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Structural Characterization of Tissue-Specific Galactan from Flax Fibers by ^{1}H NMR and MALDI TOF Mass Spectrometry

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Abstract—A high-molecular-mass polysaccharide galactan (*M* 2000 kDa) was isolated from flax at the stage of cell wall thickening of the bast fiber development. The polymer structure was studied by ^{1}H NMR spectroscopy and MALDI TOF mass spectrometry. It is built up of Gal (59%), Rha (15%), GalA (23%), and Ara (3%) residues. The galactan backbone consists of successively alternating monomer disaccharide units (⟶ 4GalA1 ⟶ 2Rha1 ⟶)$_n$ and is similar in its structure to the backbone of rhamnogalacturonan-1 (RG-I). Rhamnose residues bear in position 4 β-(1 ⟶ 4)-galactose side chains of various lengths with a polymerization degree of up to 28 or higher. A part of the side chains have branchings.

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INTRODUCTION

The plant cell wall is a complex multicomponent system that fulfills many functions and largely determines the character of processing of plant raw materials [1, 2].[2] There exist several main types of cell walls. The most known are two types of the primary cell wall, the key difference between which consists in the polymers that connect the cellulose microfibrillae between each other [2]. The composition of lignifying tissues (e.g., timber) is well characterized for the cells that form the secondary cell wall; their main, practically the only non-cellulosic polysaccharide is xylan [2]. Flax fibers belong to the cells that form a particular type of the secondary cell walls, whose peculiar features are the practically complete absence of lignin, axial disposition of cellulose microfibrillae, and a high proportion of galactose-containing polymers among the non-cellulosic polysaccharides. Such a type of cell wall is characteristic of the cells of many bast fibrillar cultures and of gelatinous fibers of tension wood [3].

An unusual readily soluble in water high-molecular polysaccharide was found in the stem of flax plants. It is present only in bast fibers and only at the stage of formation and thickening of the secondary cell wall; the fibers acquire a great rigidity due to it [4, 5]. This polymer is characterized by a great content of galactose (it was named as galactan due to this feature [4]) and is accumulated in the specialized vesicles of the Golgi apparatus [6]. The elucidation of the polymer structure is a necessary prerequisite for understanding its metabolism and function. A comparison of this polymer with the known polysaccharides of fiber cell walls [7–13] would allow the solution of the question whether it is deposited in cell walls and the determination of the structure of native polysaccharides synthesized in the Golgi apparatus. We used ^{1}H NMR spectroscopy, MALDI TOF mass spectrometry, and the results of monosaccharide analysis to get information on the polysaccharide structure.

RESULTS AND DISCUSSION

Polysaccharide isolation. A part of flax stem containing bast fibers was homogenized in 0.05 M phosphate buffer, and the substances dissolved in the buffer were precipitated with 80% ethanol. The precipitate was suspended in 0.01 M acetate buffer, and the high-molecular polysaccharides were fractioned on Sepharose 4B. Galactan was eluted in the area of 2000 kDa; it contained galactose (59%), rhamnose (15%), arabinose (3%), and galacturonic acid (23%) (Table 1).

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[2] Abbreviations: Hex, a hexose residue; RG-I, rhamnogalacturonan-1.

Table 1. Monosaccharide composition (mol %) of fractions and molelar masses of main components

Fraction	Rha	Ara	Gal	Glc	Xyl	Man	GalA	*m/z* of main fragments	Fragment composition*
Initial polysaccharide	15	3	59	tr	0	0	23		
2	7	tr	76	2	2	0	13	3282	$(Hex)_{18–28}$
3	2	tr	92	3	0	0	4	2472	$(Hex)_{13–20}$
4	tr	tr	94	4	tr	0	2	1823	$(Hex)_{9–16}$
5	1	1	95	3	tr	0	1	1175	$(Hex)_{6–11}$
6	tr	1	96	2	tr	0	1	851	$(Hex)_{4–7}$
7	tr	2	94	3	1	0	1	527	$(Hex)_{3–4}$
8	1	16	80	2	1	0	0	–	–
k1-1	43	0	5	tr	0	0	52	–	–
k1-2	46	0	2	tr	0	0	52	2295	$(GalA\text{-}Rha)_{6–8}$
k1-3	45	0	4	tr	0	0	51	1651	$(GalA\text{-}Rha)_{5–6}$
k1-4	45	0	3	2	0	0	50	1329	$(GalA\text{-}Rha)_{3–4}$
k1-5	48	0	3	tr	0	0	49	1007	$(GalA\text{-}Rha)_{2–3}$
k2-2	46	0	3	tr	0	0	52	685	$(GalA\text{-}Rha)_2$
k2-3	42	0	17	tr	0	0	41	525, 687	$(GalA\text{-}Rha)Hex_{1–2}$

* Sodium ion is not listed within the fragment composition; tr, trace amouns; Hex, hexose.

^{1}H NMR spectrum of galactan. Galactose predominates in the polymer composition (Table 1) and, consequently, exhibits the most intensive signals in its ^{1}H NMR spectrum. The signal at 4.58 ppm is assigned to H1 of β-galactose (Fig. 1). It cannot belong to the signal of H1 of α-anomer, which resonates at 5–5.5 ppm [14]. The chemical shifts in the range 3.74–3.63 ppm are assigned to H2, H3, H5, and H6 [7, 10]. The resonance at 4.12 ppm was assigned to H4 of 1 ⟶ 4 linked Gal residue, since the chemical shifts of protons of Gal residues linked in positions 2, 3, and 6 do not exceed 3.8–3.9 ppm [7, 15, 16]. The value of chemical shift of 3.86 ppm is characteristic of H4 of a terminal Gal [17] or a Gal with other types of glycoside bond [15, 16]. A high intensity of signal at 4.12 ppm and a low intensity of 3.86-ppm signal indicate that the main part of galactose contains a glycosyl residue at O4.

