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Enzymatic Transformation of Biologically Active 1,3;1,6-β-D-Glucan. Structure and Activity of Resulting Fragments

L. A. Elyakova, V. V. Isakov, L. A. Lapshina, V. P. Nagorskaya, G. N. Likhatskaya, T. N. Zvyagintseva*, and A. V. Reunov

Pacific Institute of Bioorganic Chemistry, Far East Division, Russian Academy of Sciences, pr. 100-letiya Vladivostoka 159, 690022 Vladivostok, Russia; fax: (4232) 31-4050; E-mail: zvyag@piboc.dvo.ru

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Abstract—The fragmentation of the biologically active 1,3;1,6-β-D-glucan Antivir by endo-1,3-β-D-glucanase LIV from crystalline styles of the marine mollusk *Spisula sachalinensis* was carried out. It was found that low molecular mass oligomers possessing a stabilizing effect on membranes and anti-viral activity against tobacco mosaic virus appeared in the process of enzymatic hydrolysis of Antivir. Biological activity of 1,3;1,6-β-D-glucooligo- and polysaccharides was found to be associated with molecular mass (polymerization degree (*n*) not less than 14) and with presence of intralinked β-1,6-connected monosaccharide residues. Probably, decrease in molecular mass is compensated by increase in number of intralinked β-1,6-connected monosaccharide residues.

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Key words: biologically active 1,3;1,6-β-D-glucan, 1,3-β-D-glucanase, marine mollusks, enzymatic transformation, tobacco mosaic virus, anti-viral activity, stabilizing action on membranes

In recent years, 1,3;1,6-β-D-glucans, compounds displaying anti-tumor, radioprotective, and immunomodulating activities, as well as other important properties [1-12], attract growing attention. Their ability to act as immunocorrectors in animals and plants is of great interest. These glucans can in particular induce various protective mechanisms in plants by stimulation of their natural resistance. In some instances, this property enables the use of these ecologically safe polysaccharides instead of pesticides.

Virtually all known studies on immunomodulating activity of glucans have been carried out using preparations of microbial origin. Among these studies, the work of Albersheim and Darvill [2] on elucidation of structure of resistance inducer, so-called "oligosaccharine" isolated from hydrolytic products of soybean parasite *Phytophthora megasperma* cell walls, is of great importance. This substance proved to be a branched 1,3;1,6-β-D-glucoheptasaccharide. Further studies showed that 1,3;1,6-β-D-glucans of microbial origin can induce in plants resistance to viruses [3-5].

We were the first to show that β-D-glucans obtained by enzymatic transformation of laminaran, 1,3;1,6-β-D-glucan widely spread in brown seaweeds, can serve as immunomodulators in plants. Generally laminarans are either inactive or possess moderate biological activity [6, 7]. Translam, 1,3;1,6-β-D-glucan, more branched and heavier than the initial substrate, inactive laminaran, was obtained from the laminaran by treatment with endo-1,3-β-D-glucanase Lo from the scallop *Chlamys albidus* [8]. This glucan is formed due to ability of the enzyme Lo to catalyze not only hydrolytic reaction, but also glucanosyl transferase and transglucosidase reactions [8], the distinct characteristic feature of this enzyme. Translam possesses prominent immunostimulating and radioprotective effects and is elaborated currently as a drug against acute radiation sickness [9]. Antivir, another product of the enzymatic reaction, slightly lighter than Translam, proved to be an active phytoimmunity stimulator and, together with Translam, can be used as a substance protecting plants from viral and fungal infections. This glucan taken at the concentration of 1 mg/ml, substantially (by 95-98%) inhibits the formation of local lesions on leaves of hypersensitive host infected with tobacco mosaic virus

* To whom correspondence should be addressed.

(TMV) [10]. A preparation of 1,3;1,6-β-D-glucan from *Phytophthora* kindly provided by Prof. Elbersheim was used as a positive control inhibiting virtually completely TMV development on Xanthi-nc tobacco leaves [10]. Also, the obtained 1,3;1,6-β-D-glucans were found to possess a stabilizing effect on cellular membranes [11]. At present, attempts are being made to reveal a correlation between the polysaccharide structure and their biological activity. It has been found that immunomodulating ability of glucans correlates with molecular mass and with the presence and position of β-1,6-glucoside bonds [12].

