-Glc*p*-(1→6)- and/or →3,6)-β-D-Glc*p*-(1→6)- and →6)-β-D-Glc*p*-(1→6)- and/or β-D-Glc*p*-(1→6)-. It was shown that the laminaran EbL contained about 18% of the sites, including structural fragments with the structure →3)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→, various versions of which are shown in Fig. 8 (Structure 1).

The laminaran EbL was characterized by a high content of 1,6-linked glucose residues (ratio of bonds 1,3:1,6 = 1.5:1), which are both in the branches and in the main chain of laminaran. The degree of polymerization of the fragments, building from 1,3-linked glucose residues with single glucose branches at C-6 or without it, was no more than four glucose residues (Fig. 8; Structure 2). The main part of the 1,3-linked glucose blocks were disaccharide fragments. It was suggested that 1,6-linked glucose residues were on non-reduced ends of molecules. The degree of polymerization of 1,6-linked blocks was no more three glucose residues (Fig. 8; Structure 3). Based on the results of the investigation, the laminaran can contain single glucose, laminarioligosaccharide residues (Fig. 8; Structire 1c), gentiobiose and gentiotriose residues in the branches at C-6 (Structure 4).

The anticancer activity of the native laminaran EbL and products of its enzymatic hydrolysis EbLendo1, EbLendo2, EbLexo′1 and EbLexo1 was examined on SK-MEL-28 human melanoma and DLD-1 human colon cancer cells. It was shown that all of the samples inhibited cell transformation of both cell lines. The potency of inhibition of DLD-1 cell transformation by the laminaran and glucooligosaccharides was higher than SK-MEL-28 cells. Thus, some correlations of the anticancer effect of the oligo- and polysaccharides, obtained from the brown alga *E. bicyclis*, and their structural characteristics were determined. Decreasing the native laminaran molecular weight to a determined limit and increasing the content of 1,6-linked glucose residues increased the anticancer effect. Thus, the most perspective anticancer agent was a fraction of the 1,3;1,6-β-D-glucooligosaccharides EbLendo1.

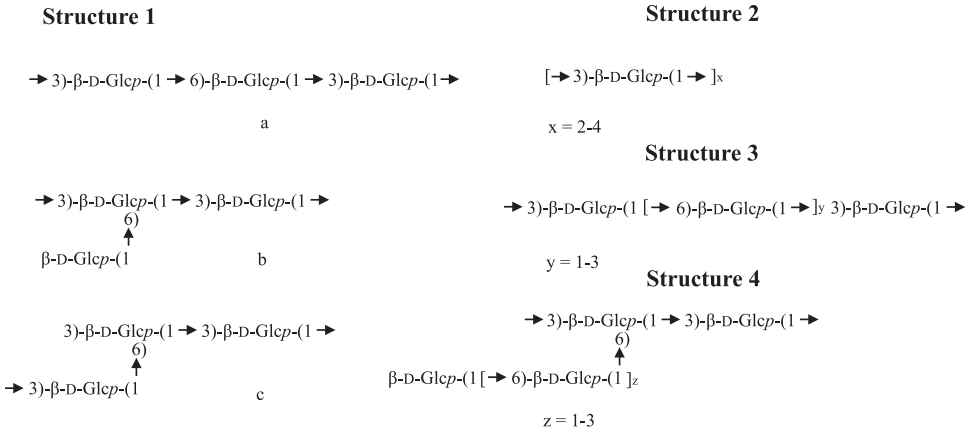

Fig. 8. Fragments of structure of the laminaran EbL.

Acknowledgements

This work was supported by the grants of REBR (11-04-98514-a, 12-04-00669-a and 12-04-31713-mol_a), FEB RAS, and the Program "Molecular and Cellular Biology".

References

Bezukladnikov, P. W. (1990). Not the 1st. *Nature*, *348*, 480.

Bezukladnikov, P. V., Elyakova, L. A., Zvyagintseva, T. N., & Mirgorodskaya, O. A. (1989). Study of reactions catalyzed by carbohydrases by the mass-spectrometry method with diluted ion extraction at atmospheric-pressure (in Russian). *Khimiya Prirodnykh Soedinenii*, *1*, 54–59.

Bjorndal, H., Hellerquist, C. G., Lindberg, B., & Svensson, S. (1970). Gas–liquid chromatography and mass spectrometry in methylation analysis of polysaccharides. *Angewandte Chemie*, *9*, 610–619.

