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Synthesis of Sulfated Pectins and Their Anticoagulant Activity

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Abstract—The following pectins were sulfated: bergenan BC (the pectin of *Bergenia crassifolia* L), lemnan LM (the pectin of *Lemna minor* L), and galacturonan as a backbone of pectins. Pyridine monomethyl sulfate, pyridine sulfotrioxide, and chlorosulfonic acid were used as reagents for sulfation. Chlorosulfonic acid proved to be the optimal reagent for sulfation of galacturonan and other pectins. Galacturonan and pectin derivatives with different degrees of sulfation were synthesized and their anticoagulant activities were shown to depend on the quantity of sulfate groups in the pectin macromolecules.

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It is always relevant to search for preparations that have therapeutic effect but no side effects on an organism. This includes the search for anticoagulants, i.e. substances that reduce blood coagulability by suppressing the biosynthesis of fibrin. Anticoagulants can be direct-acting (reduce the activities of thrombin and other serine proteases in blood) or indirect-acting (disturb production in the liver of prothrombin participating in blood coagulation) [1].

Indirect-acting anticoagulants include medicinal preparations of the coumarine and indandione series, but they have many side effects such as toxicity, long latent period, susceptibility to accumulation, long-term effects, etc. These factors are reasons for restricted application of such preparations in anticoagulant therapy [2].

Heparin preparations have a narrower spectrum of side effects: nonfractionated heparin and low molecular weight heparin (Fraxiparine, enoxaparine, deltaparine, etc.), an acidic sulfated linear polysaccharide isolated from animal tissue, a direct-acting anticoagulant that inhibits blood coagulation factors IX-XII and blocks the biosynthesis of thrombin [3, 4].

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However, the average and low molecular weight heparin fractions have a number of side effects such as hemorrhages, allergic reactions, and thrombocytopenia. Besides, the application of heparins, being exclusively animal polysaccharides, is associated with possible transmission of prions from animals to humans and bovine encephalopathy infection [5, 6].

The anticoagulant activity of heparin is associated with the peculiarities of its molecular structure: the size and presence of sulfate groups. Besides, the unique pentasaccharide sequence activating conformational changes in the plasmatic inhibitor of serine proteases of the blood coagulation system, antithrombin, is of prime importance. It was shown that the activity of heparin fractions with four sulfate groups per disaccharide structural unit was 1.5-fold higher than the activity of heparin fraction with three sulfate groups [7].

Sulfation of polysaccharides such as cellulose, chitosan, and dextran galactomannans was shown to impart anticoagulant properties [8-11].

Pectins form a large class of plant polysaccharides; they are present in almost all higher plants, usually have no sulfate groups in their composition, and show diverse physiological activities: immunomodulating, antidote, antioxidant, and gastroprotective [12-16]. The effect of

introduction of sulfate groups into pectin macromolecules on the physiological activity and anticoagulant properties of the pectins has not been investigated up to now.

The conditions of sulfation of the following physiologically active pectins have been studied in the this work: lemnan LM (the pectin of *Lemna minor* L), bergenan BC (the pectin of *Bergenia crassifolia* L), and galacturonan as the pectin backbone; the degree of sulfation has been shown to depend on the reaction conditions and sulfating agent; the correlation of anticoagulant properties of the sulfated pectins and their sulfation degree is revealed.

MATERIALS AND METHODS

General analytical methods. *The glycuronic acid content* was determined by the reaction with 3,5-dimethyl phenol in the presence of concentrated sulfuric acid and a calibration plot was constructed for D-galacturonic acid; photocolorimetry was carried out at the following two wavelengths: 400 and 450 nm [17].

Content of sulfate groups was assayed by Dodgson's method and the calibration curve for potassium sulfate; photocolorimetry was performed at 360 nm [18]. Spectrophotometric measurements were made with an Ultrospec 3000 spectrophotometer (England).

Qualitative and quantitative assay of neutral monosaccharides as the corresponding alditol acetates was carried out by gas-liquid chromatography (GLC) on a Hewlett-Packard 4890A chromatograph (USA) equipped with a flame ionization detector and an HP 3395A integrator using a capillary column RTX-1 (0.25 mm × 30 m) (Restek) and with helium as the carrier gas. GLC of alditol acetates was carried out under temperature programming from 175°C (1 min) to 250°C (2 min) at the rate of 3°C/min. The content of monosaccharides in the percent of the total preparation mass was calculated from the area of peaks using the coefficients of detector response [19]. *Myo*-inositol was used as an internal standard.

The amount of methoxyl groups was determined by a previously described method [20] and from the calibration plot constructed for methanol; photocolorimetry was carried out at 412 nm.

Molecular weights of samples were determined by high-performance liquid chromatography (HPLC). A sample (2 mg) was dissolved in 0.15 M NaCl (1 ml) and the solution was filtered. The analysis was performed in the following chromatographic system (Shimadzu, Japan): LC-20AD pump, DGU-20A_3 degasser, RID-10A refractometer, CTO-10AS thermostat, Shodex Asahipak GS-620HQ column (7.5 mm × 30 cm), and Shodex GS-26 7B pre-column (7.6 mm × 5 cm). The column was eluted with 0.15 M NaCl at 40°C at a flow rate of 0.3 ml/min. The column was calibrated using samples of sulfated dextrans with molecular weights in the range of 36-50, 100, 400-600, and 1400 kDa (Sigma, USA). The content of components in the percent of the total preparation mass was calculated from the area of peaks.