The aim of present work was to elucidate structural features of the minimal molecular fragment of Antivir responsible for the biological activity of this glucan. Antivir was fragmented with endo-1,3-β-D-glucanase LIV from crystalline style of the mollusk *Spisula sachalinensi*s, an enzyme with well-studied substrate specificity [13].

MATERIALS AND METHODS

Materials. Highly purified endo-1,3-β-D-glucanases LIV and Lo were isolated from crystalline styles of the marine mollusks *S. sachalinensi*s and *C. albidus* using published methods [13, 14]. Laminaran was isolated from brown seaweed *Laminaria cichorioides* as described earlier [15]. Antivir was produced by enzymatic transformation of laminaran from *L. cichorioides* by the method described in [8]. Dextrans with molecular mass of 10, 20, and 40 kD, standard proteins (BSA, myoglobin, ovalbumin, bovine γ-globulin, and catalase), and chromatography media were purchased from Sigma (USA) or ICN (USA); reagents and Polychrome-1 were from Reakhim (Russia).

Main analytical methods. Neutral sugars were determined by the phenol–sulfuric acid method [16] and reducing sugars by Nelson' method [17]. Oligosaccharide composition was analyzed using a Jeol-JLC-6AH liquid chromatograph (Biogel P-2 column, 1 × 100 cm; detection with orcinol–sulfuric acid reagent [18]). Mono- and oligosaccharide composition was determined by HPLC using a IC-5000 Biotronik (Germany) carbohydrate analyzer (Durrum DA-X8-11 resin, column 385 × 3.2 mm, 60°C; detection by bicinchoninate method with Shimadzu (Japan) C-R2 AX integrating system). Protein concentration was determined by the Lowry method [19] in solutions and by absorption at 280 nm during chromatography with IC-5000 analyzer.

Determination of enzymatic activity. Standard reaction mixture for determination of glucanase activity contained 20 μl of the enzyme solution in 0.05 M succinate buffer, pH 5.2, and 480 μl of the substrate solution (1 mg/ml). The sample was incubated for 20 min at room temperature. Enzymatic activities were determined by the increase in concentration of reducing sugars. The amount of enzyme catalyzing the formation of 1 μmol of glucose during 1 min under the determination conditions was taken for one unit of enzymatic activity (U). Specific activity was calculated in units per mg of protein.

Derivation of products of enzymatic hydrolysis of Antivir. To hydrolyze Antivir by 1,3-β-D-glucanase LIV, an aliquot of the preparation (5.2 g) was dissolved in 260 ml H_2O; then 24 ml of LIV solution ($5 \cdot 10^{-1}$ U/ml) was added to 200 ml of this solution. Aliquots of the incubation mixture (50 ml) (1 g of products) were taken after 25 min (P1), 45 min (P2), 2 h (P3), and 4 h (P4) of incubation. The enzymatic hydrolysis was stopped by heating the sample at 100°C for 5 min. Characteristics of the reaction products obtained at different hydrolysis depths (P1-P4) are given in Table 1. The products comprising each sample (1 g, P1-P4) were separated on a column with Polychrome-1 (polytetrafluoroethylene, 3 × 40 cm). The incubation mixture (50 ml) was loaded on the Polychrome-1 column and then eluted with water and then stepwise by a gradient of ethanol solution in water (2.5-, 5-, 7.5-, and 10% ethanol solution in water). Fractions were analyzed by the phenol–sulfuric acid method. Sugar-containing fractions were lyophilized. Polychrome-1 was regenerated with ethanol and water gradually.

