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Branching of the Galacturonan Backbone of Comaruman, a Pectin from the Marsh Cinquefoil *Comarum palustre* L.

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Abstract—Galacturonan, the main constituent of the backbone (core) of the comaruman macromolecule, a pectin from the marsh cinquefoil *Comarum palustre* L., was obtained on partial acid hydrolysis of the pectin. Using atomic force microscopy and methylation analysis of the galacturonan, the backbone of the comaruman macromolecule was shown to contain branches as side chains consisting of α-1,4-linked residues of D-galactopyranosyl uronic acid attached to the 2- and 3-positions of the galacturonic acid residues of the core, in addition to linear regions of α-1,4-D-galacturonan. A few side chains appear to attach to 2,3-positions of the D-galacturonic acid residues.

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The pectin comaruman was first isolated [1] from the marsh cinquefoil *Comarum palustre* L., which is widespread in the north of Europe. The backbone (the core of the macromolecule) of comaruman was shown to be constructed of α-1,4-D-galacturonan consisting of numerous separate sections interconnected by L-rhamnopyranose residues involved in α-1,2-linkages. The ramified region of the macromolecule contains rhamnogalacturonan I (RG-I) [2, 3] with branched side chains of arabinogalactan having a backbone of β-1,4-linked D-galactopyranose residues, which are attached to 4-position of the L-rhamnose residues of the macromolecule core. On the basis of data from methylation analysis of comaruman, a possible branching of the macromolecule core was suggested [1]. Similar assumptions have been made earlier [4] in relation to some other pectic polysaccharides, but they require experimental confirmation.

Application of atomic force microscopy (AFM) to elucidation of the architectonics of the pectic polysaccharide macromolecules [5-7] provided some attractive data concerning branching of pectins and suggested [6] the occurrence in tomato pectin of side chains composed of long regions of polygalacturonic acid attached to the core by linkages of unascertained type. However, this suggestion needs additional evidence.

The present paper provides experimental proof of the presence of branched galacturonan backbone in comaruman, a pectic polysaccharide of *C. palustre* L.

MATERIALS AND METHODS

Isolation of pectic polysaccharides. Comaruman was extracted from the aerial part of *C. palustre* L. freshly collected near Vizinga, Sysola Region (Komi Republic, Russia) using aqueous ammonium oxalate as described earlier [8]. As a result, comaruman, $[\alpha]_D^{20}$ +192° (*c* 0.1; H_2O), content of OCH_3 groups 3.9%, yield 4% air-dried raw materials, was obtained.

Isolated comaruman (1 g) was subjected to a partial acid hydrolysis with 2 M TFA (200 ml) for 5 h at 100°C to

Abbreviations: AFM) atomic force microscopy.
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obtain galacturonan. A precipitate was separated by centrifugation, washed with 96% ethanol, and dissolved in aqueous ammonia while adjusting the pH of the solution to 4.0-4.5. The solution was dialyzed in ultrafiltration units (Millipore, USA) through membrane (polysulfone, 100 kD) against water changed constantly up to complete disappearance of sugars in the dialyzate followed by lyophilization of the solution to yield 250-270 mg of galacturonan, $[\alpha]_D^{20}$ +237.6° (*c* 0.17; H_2O), galacturonic acid 99%, trace of rhamnose (less than 0.5%), OCH_3 groups and protein admixtures being absent.

General analytical procedures. Contents of glycuronic acids were determined using samples previously dried over P_2O_5 in vacuum and reaction with 3,5-dimethyl phenol in the presence of concentrated sulfuric acid [9] (a standard curve was plotted for D-galacturonic acid); protein contents were estimated accordingly to the Lowry procedure [10] (a standard curve for BSA was used); methoxyl group contents were calculated in accord with a published procedure [11] (a calibration curve for methanol was employed). All the spectrophotometric measurements were performed on an Ultrospec 3000 spectrophotometer (England). Specific optical rotations were determined on a Polartronic MHZ polarimeter (Germany).

Sugars were identified using descending paper chromatography on Filtrak FN-12 and FN-13 paper in solvents butan-1-ol–pyridine–H_2O (6 : 4 : 3 v/v). The sugars were detected by spraying with hydrogen aniline phthalate at 105°C.

Neutral sugars were qualitatively and quantitatively analyzed using GLC as the corresponding alditol acetates. GLC was performed on a Hewlett-Packard 4890A chromatograph (USA) equipped with flame-ionization detector and an HP 3395A integrator on an RTX-1 capillary column (0.25 mm × 30 m, Restek) (using argon as the carrier gas) in the following program: from 175°C (1 min) to 250°C (2 min) with a rate of 3°C/min. The percentage of monosaccharides in the total sample was calculated from the peak areas using coefficients of detector response [12].