Specific optical rotation of galacturonan was determined on a Polartronic MHZ polarimeter (Germany) at 20°C.

IR spectra of samples dehydrated in a Fisher pistol in vacuum over P_2O_5 at 60°C were recorded using a Specord M80 spectrophotometer ($\nu_0 = 4000$ cm^{-1}, $\nu_k = 400$ cm^{-1}, IT = 1). Samples (2 mg) were analyzed as pellets obtained by pressing with KBr [21].

NMR spectral data were recorded using DRX-500 spectrometer (Bruker, Germany) for 3-5% solutions of the samples in D_2O at 303°K (acetone as an internal standard, δ_H 2.225 ppm, δ_C 31.45 ppm).

Complete acid hydrolysis. One milliliter of 2 M trifluoroacetic acid (TFA) containing *myo*-inositol (1 mg/ml) was added to the sample under study (4-6 mg). The mixture was incubated for 5 h at 100°C. Excess acid was removed by repeated evaporation of hydrolysate with methanol. The resulting monosaccharides were identified by GLC as respective alditol acetates [19].

Galacturonan production. TFA (50 ml of 2 M solution) was added to the bergenan BC sample (250 mg); the mixture was incubated for 2 h at 100°C and centrifuged for 20 min at 4°C and 7943*g* (r_{av} 9.2 cm). The precipitate was dissolved in water with the addition of 2 M ammonia solution to pH ~ 5; the solution was dialyzed and lyophilized. As a result, galacturonan BCH was obtained with the yield of 100 mg, $[\alpha]^{20}_{589.44}$ +246° (0.1 g per 100 ml of aqueous ammonia); the content of D-GalA residues was 98.6%.

Triethylammonium salt of galacturonan was obtained as described previously [19]. Galacturonan was dissolved in distilled water and dialyzed against 1% aqueous solution of triethylamine hydrochloride for 24 h and then against distilled water for 24 h. The galacturonan triethylammonium salt solution was lyophilized and dehydrated in a Fisher pistol over P_2O_5 at 60°C.

Production of pyridine monomethyl sulfate. Pyridine monomethyl sulfate was obtained as described previously [22]. Concentrated sulfuric acid (48 ml) was slowly poured into cooled absolute methanol (88 ml) under continuous stirring; the resulting mixture was kept at 4°C for 24 h. Then pyridine was added to the reaction mixture at 10-15°C to pH ~ 7 and kept at 4°C for 12 h. The precipitate was separated by filtration and washed with pyridine (5 × 20 ml). The obtained white crystals of pyridine monomethyl sulfate were dried in a Fisher pistol over P_2O_5 at 60°C. The yield of pyridine monomethyl sulfate was 46.6 g.

Sulfation of galacturonan with pyridine monomethyl sulfate. Sulfation with pyridine monomethyl sulfate was performed by the method described previously [22]. Dimethylformamide (DMF) (15 ml) was added to the triethylammonium salt of galacturonan (66.0 mg); the mixture was stirred at 60°C for 0.5 h followed by the addition

of different amounts of pyridine monomethyl sulfate (458.0 or 733.0 mg). Then the reaction was carried out at 80°C for a certain period of time: 0.5, 1.5, and 3 h. The reaction mixture was cooled, 0.5 M NaOH was added to pH ~ 6-7; the mixture was dialyzed against distilled water and lyophilized. As a result, six samples of sulfated galacturonan with different degrees of sulfation were obtained (yields are given in Table 1).

Sulfation of galacturonan with pyridine sulfotrioxide. Sulfation with pyridine sulfotrioxide was performed by the method described previously [23]. Pyridine sulfotrioxide was obtained as follows: concentrated chlorosulfonic acid (0.08 ml) was slowly added to pre-cooled pyridine (0.4 ml) under continuous stirring. The resulting mixture was kept at 4°C for 24 h to obtain pyridine sulfotrioxide as white crystals, which were dissolved in 1-2 ml of DMF and used for galacturonan sulfation.

Galacturonan was sulfated as follows: DMF (15 ml) was poured into its triethylammonium salt (52.3 mg); the mixture was stirred at 60°C for 0.5 h, followed by addition of the whole pyridine sulfotrioxide solution obtained previously; then the reaction was carried out for 4 h at different temperatures: 30, 50, 60, and 70°C. The reaction mixture was cooled; 0.5 M NaOH was added to pH ~ 6-7; the mixture was dialyzed against distilled water and lyophilized. As a result, four samples of sulfated galacturonan were obtained with different degrees of sulfation (yields are given in Table 2).

Table 1. Conditions and results of galacturonan sulfation by pyridine monomethyl sulfate

T, h	Excess, mole[1]	Yield, %[2]	$-SO_3^-$, %[3]	GalA, %[3]	M_r, kDa	S, %[4]
0.5	5	76	9	81	>100 <50	72.3 27.7
1.5	5	60	12	84	>100 <50	80.9 19.1
3	5	55	18	80	>100 <50	88.7 11.3
0.5	8	62	10	80	>100 <50	76.7 23.3
1.5	8	62	14	76	>100 <50	87.7 12.3
3	8	71	21	66	>100 <50	81.6 18.4

[1] Excess of pyridine monomethyl sulfate per OH-group of GalA residue.
[2] Yield is calculated from the initial weight of galacturonan.
[3] Mass percents.
[4] Percentage of total area of peaks on elution curve of HPLC chromatogram.