Characteristics of hydrolysis products of Antivir. The composition of the enzymatic hydrolysis products was analyzed using a Biotronik carbohydrate analyzer (Durrum-X4-20; 0.63 × 30 cm; 60°C; by bicinchoninate method; Shimadzu C-R2AX detector) and using a Jeol-JLC-6AH liquid chromatograph (Biogel P-2; 1 × 100 cm; by orcinol–sulfuric acid method), the depth of Antivir hydrolysis being monitored by determination of reducing ability of solutions by Nelson's method [17]. ^{13}C- and ^{1}H-NMR spectra were recorded using a Bruker (Germany) WM-250 spectrophotometer (D_2O at 20°C, with methanol as inner standard, 50.15 ppm).

Molecular mass determinations. The molecular masses of oligo- and polysaccharides were estimated on columns with Biogel P-2 (1 × 100 cm) or Toyopearl (Japan) TSK HW40 (1.5 × 100 cm). Glucose, laminarioligosaccharides, laminaran (5 kD), and dextrans with known molecular masses (10, 20, and 40 kD) were used for calibration. The yield of sugars was monitored by the phenol–sulfuric acid method [16].

Antiviral activity of glucooligo- and polysaccharides was evaluated in relation to a usual strain of TMV isolated from infected *Nicotiana tabacum* L. (Samsun) plants by the method of Otsuki et al. [20]. The study was carried out on leaves of locally affected by TMV tobacco plant (Xanthi-nc) grown in a greenhouse. The detached leaves were cut along the main vein and sprayed with Carborundum. Then the experimental half-leaves were inoculated with the viral–glucan mixture, and controls with the virus only. The treated leaves were washed with water and placed into humid chambers. Four days after inoculation the number of local lesions on leaves was counted. Ten half-leaves were used in each experiment.

The experiments were repeated five times. Antiviral activity of the preparations was estimated by their inhibitory effect on the formation of local lesions on leaves induced by the virus according the following formula: $(100 - N_o\ 100/N_c)$, %, where N_o and N_c are the mean numbers of lesions formed on experimental and control half-leaves, respectively.

Stabilizing effect of the studied fractions on cellular membranes under low temperature was estimated by the vitality of the cells after their freezing and thawing [21]. The fraction under study (100 μl) was added into all wells of a microplate and titrated by the method of double dilutions in the concentration range of 100-1.5 mg/ml. A PBS solution (pH 7.4) was used in the control (without cryoprotector). Glycerol, the well-known cryoprotector, was used as a positive control, as 20% solution in phosphate-buffered saline (pH 7.4). Then 100 μl of 2% cell suspension of Ehrlich murine carcinoma in Eagle's medium was added into the wells and mixed by pipetting. The microplate was placed into a container of foam plastic and frozen at −40°C. One day later, the microplate was thawed in a thermostat, incubated for 30 min at 37°C, and then the vitality of the cells was determined by staining with Trypan blue. All the cells were stained in the control (without cryoprotector), and 10-20% of the cells were stained in the positive control, thus corresponding to 80-90% of viable cells. The observations and cell count were carried out using a LUMAM microscope (LOMO, Russia).

RESULTS AND DISCUSSION

We showed earlier [8] that the higher molecular mass fractions (compared with the initial substrate) of β-D-glucans named Translam and Antivir are formed under the treatment of laminaran with endo-1,3-β-D-glucanase Lo from crystalline style of marine mollusk *C. albidus*. The study of Antivir (1,3;1,6-β-D-glucan, β-1,3/β-1,6 = 80 : 20) structure has demonstrated that approximately one-third of β-1,6-bound glucose residues is included into the chain of β-D-glucan molecule, whereas two-thirds are in branch points. Besides, β-1,6-bound glucose residues are concentrated near the nonreducing terminus of the glucan molecule, and the region near its reducing terminus is depleted of β-1,6-branches [8] and, taking into account the data available, is not significant for biological activity manifestation [8, 12].

So, we addressed the task of fitting conditions for enzymatic hydrolysis of the Antivir molecule region adjacent to the reducing end, to prepare products enriched in β-1,6-bound glucose residues, to test their biological activity, and to determine the correlation between their structure and activity.

The Antivir molecule was fragmented with endo-1,3-β-D-glucanase LIV from the mollusk *S. sachalinensis*. The choice of enzyme was due to its mode of action on laminaran. As we showed earlier [22], this enzyme possesses strong affinity to the reducing terminus of the substrate. The quantitative effect of endo-1,3-β-D-glucanase LIV on laminaran radioactively labeled at its reducing terminus is shown in Fig. 1.