The proton resonances of rhamnose and galacturonic acid are very weak and are overlapped with strongly broadened and intense galactose signals. The signals in the range of 4.8–5.5 ppm may be assigned to H1 of α-rhamnose or α-galacturonic acid, whereas the peak at 4.4 ppm, to H4 of O4-linked GalA residue [17]. The signal at 1.24 ppm was assigned to the methyl group of rhamnose, and the signals at 2.04 and 2.11 ppm, to methyl groups of acetyls [7, 10]. One acetyl group was estimated to be in one of six Gal residues. The resonance at 1.85 ppm was not assigned.

Obtaining of fragments. The information on the polysaccharide structure is often obtained in contemporary studies from their cleavage to oligosaccharides and the determination of the fragment molecular masses and composition. In our case, the fragments obtained by the galactan hydrolysis with a weak acidic solution were analyzed by MALDI TOF mass spectrometry [18]. The scheme of the fragment obtaining is given in Fig. 2. A partial hydrolysis with 0.1 M TFA was carried out in two stages. After the first stage at 100°C, the hydrolysate was fractioned on Biogel P-6 at elution with water (Fig. 3*a*). The monosaccharide compositions of fractions 2–8 and molecular masses of oligosaccharides (MALDI TOF MS) were determined.

Fraction 1 (eluted with void volume) was repeatedly hydrolyzed at 120°C, and the hydrolysate was chromatographed on Biogel (Fig. 3*b*). The combined acidic fractions k1 and k2 were further rechromatographed on Biogel at an elution with 0.01 M pyridine–acetate buffer, pH 4.5 (Figs. 3*c*, 3*d*). The resulting k1-1–k1-5 and k2-1–k2-3 fractions were subjected to monosaccharide analysis and mass spectrometry. Fraction k3 (Fig. 3*b*) contained galactose and its dimer and was not used in subsequent analyses.

Monosaccharide composition. The monosaccharide composition of the fragments is given in Table 1. Fractions 2–8 of Fig. 3*a* contained neutral fragments mainly consisting of galactose. Fractions k1-1–k1-5 and k2-1–k2-3 contained acidic fragments and by over 90% consisted of rhamnose and galacturonic acid in equal proportions.

MALDI TOF mass spectrometry. The time-of-flight mass spectrometry combined with laser desorption/ionization [19] showed that all the values of molecular masses of neutral fragments in fractions 2–8 of Fig. 3*a* correspond only to oligomers of hexoses (Table 1). Fraction 2 contains the most high-molecular hexose fragments (Fig. 4); the most intensive signal

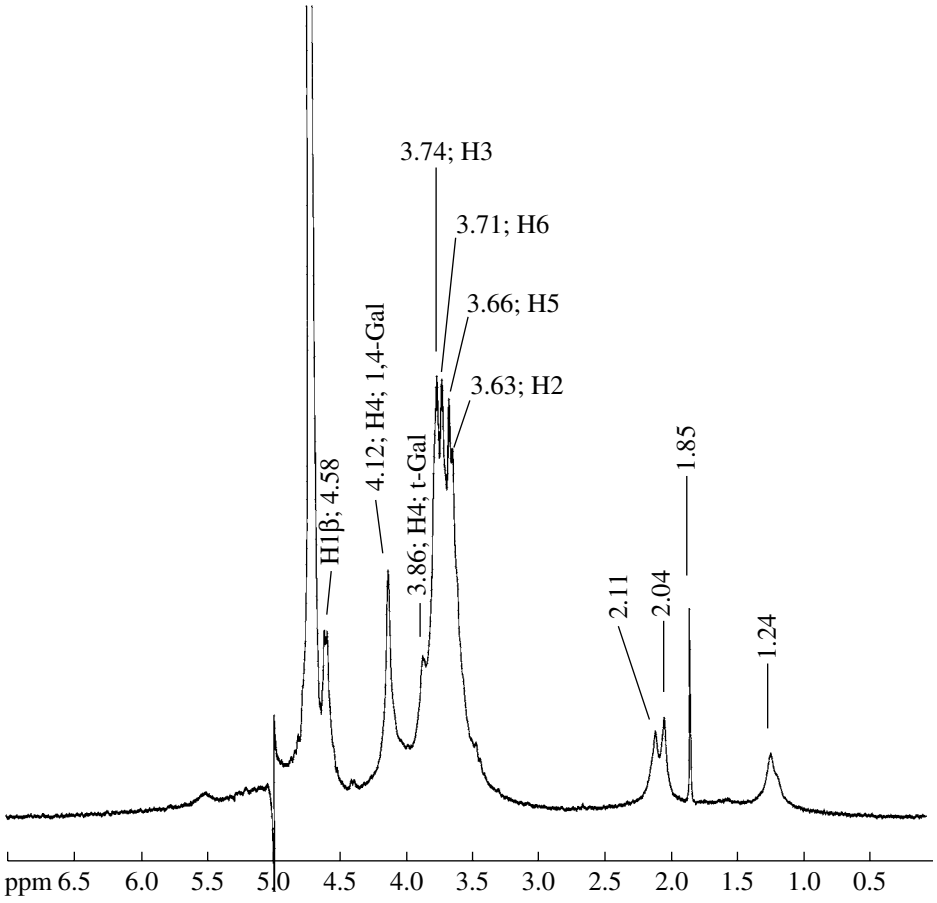

Fig. 1. ^{1}H NMR spectrum of galactan. 1,4-Gal and t-Gal, 1,4-bound and terminal galactose residues.

belongs to the fragment with *m/z* 3282 corresponding to the oligomer from 20 hexose residues $[Hex_{20}Na]^+$. Fraction 2 contains the most high-molecular of the found fragments with *m/z* 4578 ($[Hex_{28}Na]^+$). The less intensive peaks with lower *m/z* values (1823, 1985, 2147, 2309, 2471, 2633, and 2795) that correspond to hexose oligomers $Hex_{11–17}$ are also represented in the mass spectrum. Along with them, the signals $M_{(Hex11–17)} - 18$ (*m/z* 1805.7, 1967.8, 2129.8, 2291.8, 2453.8, 2615.9, and 2778.0) are also present (Fig. 4). Calculations show that the fragments may correspond to the following structures: Hex_nPen_6, $GalA_1Rha_2Hex_n$, $GalA_2Rha_1Pen_1Hex_n$, and $GalA_3Pen_2Hex_n$; the available data do not allow us to choose the only variant.