Table 2. Conditions and results of galacturonan sulfation by pyridine sulfotrioxide

T, °C	Yield, %[1]	$-SO_3^-$, %[2]	GalA, %[2]	M_r, kDa	S, %[3]
30	95	20	75	>100 <50	66.3 33.7
50	94	23	74	>100 <50	69.0 31.0
60	92	28	70	>100 <50	55.1 44.9
70	95	23	73	>100 <50	64.5 35.5

[1] Yield is calculated from the initial weight of galacturonan.
[2] Mass percents.
[3] Percentage of total area of peaks on elution curve of HPLC chromatogram.

Sulfation with chlorosulfonic acid was carried out by the method described previously [24]. DMF (15 ml) was poured into the triethylammonium salts of galacturonan and bergenan BC (66.0 mg of each); *p*-toluene sulfonic acid was added (33 mg); the mixture was stirred at 60°C for 0.5 h; chlorosulfonic acid was added (0.03, 0.07, and 0.09 ml); and the reaction was carried out at 25°C for 1 h. The reaction mixture was cooled and 0.5 M NaOH was added to pH ~ 6-7; then the mixture was dialyzed against distilled water and lyophilized.

Galacturonan was further sulfated with addition of 0.09 ml of chlorosulfonic acid for 1 h at 40 and 60°C and lemnan LM at 25°C.

As a result of sulfation, five samples were obtained for galacturonan (yields are presented in Table 3), three samples for bergenan BC (BC-SO_3-1, BC-SO_3-2, BC-SO_3-3), and one sample for lemnan LM (LM-SO_3) (yields are given in Table 4).

Specific antithrombin (aIIa) activity was assayed by the methods of [25]. Antithrombin (Behring, USA-Germany, or Sigma, USA) (1 unit/mg) in 250 μl of the buffer (0.05 M Tris-HCl, 0.0075 M Na_2EDTA, 0.175 M NaCl, pH 7.4) was incubated for 3 min at 37°C with pectin samples at the final concentration of 0.01-240 μg/ml, followed by addition of 100 μl (2 NIH U/mg) of bovine thrombin solution (Behring or Sigma) in 2 mM Tris-EDTA buffer. After 30 sec, 200 μl of solution of the chromogenic substrate for thrombin S-2238 (Behring or Sigma) in 2 mM of the Tris-EDTA buffer was added. Sulfated pectin activity was estimated by change in the optical density of the solution at 405 nm after 1 min. The activity was estimated by plotting the calibration dependence in accordance with the 5th International Standard for Unfractionated Heparin (UFH).

Effect on the general pathway of blood coagulation was estimated by the thrombin time (TT) [26]. Thrombin (Renam, Russia) (0.1 ml) was added to 0.1 ml of aqueous

Table 3. Conditions and results of galacturonan sulfation by chlorosulfonic acid

T°C	Excess[1], mole	Yield, %[2]	GalA, %[3]	$-SO_3^-$, %[3]	M_r, kDa	S, %[4]
25	1	45	84	9	>100 <50	98.2 1.8
25	2	75	69	31	>100 <50	79.9 20.1
25	3	65	61	39	>100 <50	70.2 29.8
40	3	55	61	39	>100 <50	69.0 31.0
60	3	61	61	39	>100 <50	67.4 32.6

[1] Excess of chlorosulfonic acid per OH group of the GalA residue.
[2] Yield is calculated from the initial weight of galacturonan.
[3] Mass percents.
[4] Percentage of total area of peaks on elution curve of HPLC chromatogram.

Table 4. Characteristics of bergenan BC, lemnan LM, and their derivatives

Polysaccharide	Yield, %[1]	(Gal A) : (SO_3)	Content, %[2]							
			$-SO_3^-$	GalA	Rha	Ara	Xyl	Glc	Gal	Api
BC	–	–	–	84.0	3.7	1.9	–	1.0	2.3	–
BC-SO_3-1	82	10 : 3	12.8	68.4	1.6	2.3	–	0.7	1.3	–
BC-SO_3-2	90	10 : 10	26.2	62.2	2.0	2.9	–	0.7	1.7	–
BC-SO_3-3	96	10 : 20	44.8	48.0	1.8	1.7	–	1.6	1.6	–
LM	–	–	–	69.0	2.0	1.6	2.8	1.2	2.2	25.5
LM-SO_3	60	10 : 20	45.7	35.1	1.2	0.9	1.2	1.0	1.3	8.2

[1] Yield is calculated from the initial weight of galacturonan.
[2] Mass percents.

solution of the mixture of lyophilized human plasma (Renam) with pectin sulfates at final concentrations of 0.02-31.0 μg/ml, and the clotting time was registered by a fibrin timer (Dade Behring, USA-Germany).

Effect on the extrinsic pathway of blood coagulation was estimated by the prothrombin time (PT) [27]. Pectin sulfates and unfractionated heparin at final concentrations of 0.001-0.630 μg/ml were incubated for 1 min at 37°C with 0.1 ml of the solution of lyophilized human plasma (Renam) followed by the addition of 0.2 ml of solution of Ca-thromboplastin (Renam), and the clotting time was registered by the fibrin timer.