The effect of endo-1,3-β-D-glucanase Lo from crystalline style of marine mollusk *C. albidus* is characterized by far less terminal effect [22]. Besides, the study of molecular mass distribution of the product demonstrated that a portion of the inner hydrolyzed bounds (i.e. endo-action itself) for LIV is less than for Lo. The effect of Lo on laminaran is characterized, as compared with LIV, by the formation of larger portion of glucooligosaccharides of higher molecular mass in the products at the depth of the product hydrolysis of 4, 6, or even 10%. Fractions of higher molecular mass than the initial substrate, like Translam and Antivir, are virtually not formed in the presence of LIV [6, 23].

Two tests were used to evaluate biological activity of Antivir and its enzymatic fragmentation products: antiviral activity to usual strain of TMV and stabilizing effect on membranes under low temperature, which was evaluated by viability of cells after freezing and thawing.

The reaction course for Antivir hydrolysis with LIV is demonstrated in Fig. 2. As seen from Fig. 2, the dependence of product accumulation rate on the substrate hydrolysis depth is directly proportional. The analysis of biological activity of total fractions of products sampled from the incubation mixture during Antivir hydrolysis demonstrated insignificant decrease in biological activity of the samples up to the substrate hydrolysis depth of 12% (Table 1). Note that the Antivir molecular mass is 6-8 kD ($n \sim 40$). Hence, approximately five glycoside bonds per molecule are cleaved at the substrate hydrolysis depth of 10-12%. As seen from Table 1, the antiviral activity of sugars was no less than 92% for all stages of the hydrolysis at the sugar concentration of 2 mg/ml. When the sugar concentration was decreased, a decrease in their inhibiting effect on TMV was observed. So, when the concentration of products P1, P3, and P4 was 1 mg/ml, their inhibitory effects were 57, 53, and 72%, respectively.

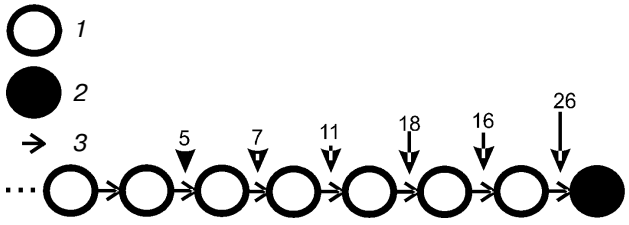

Fig. 1. Effect of endo-1,3-β-D-glucanase LIV from *S. sachalinensis* on laminaran radioactively labeled by its reducing terminus. Designations: *1* and *2*) glucose and radioactively labeled glucose residues, respectively; *3*) β-1,3-glucoside bound; numerals are frequencies of break (in %) of the connections at the reducing terminus of the Antivir molecule.

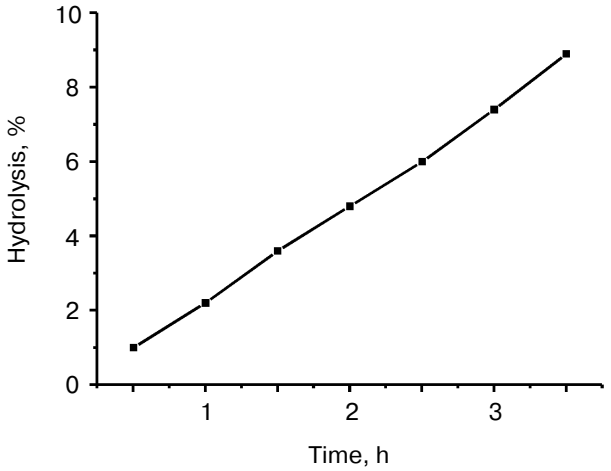

Fig. 2. Time dependence of Antivir hydrolysis under the action of endo-1,3-β-D-glucanase LIV from *S. sachalinensis*.