Fractions 3–7 also contain oligohexose fragments, with the maximally intense signals corresponding to the polymerization degrees of 15, 11, 7, 5, and 3. In the range below 700 Da, a great number of intense peaks from matrix appear, which hinders the detection of signals from low-molecular carbohydrates in fraction 8 (di- and monosaccharides). A number of neutral fractions exhibit weak signals from hexose fragments with one, two, and four pentoses: Hex_aPen, Hex_bPen_2, and Hex_cPen_4 (data not shown). Both arabinose and xylose may be the pentoses; neutral fractions contain them in trace amounts.

Galactose responds for the main portion of neutral fractions, and, therefore, fractions 2–8 are mainly its oligomers.

The structures of acidic fragments $(GalA\text{-}Rha)_n$, where n = 2–8, are aliquot to the dimer structure (Table 1). Fraction k1-2 contains the largest of the fragments we found. Its molecular mass is 2617 ($[GalA\text{-}Rha]_8Na^+$) (Fig. 5). The molecular masses of the main fragments of all the acidic fractions (2617, 2295, 1973, 1651, 1329, 1007, and 685) differed by the value corresponding to the molecular mass of the GalA-Rha disaccharide unit. The signals of low intensity correspond to the composition $(GalA\text{-}Rha)_nHex$ (Table 2). The signals of high-molecular oligosaccharides were not registered in the mass spectrum of fraction k1-1, which is probably due to a lesser volatility of these compounds. Fraction k2-1 corresponds to the fraction k1-5 on chromatogram and was not analyzed. Peaks of diminishing intensity accompany the main signal $[(GalA\text{-}Rha)_nNa]^+$; they are related to $(GalA\text{-}Rha)_n$ fragments with different combinations of sodium and potassium ions (Fig. 6). However, the peaks with the difference of 60 a.e.m.

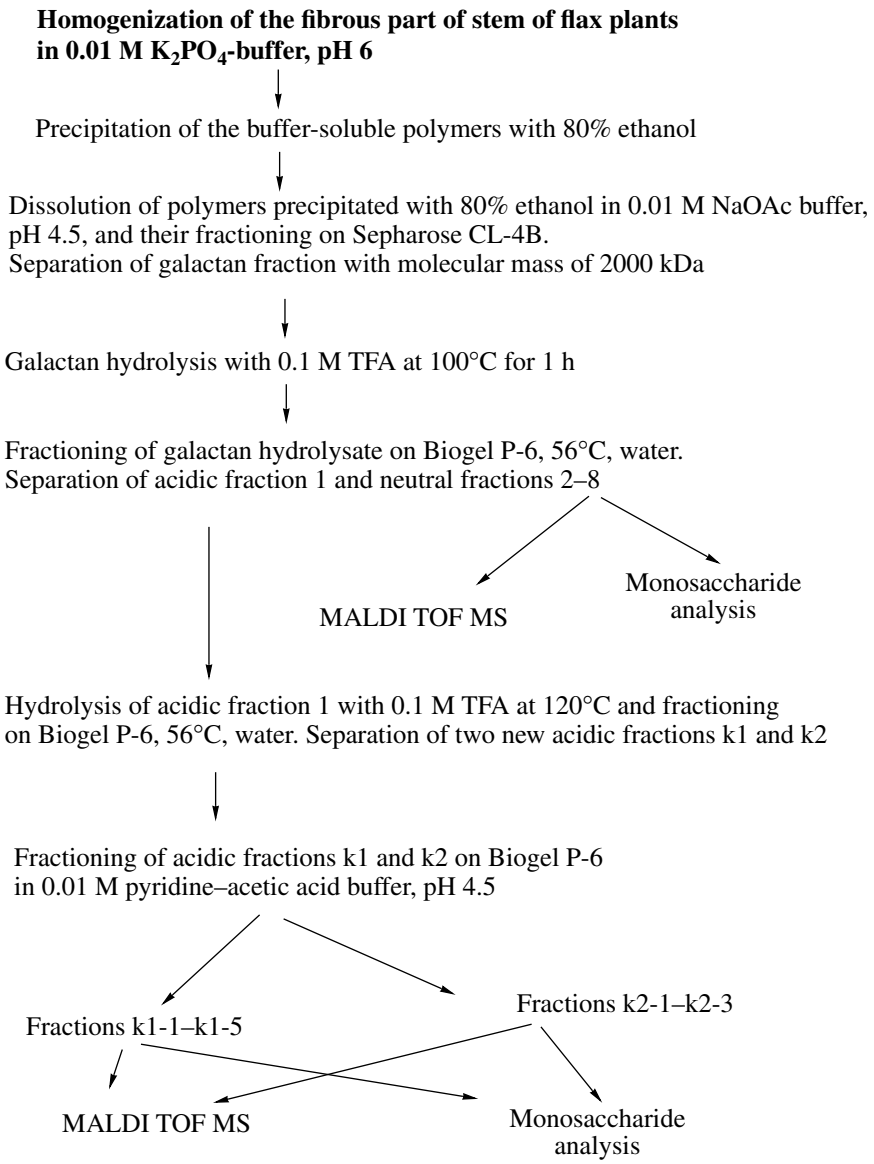

Fig. 2. Scheme of the obtaining of the galactan fragments from its hydrolysate

could also be assigned to the fragments $GalA_2(GalA\text{-}Rha)_n$.