Anti-factor Xa activity (aXa) was determined by the methods of [28]. The mixture of 8 U/ml factor Xa (Behring; Trinity Biotech, Ireland) and 0.02 mg/ml dextran sulfate (Behring; Trinity Biotech) in Tris-EDTA buffer (250 μl) was added to 250 μl of 0.05 M Tris-EDTA buffer, 0.0075 M Na_2EDTA, and 0.175 M NaCl, pH 8.4, containing 1 U/ml of antithrombin (Behring; Trinity Biotech) and pectins at final concentration of 0.01-1000 μg/ml and incubated for 3 min at 37°C. Then the residual activity of factor Xa was determined by addition of 50 μl of chromogenic substrate S-2222 (Behring; Trinity Biotech). The activity was determined by measuring the optical density of the solution at 405 nm. The 5th International UFH Standard was used for plotting the calibration curve.

Antithrombin (aIIa) activity was determined by the activated partial thromboplastin time (APTT) test by the method of [29]. Pectin sulfates at final concentrations of 0.0001-1000 μg/ml and 0.1 ml of the mixture of ellagic acid (Trinity Biotech; Renam) and phospholipids were added to 0.1 ml of solution of lyophilized human plasma (Renam). After 3 min of incubation at 37°C, 0.1 ml of 0.025 M $CaCl_2$ solution was added and the clotting time was registered by the fibrin timer. The aIIa activity was calculated using the calibration curves of the 5th International UFH Standard.

Determination of aXa activity by inhibition of fibrinogen-coagulating activity of factor Xa [30]. The plasma with pectin sulfates at final concentration of 0.01-10,000 μg/ml (0.1 ml) was incubated for 1 min at 37°C. Then 0.05 ml of the mixture of phospholipids and factor Xa (Trinity Biotech; Renam) was added. After 3 min of incubation at 37°C, 0.05 ml of 0.035 M $CaCl_2$ solution was added and the plasma coagulation time was registered by a coagulometer. The aXa activity was calculated using the calibrations curves of the 1st International Standard for Low Molecular Weight Heparin or the 5th International UFH Standard.

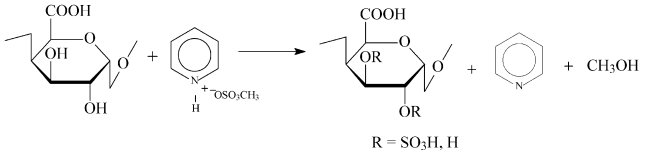
RESULTS AND DISCUSSION

The conditions for pectin sulfation were selected using galacturonan BCH obtained from bergenan by partial hydrolysis. The high positive specific rotation of the produced galacturonan indicates the D-configuration of galacturonic acid residues. Galacturonan BCH has a low degree of methyl etherification of the carboxyl groups of D-galacturonic acid residues. The content of methoxyl groups is 1.4%.

The ^{13}C-NMR spectrum of galacturonan BCH (Fig. 1) demonstrates the presence in its carbohydrate chain of a fragment →4α-D-GalpA-1→ that gives the signal of anomeric carbon atom at 100.8 ppm. Signals of the other atoms of D-galacturonic acid residues corresponded to the ^{13}C-NMR spectrum of the 1,4-α-D-galactopyranosyluronan [31, 32]. In addition, the ^{13}C-NMR spectrum of galacturonan BCH (Fig. 1) demonstrated the presence of unmethoxylated galacturonic acid residues, because the signal of atom C6 of the galacturonic acid residues of galacturonan BCH is present at 175.5 ppm. It is known [31] that atom C6 of methyl etherified galacturonic acid gives a signal at 172.4 ppm in addition to the signal of C-atom of methoxyl group at 54.1 ppm. The homogeneity of galacturonan BCH was proved by HPLC (M_r ~100 kDa).

Galacturonan was sulfated by three sulfating reagents: pyridine monomethyl sulfate, pyridine sulfotrioxide, and chlorosulfonic acid [22-24]. For the enhancement of solubility, galacturonan was preliminarily converted into a saline form (triethylammonium salt).

Galacturonan sulfation with pyridine monomethyl sulfate proceeds by the following scheme:

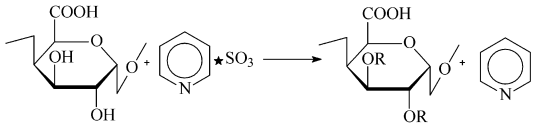


R = SO_3H, H

Galacturonan sulfation conditions were selected by varying the time and the quantity of sulfating reagent: five- and eight-fold excess of pyridine monomethyl sulfate per hydroxyl group of the galacturonic acid residue and different reaction times (0.5, 1.5, and 3 h). As a result, it was established that the degree of substitution for galacturonic acid residues in galacturonan (6 moles -SO_3^- per 10 moles of galacturonic acid residues) was maximal with the eight-fold excess of pyridine monomethyl sulfate per hydroxyl group of the galacturonic acid residue and during sulfation for 3 h at low pressure and 80°C (Table 1).

HPLC data demonstrate that the sulfation with pyridine monomethyl sulfate led to the partial destruction of galacturonan (~30%) accompanied by the formation of fragments with M_r below 50 kDa.