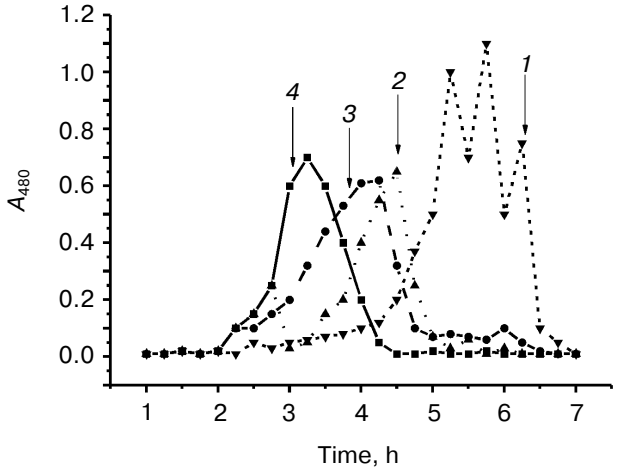

Fig. 3. P-2 (1 × 100 cm) chromatography of fractions obtained after the separation of P4 products on Polychrome-1. Elution with water (*1*) and stepwise gradient of ethanol in water: 2.5% (*2*), 5% (*3*), and 7.5% (*4*).

The total fractions P1, P2, P3, and P4 (Table 1) sampled during the Antivir hydrolysis and representing multicomponent mixtures of glucose, 1,3- and 1,3;1,6-β-D-glucooligo- and polysaccharides of various polymerization degree were sequentially separated on hydrophobic sorbent Polychrome-1 by stepwise gradient of ethanol in water (Fig. 3) with the aim of isolating biologically active oligomers. As we have shown earlier [15], the sorption force of 1,3;1,6-β-D-glucooligo- and polysaccharides to Polychrome-1 is reduced with decrease in the oligomer polymerization degree (*n*) and the content of β-1,6-bound glucose residues within it. The results of fractionation of total products on Polychrome-1, characteristics, and biological activity of distinct fractions are presented in Table 2.

Table 1. Antiviral activity of products obtained at various degrees of hydrolysis of Antivir by LIV

Product	Hydrolysis degree, %*	TMV inhibition**
P1	0.8	98.3/2, 57/1
P2	2.1	92/2
P3	6.2	96/2, 53/1
P4	12	97/2, 72/1, 47/0.5
Antivir	0	(92-98)/1
Translam	0	(98-100)/1
Laminaran	0	30/1

* Hydrolysis degree is expressed in percentage of glycoside bonds hydrolyzed by the enzyme versus the total number of bonds in the substrate.

** Inhibition degree (in %) is given before the slash, and concentration of product (in mg/ml) is given after the slash.

The yield of fractions obtained from the chromatography on Polychrome-1 of the initial Antivir and of the mixture of P1 products (0.8% hydrolysis of Antivir) was virtually the same (Table 2). The transformation of initial high molecular mass substances into the low molecular mass ones occurred thereafter under the action of the enzyme (see Table 2). Fractions of average molecular masses began to accumulate in the hydrolysis products P2 and P3; finally, in products P4 (12% hydrolysis of Antivir), the content of average and large fractions eluted with 7.5 and 10% ethanol in water became drastically decreased, and many low molecular mass sugars appeared (Table 2). Thus, the hydrolysis course was as anticipated—Antivir molecules became gradually shortened.

The results of study on protective effect of fractions of 1,3;1,6-β-D-glucooligo- and polysaccharides obtained from Polychrome-1 against TMV on tobacco plants are given in Table 2. Notably, the fractions of lower molecular masses acquire the ability to stimulate the plant immunity against TMV induced by Antivir (fractions K(7.5) and K(10)) as its enzymatic hydrolysis advances. Possibly, this is ascribable to the shortening of the Antivir molecule due to the decomposition of the β-1,6-bound glucose residue-depleted part of its molecule and the transition of its biologically active branched part into the fractions of average molecular masses after this shortening. The fraction P2(7.5) (92% protective effect at the concentration of 1 mg/ml) could be diluted twice without notable loss of biological activity (82%), and 4-fold dilution resulted in the decrease in its activity to 48%.