Burtseva, Y. V., Verigina, N. S., Sova, V. V., Pivkin, M. V., & Zvyagintseva, T. N. (2003). Filamentous marine fungi as producers of O-glycosylhydrolases. β-1,3-Glucanase from *Chaetomium indicum*. *Marine Biotechnology*, *5*, 1–11.

Ciucanu, I., & Kerek, F. (1984). A simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research*, *131*, 209–217.

Colburn, N. H., Wendel, E. J., & Abruzzo, G. (1981). Dissociation of mitogenesis and late-stage promotion of tumor cell phenotype by phorbol esters: Mitogen-resistant variants are sensitive to promotion. *Proceedings of the National Academy of Sciences of the United States of America*, *78*, 6912–6916.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Elyakova, L. A., Isakov, V. V., Lapshina, L. A., Nagorskaya, V. P., Likhatskaya, G. N., Zvyagintseva, T. N., et al. (2007). Enzymatic transformation of biologically active 1,3;1,6-β-D-glucan. Structure and activity of resulting fragments (in Russian). *Biokhimia*, *72*, 29–36.

Ermakova, S., Men'shova, R., Vishchuk, O., Kim, S. M., Um, B. H., Isakov, V., et al. (2013). Water-soluble polysaccharides of brown algae *Eisenia bicyclis*: Structural characteristics and antitumor activity. *Algal Research*, *2*, 51–58.

Maeda, M., & Nishizawa, K. (1968). Fine structure of laminaran of *Eisenia bicyclis*. *Journal of Biochemistry*, *63*, 199–206.

Menshova, R. V., Ermakova, S. P., Um, B. H., & Zvyagintseva, T. N. (2013). The composition and structural characteristics of polysaccharides of the brown alga *Eisenia bicyclis*. *Russian Journal of Marine Biology*, *39*, 208–213.

Nanjo, F., Usui, T., & Suzuki, T. (1984). Mode of action of an exo-β-(1-3)-D-glucanase on the laminaran from *Eisenia bicyclis*. *Agricultural and Biological Chemistry*, *48*, 1523–1532.

Nelson, N. J. (1944). A photometric adaptation of the Somogyi method for the determination of sugars. *Journal of Biological Chemistry*, *153*, 375–381.

Pang, Z., Otaka, K., Maoka, T., Hidaka, K., Ishijima, S., Oda, M., et al. (2005). Structure of β-glucan oligomer from laminarin. *Bioscience, Biotechnology, and Biochemistry*, *69*, 553–558.

Shirokova, N. I., & Elyakova, L. A. (1983). Mechanism of action and specificity of exo-β-1,3-glucanase from the mollusk *Eulota maakii* (in Russian). *Biokhimia*, *48*, 83–91.

Sova, V. V., Elyakova, L. A., & Vaskovcky, V. E. (1970). Purification and some properties of β-(1→3)-glucan glucanohydrolase from the crystalline style of bivalvia, *Spisula sachalinensis*. *Biochimica et Biophysica Acta*, *212*, 111–115.

Tissot, B., Salpin, J. Y., Martinez, M., Gaigeot, M. P., & Daniel, R. (2006). Differentiation of the fucoidan sulfated L-fucose isomers constituents by CE-ESIMS and molecular modeling. *Carbohydrate Research*, *341*, 598–609.

Usui, T., Toriyama, T., & Mizuno, T. (1979). Structural investigation of laminaran of *Eisenia bicyclis*. *Agricultural and Biological Chemistry*, *43*, 603–611.

Zvyagintseva, T. N., Shirokova, N. I., & Elyakova, L. A. (1994). The structure of laminarins from some brown algae (in Russian). *Bioorganicheskaya Khimiya*, *20*, 1349–1358.

Zvyagintseva, T. N., Elyakova, L. A., & Isakov, V. V. (1995). The enzymic transformation of laminarans in 1→3;1→6-β-D-glucans with immunostimulating activity (in Russian). *Bioorganicheskaya Khimiya*, *21*, 218–225.

Zvyagintseva, T. N., Shevchenko, N. M., Popivnich, I. B., Isakov, V. V., Scobun, A. S., Sundukova, E. V., et al. (1999). A new procedure for the separation of water-soluble polysaccharides from brown seaweeds. *Carbohydrate Research*, *322*, 32–39.