Liquid chromatography of galacturonan samples was run as described earlier [13]. The sample (3 mg) was dissolved in 0.15 M NaCl (1 ml) in bidistilled water, and then the solution obtained was filtered. The following chromatographic equipment was used for analysis: SD-200 pump (Dynamax, USA), Shodex Asahipak GS-620 HQ column (7.6 mm × 30 cm) and Shodex GS-26 7B pre-column (7.6 mm × 5 cm), CTO-10AS thermostat, and RID G 136 A refractometer (Shimadzu, Japan) as the detector. Elution was carried out with 0.15 M NaCl at 40°C with a flow rate of 0.5 ml/min. The column was calibrated using dextran sulfates with molecular masses in the range of 36-50, 400-600, and 1400 kD (Sigma, USA).

AFM measurements were run on a Solver-Bio P47 instrument (NT-MDT, Russia). All AFM images were obtained in broken contact regulations as analog of tapping mode using supersharp cantilevers of high resolution (Nanotuning, Russia). The rate of scanning was 0.5-1.0 Hz, and the angle of scanning was 0-90°. All the images were obtained in air with uncontrolled humidity of surrounding air.

Highly oriented pyrolytic graphite (HOPG) surface was modified as follows: a drop of GM graphite modifier (Nanotuning) was applied on the HOPG surface, kept for 5 min, and blown off with a stream of argon. Aqueous solution of galacturonan (10 μl) as a drop with concentration of 1.5 μl/ml was applied on the modified HOPG surface. After 10 min adsorption, the sample surface was washed with water for 15 min and dried with a stream of argon. The mean length of the galacturonan macromolecule were measured on AFM images obtained (Fig. 1) followed by representation as the corresponding histogram (Fig. 2).

GLC-MS of the partially methylated alditol acetates was carried out on a Carlo Erba Finnigan 4200 instrument using a DB-5 capillary column (0.25 mm × 30 m, 72W Scientific) (helium carrier gas) over the temperature gradient of 150-280°C with a rate of 5°C/min. MS was measured with a Finnigan MAT ITD-700 ion trap (England), mass range from m/z 44 to 500. The energy of ionizing electrons was ~70 eV. The temperature of the interface was 220°C, scanning frequency 1 scan/sec, and acquisition delay 250 sec.

All the aqueous solutions were concentrated in vacuum at 40-45°C followed by centrifugation at 7000-8000*g* for 10-20 min, and then the samples were lyophilized on a Virtis instrument (USA).

Synthesis of the salt of galacturonan with triethylamine. Galacturonan (20 mg) was dissolved in distilled water and dialyzed against 1% aqueous triethylamine hydrochloride for three days, changing the solution of triethylamine hydrochloride several times. The solute of the galacturonan triethylamine salt obtained was dialyzed against distilled water, lyophilized, and dried over P_2O_5 in vacuum at 60°C. As a result, the anhydrous salt of galacturonan with triethylamine easily soluble in dimethyl sulfoxide was obtained and used for methylation analysis [12].

Methylation analysis of galacturonan. The salt of galacturonan (3-5 mg) was mixed vigorously with tetrahydrofuran (1 ml), excess of $LiBH_4$ was added, and the resulting mixture was heated at 70°C for 1 h, neutralized with 10% acetic acid in methanol, dialyzed, and lyophilized to give galactan [14] that was permethylated in accord with Hakomori as described earlier [12]. Methylation was complete after full disappearance of the absorption band of hydroxyl groups in the IR spectrum of the biopolymer. The bands of -COOH and -$COOCH_3$ groups were also absent from the IR spectrum. The sample of the resulting galactan was permethylated repeatedly to give the second sample of permethylated galactan. The samples of galactan obtained after the first and

repeated permethylation were hydrolyzed with 2 M TFA (0.5-1 ml) at 100°C for 5 h. Excess acid was removed by multiple evaporation with methanol in vacuum at 40°C, and the methylated sugars were reduced with $NaBH_4$ to the corresponding alditols followed by acetylation with acetic anhydride in pyridine. The mixture of methylated alditol acetates obtained was analyzed using GLC-MS.