We found earlier that both laminaran and the products of its enzymatic transformation Translam and Antivir displayed stabilizing effects on cellular membranes at low temperature [24]. The stabilizing effect of glucans corre-

Table 2. Yield, characteristics, and biological activity of fractions obtained by separation of enzymatic hydrolysis products P1, P2, P3, and P4 on Polychrome-1

Product/hydrolysis degree, %	Polychrome-1, fraction, (ethanol, %)	Yield, %	[α]D	*n**	β-1,3 : β-1,6**	TMV inhibition***	Membrane stabilization, % (products, 50 mg/ml)
	K(H_2O)	trace		n.d.	n.d.	n.d.	0
Antivir	K(2.5)	trace		n.d.	n.d.	0/2	0
	K(5)	7	–22	n.d.	n.d.	33/1	0-10
K/0	K(7.5)	24	–22	30	7.6 : 1	57/1	60
	K(10)	56		35	9.6 : 1	72/1	90
	P1(H_2O)	0.7		n.d.	n.d.	n.d.	0
	P1(2.5)	1	–14.5	6	n.d.	0/2	0
P1/0.8	P1(5)	7.5	–20	n.d.	n.d.	28/1	50
	P1(7.5)	23	–20	n.d.	6.6 : 1	84/1	60
	P1(10)	51		n.d.	7.6 : 1	35/1	90
	P2(H_2O)	1.7		n.d.	n.d.	n.d.	0
	P2(2.5)	2	–13	5	n.d.	0/1	0
P2/2.1	P2(5)	5	–26	7-8	n.d.	21/1	60
	P2(7.5)	24	–18	20	7 : 1	92/1, 82/0.5, 48/0.25	70
	P2(10)	50	–14	25	7.8 : 1	36/1	90
	P3(H_2O)	11		n.d.	n.d.	n.d.	n.d.
	P3(2.5)	3	–15	5	n.d.	0/1	n.d.
P3/6.2	P3(5)	6	–25	7	n.d.	36/1	n.d.
	P3(7.5)	27	–19.6	14	6 : 1	64/1, 60/0.5, 52/0.25	70
	P3(10)	35		15	5 : 1	80/1	90
	P4(H_2O)	20		n.d.	n.d.	n.d.	0
	P4(2.5)	15	–20	5-6	7 : 1	0/2	50
P4/12	P4(5)	15	–24.3	8-9	5.5 : 1	38/1	n.d.
	P4(7.5)	9	–14	n.d.	6.3 : 1	89/1, 45/0.5	70
	P4(10)	26		14	6.3 : 1	63/1	80

Note: n.d., not determined.

* Polymerization degree was determined by gel-permeation chromatography.

** Ratio of bonds is determined from NMR spectra.

*** Inhibition degree (in %) is given before the slash, and concentration of product (in mg/ml) is given after the slash.

lated with the degree of branching of their molecules. Low-branching laminarans displayed low stabilizing activity, and highly branching glucans possessed higher activity [11, 12]. This test was used in our work for determining the activity of fractions obtained by the product separations on Polychrome-1. The activity was not detected in low molecular mass fractions of the Antivir obtained from its chromatography on Polychrome-1. The activity of fraction P1(5) was substantially increased in comparison with the fraction K(5) at the hydrolysis extent of 0.8%. The activity of fractions eluted with 5% ethanol was retained with the increase in the hydrolysis extent, and even the low molecular mass fraction P4(2.5) demonstrated activity at the hydrolysis extent of 12% (Table 2).

Thus, low molecular mass oligomers showing membrane stabilizing capability and antiviral activity appeared in the process of enzymatic hydrolysis of Antivir.