One can suppose on the basis of studying the monosaccharide composition and type of glycoside bonds [4] that the polysaccharide is built up similarly to RG-I, whose backbone consists of the successively alternating monomers of rhamnose and galacturonic acid, whereas its side chains attached to rhamnose consist of galactose and arabinose [2, 20, 21]. It is necessary to prove a polymer chain from the alternating residues of galacturonic acid and rhamnose and neutral galactooligomeric chains to confirm such a structure of the flax galactan.

In fact, the acidic fragments contain equal numbers of the galacturonic acid and rhamnose residues (Table 1). The least fragment found in a significant amount is $(GalA\text{-}Rha)_2$; therefore, the carbohydrate chain can be represented as an alternation of either monosaccharides or dimeric units of GalA and dimeric units of Rha. The glycoside bond of galacturonic acid with an adjacent monosaccharide (GalA1-monosaccharide) is known to be much more rigid than that of rhamnose with an adjacent monosaccharide (Rha1-monosaccharide) [1]. Therefore, only the fragments $(GalA\text{-}Rha)_n$ with even n values (2, 4, 6, and 8) and the corresponding fragments containing by one Rha residue less should exist in the case of alternation of dimeric chains of galacturonic acid with the dimeric chains of rhamnose. In our case, the fragments with the even and odd *n* values were formed. Such fragments arise only from a carbohydrate sequence with alternating residues GalA and Rha. The previous studies of the

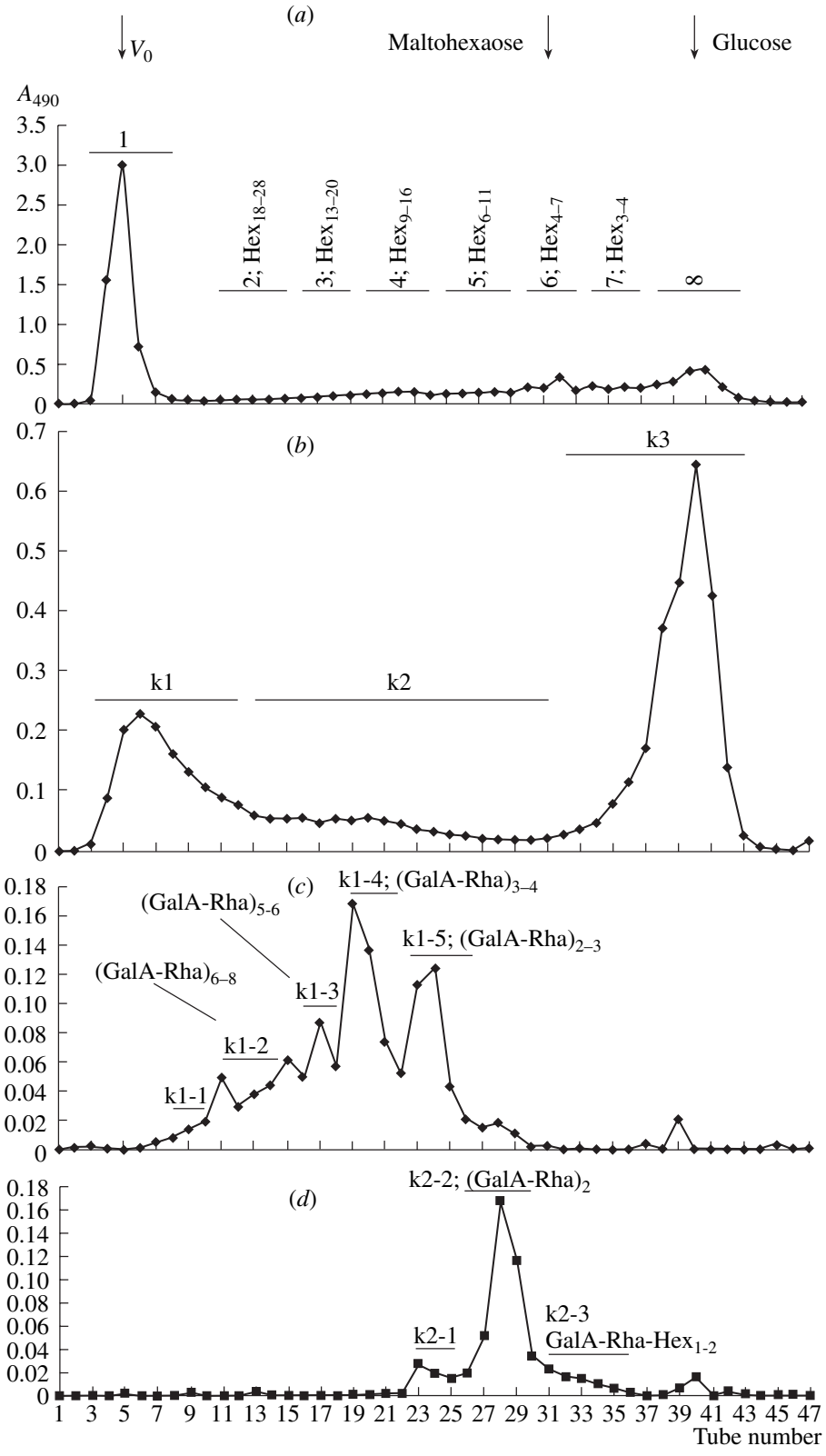

Fig. 3. (*a*) Chromatography on Biogel P-6 (column 2 × 60 cm) of galactan fragments after its hydrolysis with 0.1 M TFA at 100°C; rechromatography on the same column of (*b*) fraction 1 after its repeated hydrolysis with 0.1 M TFA at 120°C and fractions (*c*) k1 and (*d*) k2. Elution at 56°C with (*a*, *b*) water and (*c*, *d*) 0.01 M pyridine–acetate buffer (pH 4.5) at a rate of 15 ml/h. Volume of eluate in each test tube was 2 ml. Lines show combining of fractions.