Galacturonan sulfation with pyridine sulfotrioxide proceeds by the following scheme:



R = H, SO_3H

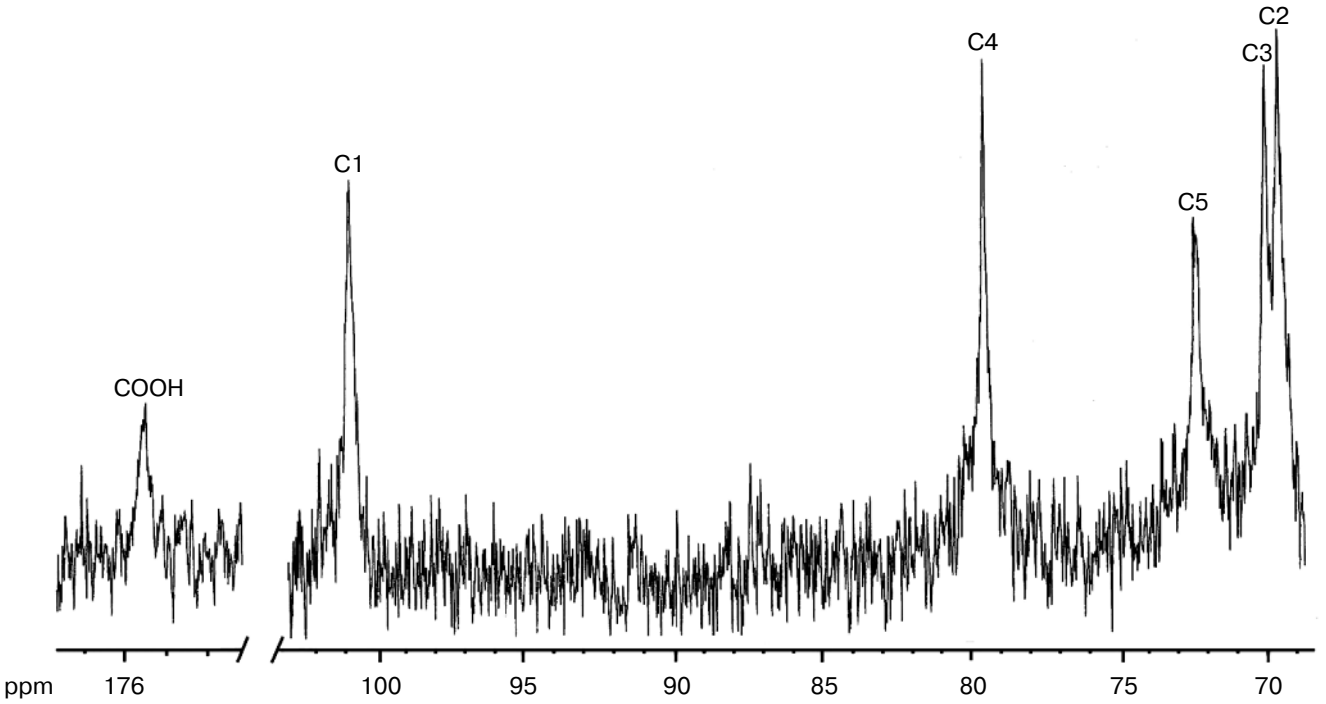
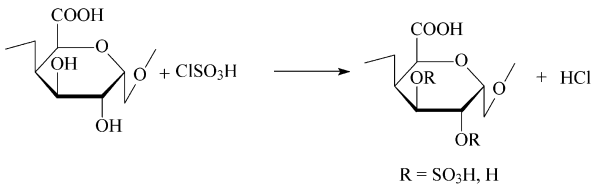


Fig. 1. ^{13}C-NMR spectrum of galacturonan.

Galacturonan sulfation conditions were selected by varying the temperature (30, 50, 60, and 70°C). Sulfation was carried out for 4 h with a three-fold excess of pyridine sulfotrioxide per hydroxyl group of the galacturonic acid residue. As a result, it was shown that reaction temperature increase had no substantial effect on the degree of galacturonan sulfation, and the degree of substitution during galacturonan sulfation with pyridine sulfotrioxide was 6-8 moles of $-SO_3^-$ per 10 moles of galacturonic acid (Table 2).

HPLC data demonstrate that galacturonan sulfation with pyridine sulfotrioxide, as well as sulfation with monomethyl sulfate, led to the partial destruction of galacturonan (~40%) accompanied by the formation of fragments with M_r below 50 kDa.

Galacturonan sulfation with chlorosulfonic acid proceeds by the following scheme:



Galacturonan sulfation conditions were selected by varying the quantity of sulfating reagent. The reaction was carried out for 1 h with a one-, two-, and three-fold excess of chlorosulfonic acid per hydroxyl group of the galacturonic acid residue. As a result, it was established that the three-fold excess of sulfating reagent substantially increased the degree of galacturonan sulfation (from 2 to 15 moles $-SO_3^-$ per 10 moles of galacturonic acid), while the increase of reaction temperature from 25 to 60°C had no effect on sulfation degree (Table 3). The maximum degree of galacturonan substitution (15 moles $-SO_3^-$ per 10 moles of galacturonic acid residue) was obtained with the three-fold excess of chlorosulfonic acid per hydroxyl group of the galacturonic acid residue.