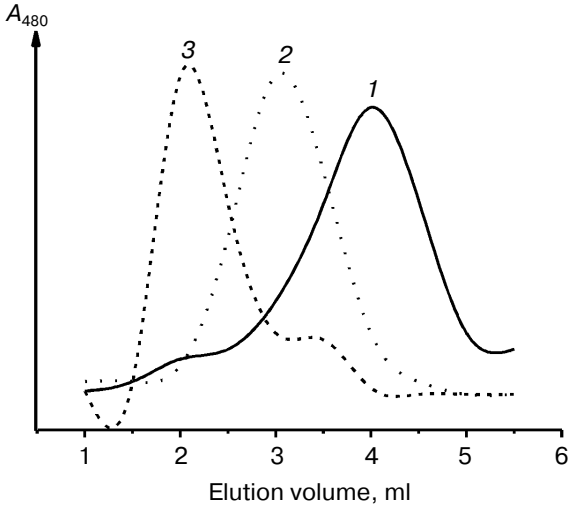

Fig. 4. Toyopearl TSK HW40 (2 × 40 cm) chromatography of P4(10) (*1*) and P2(10) (*2*) fractions, obtained from Polychrome after elution with 10% ethanol in water, and of Antivir (*3*).

We have chosen various fractions first of all possessing the most interesting biological activity for further structural studies. These include the fractions eluted with 5 and 7.5% ethanol in water (Table 2). The structural study was carried out using NMR spectroscopy, mass-spectrometry, HPLC, gel-permeation chromatography, and polarimetry. Also, the structure of fractions similar to these was studied earlier [25-28].

Short (n = 2-4), mainly unbranched oligosaccharides and glucose were detected in fractions P1(H_2O)-P4(H_2O) (data from Biotronik and Jeol carbohydrate analyzers) [22-24]. Fractions P1(2.5)-P4(2.5) contained higher molecular mass glucooligosaccharides (n = 5-6) (Table 2). Their composition was characterized earlier by FAB-mass-spectrometry: oligomers were detected consisting of two, three, four, and five glucose residues [26-28].

Structural characteristics of the following compounds (fractions (P1(5)-P4(5)) were determined from NMR spectroscopy data and expanded with evidence on their molecular masses. So, gel filtration on Biogel P-2 showed that the fractions eluted with 5% ethanol have polymerization degree (n) of 7-9 (for instance, for P4, Fig. 3). This data is in agreement with the results of ^{13}C- and ^{1}H-NMR spectroscopy (Table 2).

The common feature for fractions eluted with 7.5 and 10% ethanol in water is the distinct, as compared with the initial Antivir, reduction of n with increase in hydrolysis degree (Table 2 and Fig. 4). The decrease in polymerization degree is due to the shortening of the lin-

Table 3. Characteristics of enzymatic reaction products on the basis of analysis of integral signal intensities on anomeric protons in PMR spectra

Product	Fraction	n	β-1,3 : β-1,6	β-1,3; 6 : β-1,6	Structural fragments (Fig. 5)
P1	P1(7.5)	n.d.	6.6 : 1	1.6 : 1	A,B,C,D,E; D + E > C
	P1(10)	n.d.	7.6 : 1	2 : 1	A,B,C,D,E; D + E > C
P2	P2(7.5)	n.d.	7 : 1	1.8 : 1	A,B,C,D,E; D + E > C
P3	P3(10)	15	5 : 1	1.3 : 1	A,B,C,D,E; D + E > C
P4	P4(2.5)	5	7 : 1	1 : 1	A,B,C,D; D ≈ C
	P4(5)	9	5.5 : 1	1 : 1	A,B,C,D; D > C

Table 4. Magnitudes of chemical shifts in signals from ^{1}H and ^{13}C in monosaccharide fragments in 1,3;1,6-β-D-glucooligo- and polysaccharide spectra

Fragment	Structure	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
A	Glcβ1→3	4.74/103.95	3.40/75.02	3.54/77.07	3.45/70.92	3.50/77.23	3.93; 3.74/62.01
B	→3Glcβ1→3	4.79/103.68	3.58/74.39	3.80/85.54	3.53/69.04	3.54/76.86	3.94; 3.77/62.01
C	Glcβ1→6	4.52/103.95	3.33/74.18	3.51/77.07	3.43/70.92	3.51/77.23	3.92; 3.75/62.01
D	→3Glcβ1→6	4.56/103.77	3.53/74.18	3.75/85.84	3.53/69.04	3.53/76.86	3.93; 3.75/62.01
E	→3,6Glcβ1→3	4.77/103.77	3.59/74.58	3.80/85.84	3.58/69.04	3.70/75.79	4.22; 3.89/70.1