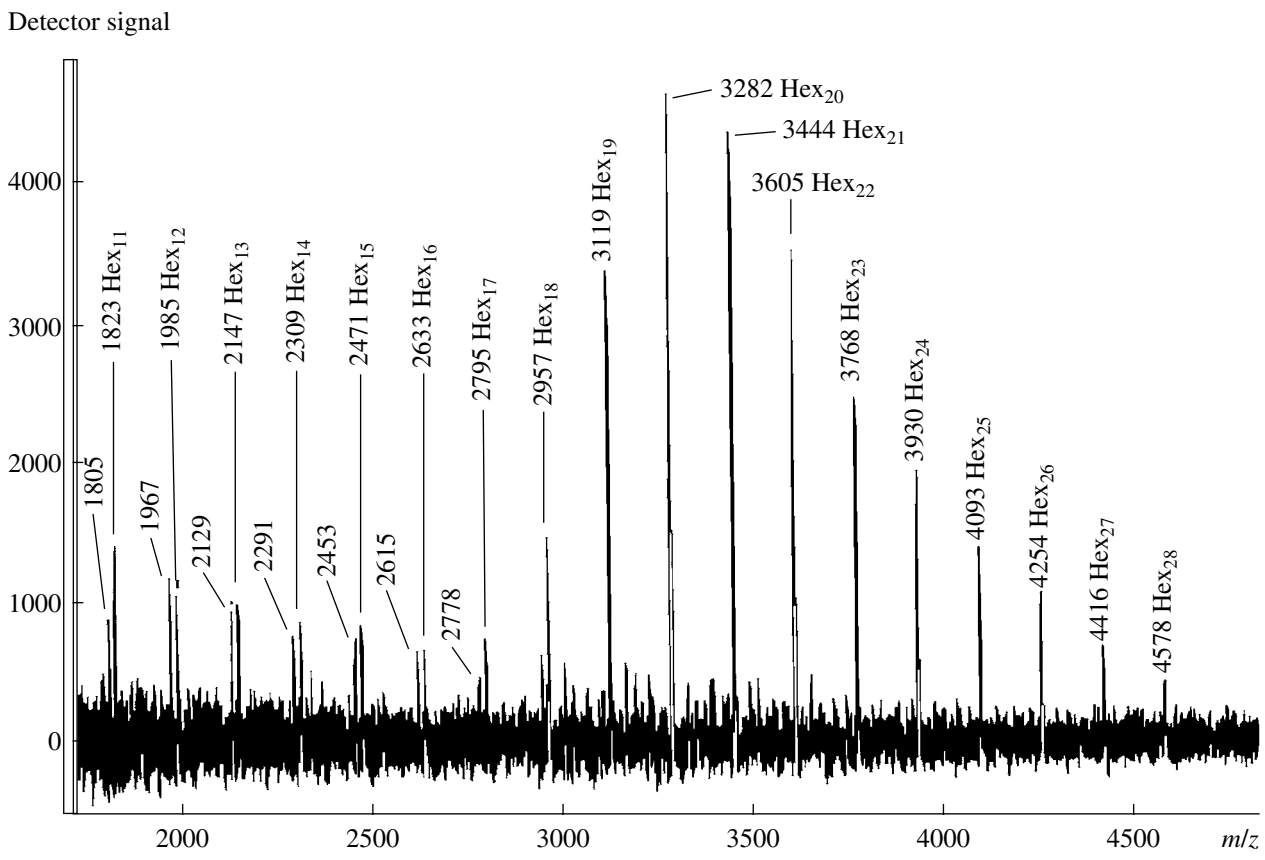

Fig. 4. MALDI TOF MS of fraction 2 (Fig. 3*a*).

polysaccharide have showed that rhamnose contains substituents only in positions 2 and 4 [4]. The presence in galactan of the 2-Rha and 2,4-Rha residues and the alternation of the residues of GalA and Rha suggest that the galactan backbone, like that of RG-I [2, 20, 21], is the $(\longrightarrow 4GalA1 \longrightarrow 2Rha1\longrightarrow)_n$ chain.

The neutral fragments were subjected to hydrolysis more rapidly than the acidic ones: the fragments of acidic molecules suitable for the mass spectrometry were obtained during the second stage of hydrolysis. Under these conditions, neutral fragments were destructed to monomers, which hindered the obtaining of information on the binding of the acidic backbone of galactan with the neutral side chains. Nevertheless, the presence of the binding was confirmed by the signals of acidic fragments of low intensity containing hexose in their composition (Tables 1, 2); They were present in all the acidic fractions. The sorption of galactan on Dowex-1, an anion exchanger, in a buffer with a low ionic strength (data not given) are also in accord with the binding of neutral galactose oligosaccharides with the acidic backbone. In the opposite case, neutral carbohydrates should pass through the anion exchanger and separate from acidic sugars.

An intensive signal at 4.12 ppm characterizes a high content of 1 $\longrightarrow$ 4 linkage between Gal residues, which is in accord with the previous study where the predominance of 4Gal residues (65%) in the monosaccharide composition of galactan had been shown [4]. Thus, a linear β-1,4-galactose chain is the major portion of galactan.

The mean polymerization degree of the galactose chains can be calculated from the ratio of galactose and rhamnose in galactan (Table 1); it is approximately 4. However, judging from MALDI TOF MS (Fig. 4), the galactan contains galactose side chains with the polymerization degree being more than average (up to 28 and, possibly, higher). Therefore, another part of the

Table 2. Molecular mass and the composition of acidic fragments containing hexose

Fraction	Molecular masses and composition of fragments*	
k1-2	2459; $(GalA\text{-}Rha)_7$-Hex	2135 $(GalA\text{-}Rha)_6$-Hex
k1-3	1813; $(GalA\text{-}Rha)_5$-Hex	
k1-4	1491; $(GalA\text{-}Rha)_4$-Hex	1169 $(GalA\text{-}Rha)_3$-Hex
k1-5	1169; $(GalA\text{-}Rha)_3$-Hex	847 $(GalA\text{-}Rha)_2$-Hex
k2-2	847; $(GalA\text{-}Rha)_2$-Hex	

* Sodium ion is not listed within the fragment composition; Hex, hexose.