HPLC data demonstrate that the sulfation with chlorosulfonic acid also led to the partial destruction of the carbohydrate chain of galacturonan (~30%) accompanied by the formation of fragments with M_r below 50 kDa.

NMR and IR spectroscopy of maximally sulfated galacturonan (degree of substitution: 15 moles $-SO_3^-$ per 10 moles of the galacturonic acid residue) obtained during the treatment with chlorosulfonic acid provided important additional information.

The presence of an intensive absorption band in the region of 1230-1260 cm^{-1} in the IR spectrum of sulfated galacturonan, which is typical of S=O valence vibrations, is evidence of substitution of hydroxyl groups with sulfate groups in the macromolecule (Fig. 2).

The ^{13}C-NMR spectrum of sulfated galacturonan (Fig. 3) demonstrates the presence of sulfate groups at the C2 and C3 atoms of galacturonic acid residues. The presence of sulfate group at the C2 atom shifts the signal from the anomeric carbon atom to higher field (from 100.8 ppm

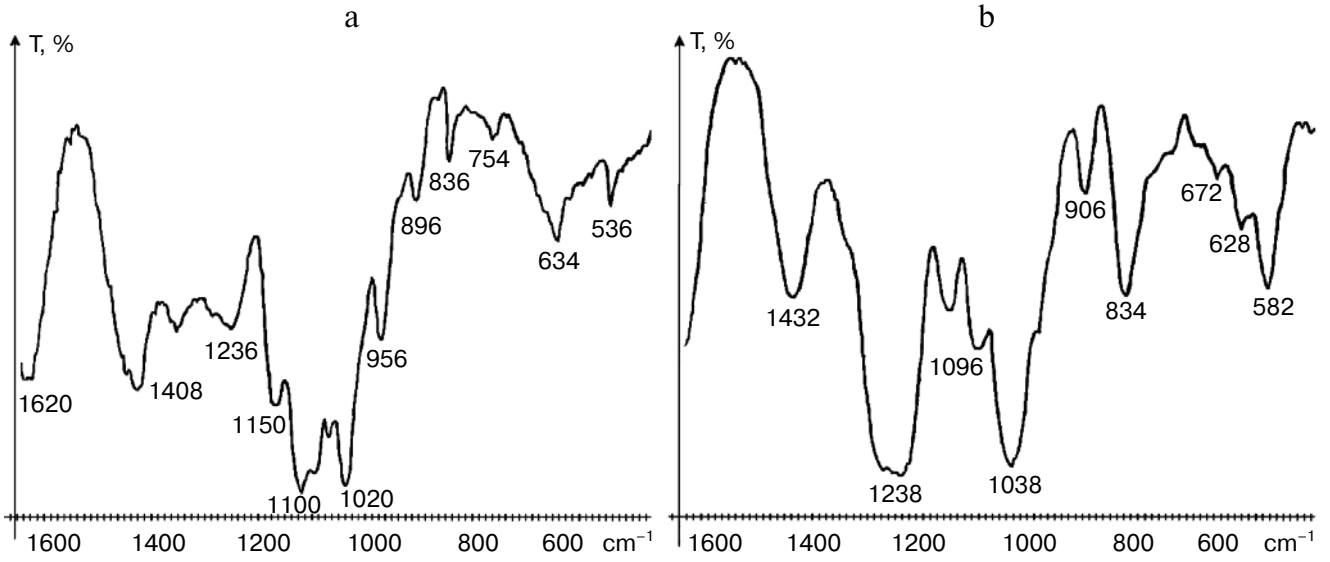


Fig. 2. IR spectra of galacturonan (a) and sulfated galacturonan (b).