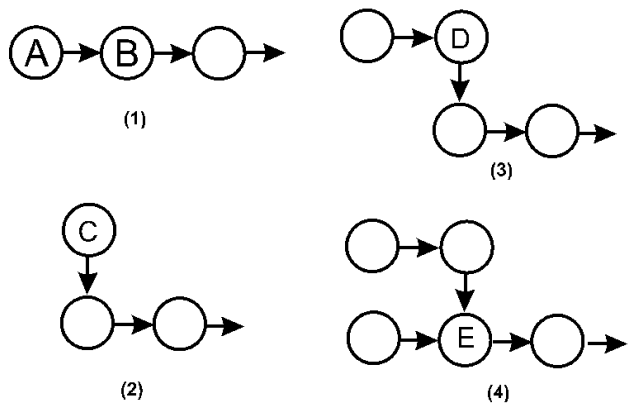

Fig. 5. Structural fragments of oligosaccharide molecule products of Antivir hydrolysis, as identified by 2D-NMR spectroscopy. Symbols ↓ and → designate β-1,6- and β-1,3-bonds between glucose residues (O).

ear part of the molecule. So, the polymerization extent is decreased from 35, for the initial Antivir, to 14, for P3 and P4 fractions eluted from Polychrome with 10% ethanol in water (Tables 2 and 3), whereas the ratio of β-1,3- to β-1,6-bound glucose residues is changed for these fractions from 9.6 : 1 to 5 : 1. A similar pattern is observed for fractions eluted with 7.5% ethanol in water (Tables 2 and 3). These fractions were subjected to gel filtration on Toyopearl (Japan) TSK HW40 with the detection of carbohydrates eluted from the column by refractometry (Fig. 4). The gradual decrease in *n* in these fractions with increase in the hydrolysis degree is seen in the figure.

The analysis of integral signal intensities of anomeric protons in PMR spectra of various fractions revealed the relative polymerization degree and the number of branching (Table 3). Note that not always the polymerization degree can be unambiguously determined, whereas the reducing termini are partially substituted with mannitol (the presence of oxymethylene group at 64.2 in ^{13}C-NMR spectra).

We carried out detailed analysis of ^{1}H and ^{13}C spectra of the fraction P2(7.5) possessing high biological activity. The use of 2D-NMR spectroscopy techniques (COSY, TOCY, HSQC) enabled the assessment of the signals of protons and C-atoms in ^{1}H and ^{13}C spectra and selection of ^{1}H and ^{13}C signals of monosaccharide residues corresponding to fragments present in molecules of the studied oligosaccharides (Table 4 and Fig. 5).

The spectra of P4(2.5) and P4(5) fractions were also analyzed to compare the structures of the fractions obtained after enzymatic hydrolysis of Antivir. As seen from Tables 2 and 3, these fractions represent a mixture of oligosaccharides. ^{1}H and ^{13}C spectra of these fractions suggest the presence of monosaccharide fragments A, B, C, D in their molecules with prevalence of β-1,3-bound monosaccharide residues. The ratio of C to D fragments in P4(2.5) fraction is 1 : 1 (Table 3), and D > C in P4(5) fraction (Table 3). There are fragments A, B, C, D in P1(7.5), P2(7.5), and P3(10) fractions of higher polymerization degree, but besides them signals are distinguishable in the spectra corresponding to fragment E, wherein D and E structural fragments are more rare than C (Tables 3 and 4, Fig. 5).

Thus, it can be concluded that the biological activity of 1,3;1,6-β-D-glucooligo- and polysaccharides is associated with the presence of intrachain β-1,6-bound monosaccharide residues (fragments D and E, Fig. 5). A low molecular mass fragment retaining biological effect of the initial molecule was obtained as a result of directed enzymatic hydrolysis of biologically active 1,3;1,6-β-D-glucan. This fragment may be the active site of the Antivir molecule.

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