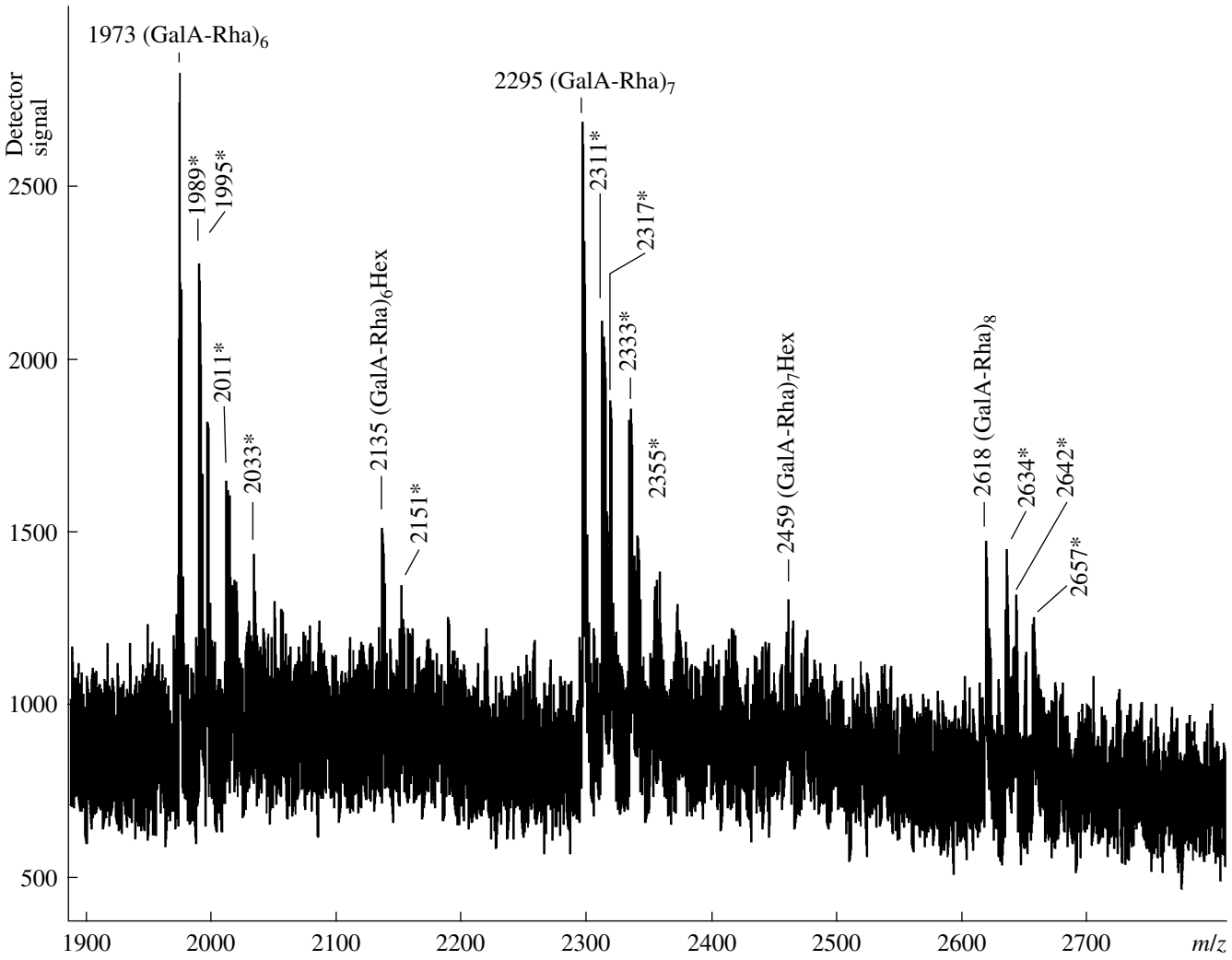

Fig. 5. MALDI TOF MS of fraction k1-2 (Fig. 3*c*). The composition corresponds to the main fragment with various combinations of sodium and potassium ions.

side galactose chains has the length below the average. Thus, the galactose chains varied in their lengths.

In addition to the main fragments with the masses from 2957 to 4578, fraction 2 contains oligohexose fragments with lesser molecular masses: 1823, 1985, 2147, 2309, 2471, 2633, and 2795 (Fig. 4). They are not the admixtures from the neighboring less low-molecular fractions, since their intensities do not drop along with a decrease in molecular masses. We believe that these are branched galactose fragments that are eluted before than the linear oligosaccharides with similar masses. 2,4-Gal and 3,4-Gal are present in small amounts in the composition of galactan [4], and this is in accord with the presumption on branched galactose chains.

The distribution of acetyl groups in galactan is unknown. They may be connected with both the neutral side galactose chain [10] and galacturonic acid [22].

Thus, we think that the galactan molecule is built up by the type of RG-I and its backbone has the $(\longrightarrow 4GalA1 \longrightarrow 2Rha1 \longrightarrow)_n$ sequence. The side linear β-1,4-galactose chains are attached to rhamnose in position 4, and their lengths vary. A part of galactose chains have branchings in positions 2 and 3. The galactan may contain galactose chains as side branchings with a pentose in its composition. According to recent information, the galactan is accumulated in special vesicles of the Golgi apparatus [6]. At the same time, no significant (in size) blocks of polygalacturonic acid or xyloglucan were found in the composition of galactan; although the complexes of them with other polysaccharides have recently been found in the structures of Golgi apparatus [23]. Therefore, such complexes are not obligate for all the secreted polysaccharides.