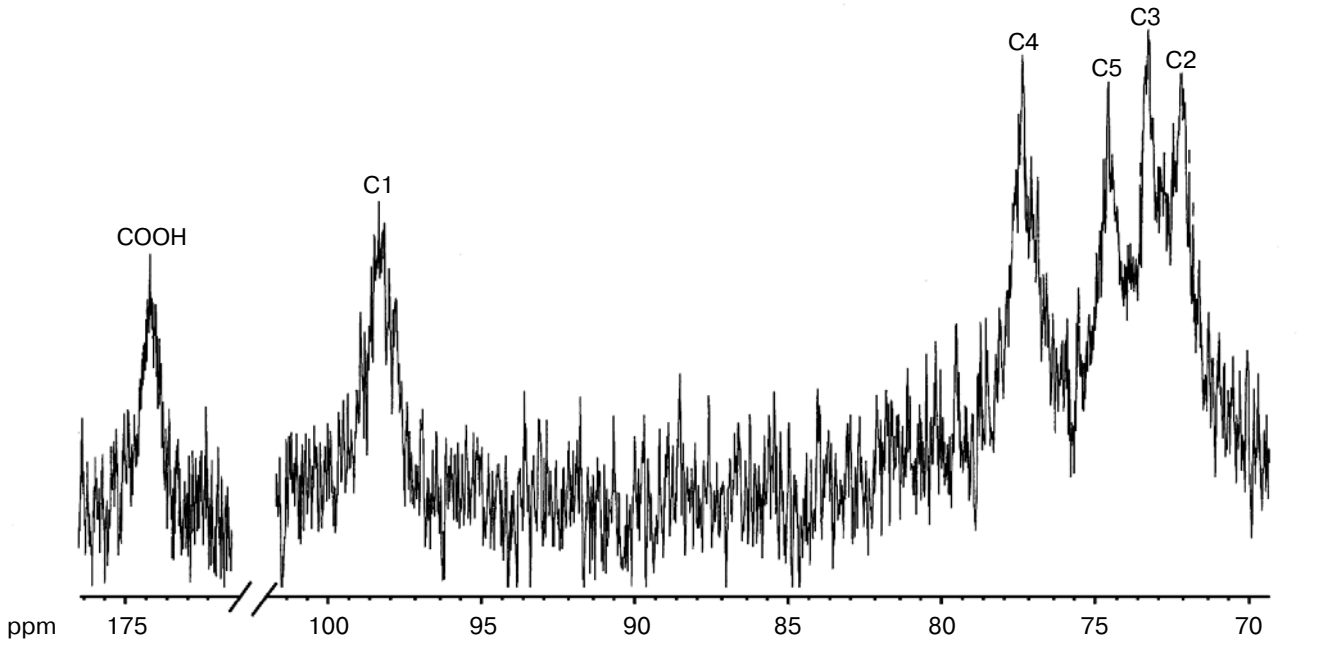


Fig. 3. ^{13}C-NMR spectrum of sulfated galacturonan.

in the initial galacturonan to 98.3 ppm in the sulfated galacturonan). The presence of sulfate group at the C3 atom is demonstrated by the shift of the signal of atom C4 to a higher field (from 79.5 ppm in the initial galacturonan to 77.4 ppm in the sulfated galacturonan) (Figs. 1 and 3).

Comparison of sulfation degree, destruction degree, and yield of sulfated derivatives depending on the type and quantity of sulfating reagent, reaction time, and reaction temperature shows that the optimal method is sulfation with chlorosulfonic acid. This method was used for sulfation of pectin polysaccharides containing, in addition to galacturonic acid residues in the main carbon chain, the residues of neutral monosaccharides as components of branched regions. The method was also successfully used for sulfation of bergenan BC with the linear region represented by galacturonan (97% of the pectin macromolecule) and the branched region represented by rhamnogalacturonan I (RG-I) [33] and lemnan LM with the linear region represented by galacturonan (84%) and

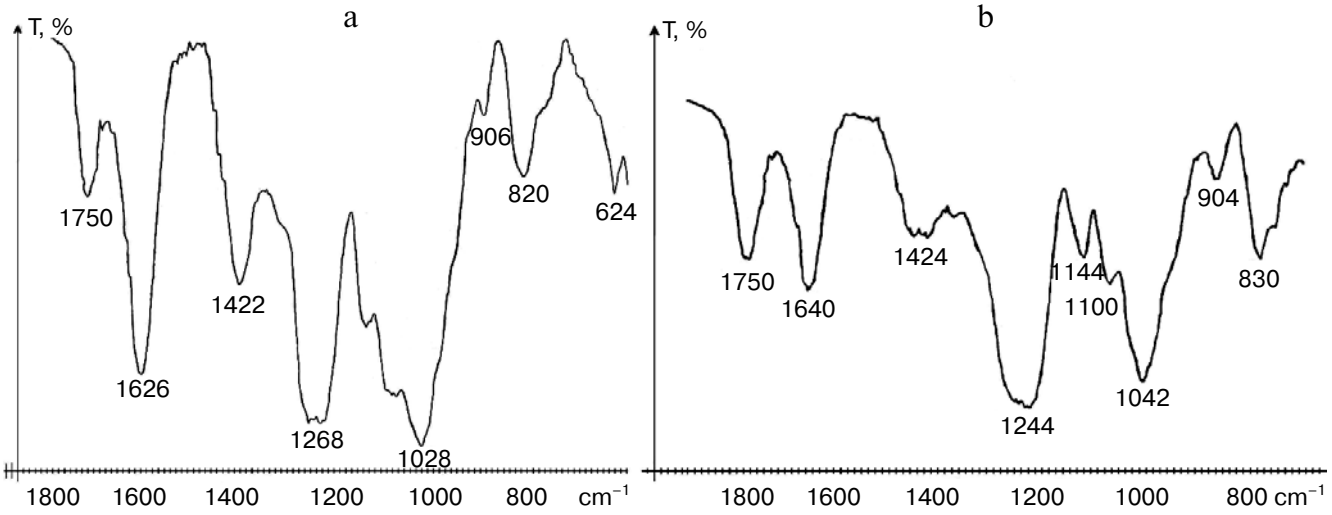


Fig. 4. IR spectra of derivatives LM-SO_3 (a) and BC-SO_3-3 (b).

the branched region represented by apiogalacturonan (15%) and RG-I [34].

Sulfation of pectins with chlorosulfonic acid. Bergenan BC was sulfated with one-, two-, and three-fold excess of chlorosulfonic acid per hydroxyl group of galacturonic acid; as a result, derivatives BC-SO_3-1, BC-SO_3-2, and BC-SO_3-3 were obtained, respectively (Table 4). Sulfation was carried out for 1 h at 25°C. The derivatives are characterized by different degrees of sulfation (Table 4).

Lemnan LM was sulfated with a three-fold excess of chlorosulfonic acid per hydroxyl group. As a result, LM-SO_3 was obtained (Table 4).