RESULTS AND DISCUSSION

Isolation of pectic polysaccharides. Comaruman was isolated from the aerial part of *C. palustre* L. as described earlier [8]. Aqueous solutions of comaruman were found to possess a very high viscosity. In addition, comaruman showed a substantial gel-forming ability [1]. The comaruman was subjected to partial acid hydrolysis (2 M TFA, 100°C, 5 h) to furnish the crude galacturonan, and further purification led to a virtually pure galacturonan, $[\alpha]_D^{20}$ +237.6° (*c* 0.17; H_2O) that contained more than 98% galacturonic acid. Rhamnose as the single neutral monosaccharide was detected as a trace (less than 0.5%). HPLC indicated that the galacturonan sample had a high degree of homogeneity indicated by the single peak corresponding to molecular masses from 100 to 300 kD observed on chromatograms.

Elucidation of galacturonan architectonics using AFM. The sample of the purified galacturonan was studied by AFM [5, 6]. An AFM image obtained for the galacturonan macromolecule is given in Fig. 1. As can be seen from Fig. 1, the galacturonan occurs as separate molecules and aggregates. The presented image is typical of a series of galacturonan samples. The contour sizes of the galacturonan macromolecules are given in a histogram (Fig. 2). As can be seen from Fig. 2, the galacturonan displays substantial polydispersity. This phenomenon was confirmed by HPLC of the galacturonan which gave a single but wide peak, which corresponded to molecular masses in the range of 100-300 kD. Lengths of the linear molecules and the linear sections of the side chains of the branched macromolecules varied within 10-100 nm and had mean equal to 30 ± 15 nm in accord with the AFM measurements. Branched molecules (Fig. 1a) represented not less than 50% of the total number of macromolecules. AFM imaging characterized separate galacturonan molecules (Fig. 1b) and their collection in the sample (Fig. 1a).

Figure 1 demonstrates clearly that many of the galacturonan macromolecules appeared to bear side chains of various sizes, including rather long ones in addition to the backbone. It must be noted that the galacturonan molecules contained one or several side chains attached to different or the same region of the backbone (Fig. 1b). Taking into account that virtually pure galacturonan contains no neutral sugar residues, it can be concluded that branching of the backbone contains fragments consisting of D-galacturonic acid residues only. Therefore, some regions of the galacturonan core of comaruman appeared to possess branched structural patterns and various extent of branching. In addition to the branched regions, linear regions can be seen in the comaruman macromolecule (Fig. 1).

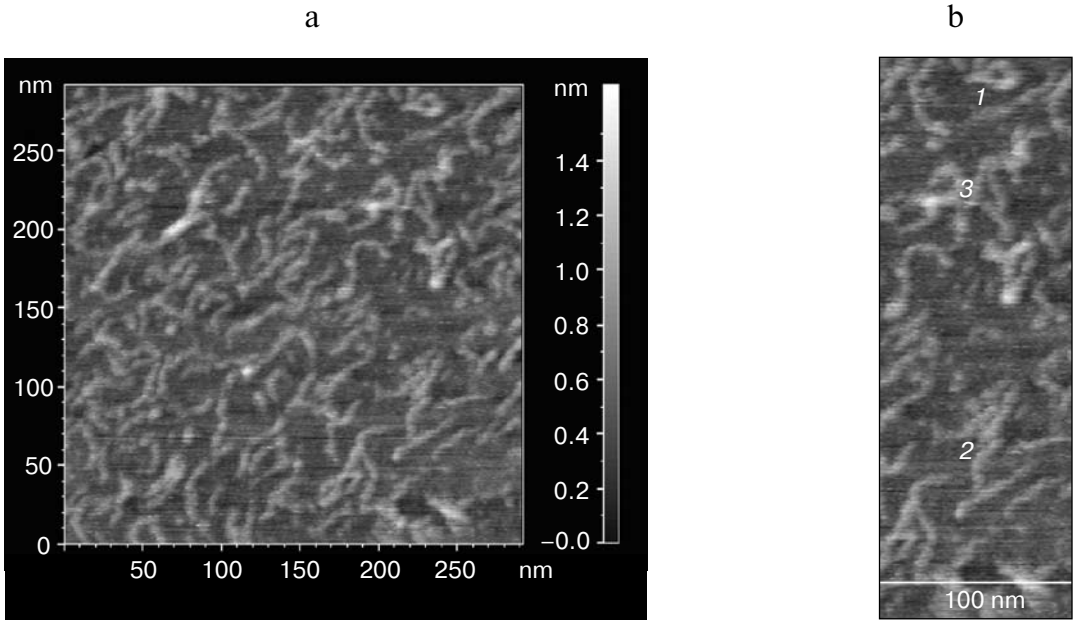

Fig. 1. AFM image of galacturonan as the main constituent of the comaruman macromolecule core. a) AFM image obtained on the instrument; b) a magnified area (171-270 nm) of AFM image. *1*) Linear molecule; *2*) molecule bearing one branching; *3*) molecule having several side chains.