The galactan is synthesized only after the end of the intrusive growth at the stage of formation of thickened secondary cell wall, which imparts the fiber an exclu-

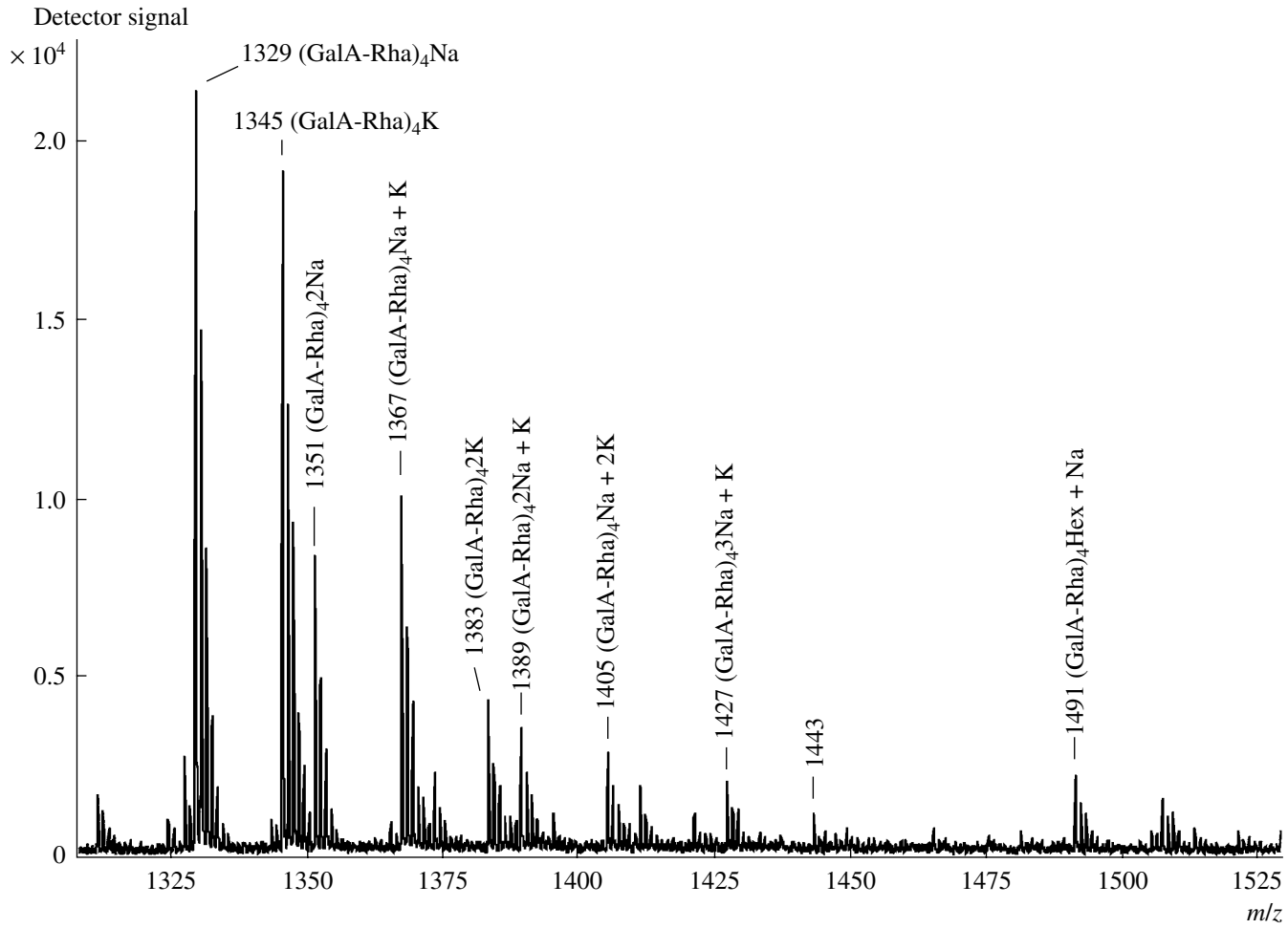

Fig. 6. MALDI TOF MS of fraction k1-4 (Fig. 3*c*).

sive rigidity. The natural presumption arises whether the galactan participates in the formation of the secondary cell wall. A study of the cell wall of flax fibers showed the existence of polysaccharides with a structure similar to that of galactan. The polymers with the structure of RG-I and a high content of linear chains built up of (1 ⟶ 4)-linked residues of β-galactose were isolated from the pectin fraction of mature flax fiber [7]. In this case, the galactose content varied in different fractions from 81 to 50%. Davis et al. [7] published the ^{1}H NMR spectrum practically identical to our spectrum; however, their polymer had a significantly lesser molecular mass (100 kDa and less) instead of 2000 kDa for the galactan from the Golgi apparatus. The polysaccharides with a similar structure were also isolated by the extraction with 90% aqueous DMSO [11] or a hot water [10].

Note that the main mass of galactose-containing polymers (>80%) may be isolated from the cell walls of flax fibers under rigorous treatment connected with a degradation of the structure of cellulose microfibrils, e.g., by hot alkali [8], hot acid [12], or enzymes hydrolyzing cellulose [3]. Under these conditions, fractions with the composition close to that of galactan were isolated. However, these conditions also resulted in the products with significantly lesser molecular masses. Therefore, it is possible to presume that the incorporation of galactan in cell wall is accompanied by its extremely rigid binding with cellulose associated with a simultaneous or subsequent partial disassembling of the molecule backbone. Taking into consideration the tissue- and stage-specificity [5] of the high-molecular galactan from the Golgi apparatus we characterized and, therefore, its particular role in the formation of flax fibers, the revealing of the character of modifications of this polymer and the enzymes (or nonenzymatic processes) providing the modifications is obviously interesting for further studies.