The presence of sulfate groups in the sulfated derivatives BC-SO_3-3 and LM-SO_3 is demonstrated by the presence of an intensive absorption band in the region of 1230-1260 cm^{-1} in their IR spectra, which is typical of S=O valence vibrations (Fig. 4).

Anticoagulant properties of sulfated pectins. Sulfated polysaccharides of animal (dermatan sulfate, chondroitin sulfate, keratin sulfate, hyaluronic acid) and plant origin (fucoidans, galactomannan and cellulose sulfates) are characterized by anticoagulant activity [8, 35-37]. However, we have found no publications on the anticoagulant properties of sulfated pectins. The effect of sulfated polysaccharides on proteases of the blood coagulation system is associated either with activation of serpins (antithrombin, heparin cofactor II, protease nexin I, plasminogen I activator inhibitor (PAI), protein C inhibitor (PCI), protein Z-dependent protease inhibitor (PZI)) or with direct inhibition of the activity or generation of coagulation factors. The anticoagulant properties of polysaccharides depend on the degree of substitution: inhibition of the activity or generation of serine proteases of the blood coagulation system increases together with enhancement of the degree of sulfation of the polysaccharides [38].

Pectin sulfates BC-SO_3-3 and LM-SO_3 increase the time of human plasma coagulation in the PT test. Double

Table 5. Specific anticoagulant activity of sulfated pectins

Polysaccharide	APTT test		ReaClot test	
	2APTT, μg/ml	aIIa, U/mg	2ReaClot, μg/ml	aXa, U/mg
BC	>500	0	>500	0
BC-SO_3-1 (10 : 3)	470.00 ± 40.00	0.71 ± 0.23	112.11 ± 18.00	0.22 ± 0.07
BC-SO_3-2 (10 : 10)	240.00 ± 3.03	1.40 ± 0.21	100.12 ± 9.23	0.25 ± 0.06
BC-SO_3-3 (10 : 20)	1.11 ± 0.06	90.33 ± 8.91	5.60 ± 0.70	5.82 ± 0.73
LM	>500	0.25 ± 0.11	>500	0
LM-SO_3	1.78 ± 0.22	64.53 ± 5.50	3.98 ± 0.96	11.92 ± 2.44
UFH st.	0.36 ± 0.05	203.10	0.02 ± 0.01	220.00

Table 6. Inhibition of amidolytic activity of factor Xa in the presence of antithrombin

Polysaccharide	IC_{50}, μg/ml	aXa, U/mg
BC	>500.000	0
BC-SO_3-1	>500.000	0.0012 ± 0.0006
BC-SO_3-2	>500.000	0.0013 ± 0.0005
BC-SO_3-3	125.000 ± 32.000	0.1432 ± 0.0251
LM	>500.000	0
LM-SO_3	63.000 ± 7.000	0.3233 ± 0.0905
UFH st.	0.051 ± 0.004	220.0000

effective concentrations increasing the coagulation time twofold compared to the control (12.5 ± 1.6 sec) are 0.34 ± 0.06 and 0.36 ± 0.09 μg/ml for pectin sulfates, respectively, i.e. fivefold higher than for UFH (0.07 ± 0.02 μg/ml).

BC-SO_3-3 and LM-SO_3 with the content of sulfate groups 44.8 and 45.7 wt.%, respectively, have the highest aIIa activity in heparin units (47.4 ± 5.5 and 32.7 ± 4.6 U/mg). Initial pectins BC and LM do not increase the time of human plasma coagulation in the TT test. Pectin sulfates increase the time of human plasma coagulation in the APTT test. The highest aIIa activity of BC-SO_3-3 and LM-SO_3 is 90.33 ± 8.91 and 64.53 ± 5.50 U/mg, respectively (Table 5). However, the aIIa activity by inhibition with the most active pectin sulfates of the rate of chromogenic substrate hydrolysis by thrombin in the presence of antithrombin only increases 1.7-fold for LM-SO_3 (100.0 ± 18.3 U/mg) and decreases 2.3-fold for BC-SO_3-3 (39.0 ± 5.2 U/mg). Probably, LM-SO_3 needs only antithrombin for realization of the antithrombin activity, while BC-SO_3-3 might need some other serpin.

It is notable that the aXa activity of LM-SO_3 in the ReaClot coagulation test is twice higher than that of BC-SO_3-3 (11.92 ± 2.44 and 5.82 ± 0.73, respectively; Table 5), though it would be logical to anticipate higher activity for bergenan pectin sulfate. Besides, lemnan sulfate inhibits the amidolytic activity of factor Xa more effectively than BC-SO_3-3 (Table 6). It is known that the monosaccharide composition of plant polysaccharides influences their anticoagulant activities [39]. Most likely, the amounts of Xyl and Api play a role in manifestation of the aXa activity. At the same time, decrease (nearly 40 times) of the aXa activity calculated in the coagulation test, compared to the aXa activity of pectin sulfates during inhibition of the rate of chromogenic substrate hydrolysis, indicates the necessity of antithrombin for the inhibition of factor Xa.

Thus, the sulfation of lemnan LM and bergenan BC leads to the manifestation of anticoagulant properties associated with the inhibition of fibrinogen-coagulating and amidolytic activities of thrombin and factor Xa through antithrombin and, probably, other serpins. The specific anticoagulant activity of sulfated pectins depends on plant species, pectin monosaccharide composition, and degree of sulfation.

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