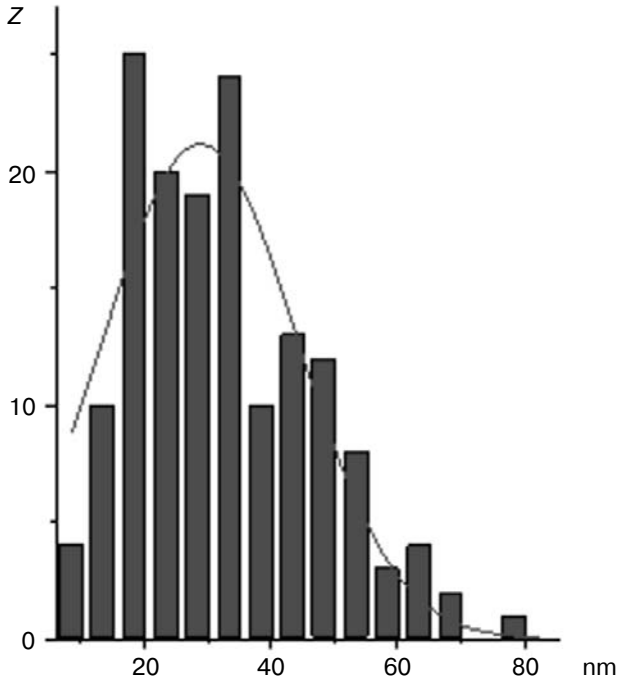

Fig. 2. Histogram of distribution of contour lengths of the galacturonan macromolecules measured using AFM. Parameters of the polysaccharide macromolecules obtained using AFM: mean length of the linear segments of the polysaccharide molecule, 30 ± 15 nm; molecule height measured using AFM, 0.6 ± 0.3 nm; width measured at half height, 4.2 ± 0.7 nm.

This structural peculiarity of comaruman is suggested to be reflected in its physiological activity. As shown earlier [15, 16], comaruman possesses a marked anti-inflammatory activity in contrast to other pectic polysaccharides. In addition, galacturonan, as the comaruman macromolecule core, was shown to possess higher activity in comparison with the parent polysaccharide [15, 16].

Structural studies of galacturonan by methylation analysis. In order to get additional data concerning branching of the backbone of comaruman and its structure, galacturonan as the main constituent of the core was studied using classic methylation analysis as described earlier [12]. To increase solubility of galacturonan in non-aqueous solvents, its salt with triethylamine was obtained [1]. The salt was dried carefully and reduced with $LiBH_4$ in tetrahydrofuran to the corresponding galactan [14] because complete permethylation of polysaccharides containing glycuronic acid residues as constituents of their sugar chains is virtually impossible. The galactan obtained was shown to afford galactose only on complete acid hydrolysis and was methylated twice according to Hakomori's method [12, 17] with methyl iodide in dimethyl sulfoxide in the presence of methyl sulfinyl carbanion [17].

Disappearance of the absorption band of hydroxyl groups in the IR spectrum of permethylated galactan was an indication of completeness of methylation. The permethylated galactan was obtained. This sample was methylated repeatedly to give the second permethylated one. Both samples of galactan were subjected to a complete acid hydrolysis, and the mixture of methylated sugars obtained was reduced with $NaBH_4$ and partially methylated alditols were acetylated with acetic anhydride in pyridine followed by analysis of the mixture of the corresponding methylated alditol acetates using GLC-MS. In the two cases identical results were obtained.

As a result of the analysis, the sugar chain of permethylated galactan was shown to be composed of 2,3,4,6-tetra- (small amounts), 2,3,6-tri- (the main constituent), 2,6-, and 3,6-di-O-methyl D-galactopyranose. The combined contents of 2,6- and 3,6-di-O-methyl derivatives were approximately equal to those of the permethylated galactopyranose. In addition, 6-O-methyl-D-galactose was detected in very small amounts. In accord with these data, galacturonan as the backbone of comaruman possessed branched structural features of the sugar chain. Taking into account data obtained previously [1], it can be concluded that the galacturonan macromolecule core represented the linear sugar chain consisting of residues of α-1,4-linked D-galactopyranosyl uronic acid. Branches are present as side chains composed of the same D-galacturonic acid residues α-1,4-linked and attached to 2-O- and 3-O-positions of the galacturonic acid residues of the galacturonan backbone. A small number of side chains appeared to attach to 2,3-positions simultaneously (see Fig. 1b).

Thus, existence of branching in the backbone of comaruman, a pectic polysaccharide of *C. palustre* L., is confirmed experimentally for the first time.

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