EXPERIMENTAL

Reagents. Acetic acid (analytical grade) was distilled over potassium permanganate [24]; hydrochloric acid (analytical grade), as an azeotrope with water [25]; and pyridine (analytical grade) was purified by an usual

distillation [24]. Water was purified by Milli-Q Ultrapure water purification system (Millipore). Bio-Gel P-6 (200–400 mesh, separation range 1–6 kDa) was from Bio-Rad, Sweden.

Flax plants (*Linum usitatissimum* L., Novotorzhskii variety) were grown under the conditions of vegetation experiment (in boxes with a soil level of 50 cm in open air at the natural illumination and daily watering). A part of stem was cut below (15 cm) the snap point [26], and the outer part of the cut-off containing the bast fibers were separated from the inner part whose main portion was xylem [4]. The fiber part was immediately frozen in liquid nitrogen, and the resulting material was store in liquid nitrogen or in a freezer at −80°C.

Isolation of galactan. A fibrous part of stem (1 g) was ground in liquid nitrogen in a mortar to a powder and suspended in 0.05 M phosphate buffer (pH 6.0, 15 ml). The precipitate was separated in a centrifuge (1200 *g*, 5 min) at 2–4°C. The supernatant was mixed with ethanol (1 : 5 vol/vol) up to the alcohol concentration of 80% to precipitate all the polysaccharides soluble in the buffer, and the solution was kept at 4°C overnight. The precipitate was separated on a centrifuge (1200 *g*, 5 min), three times washed with 80% ethanol, and dried in air. The dry precipitate was suspended in small volume of chromatographic buffer, and the mixture was centrifuged (2500 *g*, 5 min). The supernatant was fractioned on Sepharose CL-4B (Pharmacia, Sweden) [4]. The column (1.8 × 62 cm) was eluted with a buffer (0.01 M sodium acetate + 0.05% sodium azide, pH 4.5) [4]. Elution rate was 15 ml/h, fraction volume 2 ml. The sugar content was determined by the Dubois method (phenol–sulfuric acid) [27]. The fraction containing galactan with *M* 2000 kDa [5] was collected, desalted, and dried on a rotary evaporator at 40°C. The yield of galactan was 0.05% of the wet mass of the fibrous part of stem.

Galactan hydrolysis and chromatography on Biogel P-6. Galactan (0.8 mg) was hydrolyzed in 0.1 M TFA (2 ml) at 100°C for 1 h and dried in a nitrogen flow at 30–40°C. A dried hydrolysate of 6.4 mg of the galactan was dissolved in water (0.5 ml) and fractioned on a column of Biogel P-6 (2 × 60 cm) eluted with milliQ water at 56°C. The elution rate was 15 ml/h, the volume of collected fractions being 2 ml. The content of sugars was analyzed by the Dubois method. The fractions were combined according to the peaks obtained and dried on a rotary evaporator at 40°C. Fractions 2–8 were used for monosaccharide analysis and MALDI TOF MS.

Fraction 1 (void volume) was repeatedly hydrolyzed in 0.1 M TFA at elevated temperature (120°C) for 1 h and dried in a nitrogen flow at 30–40°C. The residue was dissolved in water (0.5 ml) and chromatographed on a Biogel P-6 column as described above. Combined fractions k1, k2, and k3 were obtained. The first two fractions were repeatedly chromatographed on Biogel P-6 using elution with 0.01 M pyridine–acetic acid buffer, pH 4.5. Fractions k1-1–k1-5 and k2-1–k2-3 were dried and used for the monosaccharide analysis and MALDI TOF MS.

Monosaccharide analysis. The samples were hydrolyzed with 2 M TFA at 120°C for 1 h and dried in a nitrogen flow at 30–40°C. The monosaccharide composition was analyzed by high-performance anion-exchange chromatography (HPAEC) on a CarboPac PA-1 column (4 × 250 mm, Dionex) using a pulse amperometric detector (PAD, Dionex). The elution rate was 1 ml/min, column temperature 30°C, and buffer solutions: A, milliQ water; B, 150 mM NaOH; and D, 600 μM NaOAc. The eluate was mixed with 500 mM NaOH after the output from column. Before a sample injection, column was equilibrated with a 83 : 17 buffer A–buffer B mixture. Elution was carried out by a linear gradient from A 100%; a to A 30% for 2–30 min; then to A 30% + B 35% + D 35% for 33–42 min; and to B 100% for 45–60 min; with subsequent gradient to A 83% + B 17% for 50.1–60 min. Mannitol was used as an internal standard. When plotting calibration, monosaccharide standards were preliminarily heated in 2 M TFA at 120°C for 1 h and dried in a nitrogen flow at 30–35°C.

MALDI TOF MS. A solution of matrix contained 9 mg of 2,5-dihydroxybenzoic acid (Bruker Daltonic's) in a mixture of water (700 μl) and acetonitrile (300 μl). A sample (20 μg) was dissolved in water (10 μl), and the solution (1 μl) was mixed with a matrix solution (1 μl). The resulting solution was applied onto a MALDI TOF plate (Bruker Daltonic's) and dried in a flow of warm air.

The MALDI TOF MS was carried out on an Ultraflex Bruker Daltonic's instrument equipped with a nitrogen laser (337 nm) functioning in a positive ion regime. After generation, ions were accelerated to a kinetic energy of 12 000 eV. Mass spectrometer was calibrated by a mixture of maltodextrins (molecular mass range of 365–2309 Da).

^{1}H NMR spectroscopy. A sample (10 mg) was dissolved in D_2O (99.96% D), and a spectrum was registered on a Bruker Advance DPX300 NMR instrument at 27°C. Aceton (δ 2.22 ppm) was used as an external standard.

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