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RHAMNOGLUCOURONAN FROM *Alcea rosea* STEMS

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The acidic polysaccharide rhamnoglucouronan, the structure of which was studied by chromic and periodate oxidation, methylation, partial acid hydrolysis, and ^{13}C NMR spectroscopy, was isolated from Alcea rosea *stems. The main polysaccharide chain consists of α-1→2 and α-1→4 bound rhamnopyranoses and uronic acids and is branched.*

Key words: *Alcea rosea*, rhamnoglucouronan, hydrolysis, fractional precipitation, structure.

Polysaccharides of plant origin are important high-molecular-weight compounds with a wide range of practical application. Therefore, they are of great interest. We studied stems of *A. rosea* f. nigra (black variety of hollyhock, Malvaceae) [1] collected in the plot of the Botanical Institute and the Botanical Garden of the Academy of Sciences of the Republic of Uzbekistan.

Water-soluble polysaccharides (WSPS) were isolated by the previously described method [2]. WSPS are a white amorphous powder that is very soluble in water, η_{rel} 4.8 (*c* 1.0, H_2O), with 2.5% ash content. An aqueous solution of WSPS does not give a positive iodine test for starch and contains 1.4% nitrogen from proteinaceous substances. A polysaccharide without nitrogen was obtained after removing proteinaceous substances by the Sevag method [3].

Total acid hydrolysis of WSPS produces rhamnose, arabinose, glucose, and galactose in a 22:2:5:1 ratio according to PC and GLC [4, 5]. Galacturonic and glucuronic acids, the content of which was 46% according to the carbazole method, were also isolated by PC [6].

According to gel filtration, the WSPS were heterogeneous [7].

A homogeneous polysaccharide was obtained by fractional precipitation with alcohol of an aqueous solution of WSPS [8]. Four fractions were obtained. The greatest yield came from fraction 2 (Table 1).

Fraction 2 was homogeneous according to gel filtration over Sephadex G-75 and ultracentrifugation. PC and GLC identified mainly L-rhamnose and D-glucose in a 2.5:1.0 ratio in the hydrolysate of fraction 2. The content of uronic acids was 15%. Therefore, the polysaccharide is a rhamnoglucouronan (RGU). It was further studied by extensive chemical analysis.

RGU is a white amorphous powder that dissolves well in water to give a viscous solution, η_{rel} 5.4 (*c* 1.0, H_2O), $[\alpha]_D$ +58° (*c* 1.0, H_2O), $O–CH_3$ = 1.85%.

The molecular weight determined by sedimentation is 39,000±10%, which is close to the value of 40,000 estimated from a calibration curve for dextrans (MW 80,000, 40,000, and 20,000-15,000).

IR spectrum of RGU contains absorption bands at 815 cm^{-1} (pyranose ring), 900 cm^{-1} (β-glycoside bond), 840 cm^{-1} (α-glycoside bond), 1200-1600 cm^{-1} (ester), and 3400-3600 cm^{-1} (hydroxyl).

The positive specific rotation and the IR spectra indicate that the glycoside bond between the monosaccharides has the α-configuration. This was confirmed by oxidation of the total acetylated RGU with chromic anhydride in glacial acetic acid [9]. PC of the reaction products demonstrated that rhamnose, glucose, and galacturonic acid were not oxidized. Therefore, there is an α-glycoside bond between the monosaccharides. RGU contains glucuronic acid. However, glucuronic acid was not identified among the oxidation products. This confirms that the glycoside bond to glucuronic acid has the β-configuration.

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TABLE 1. Properties of *A. rosea* Fractions

Fraction No.	Yield, % as WSPS	Monosaccharide ratio						
		Rha	Ara	Xyl	Man	Glu	Gal	UAC
1	8.00	4.0	1.3	1.3	Tr.	1.0	1.0	+
2	70.00	2.5	Tr.	Tr.	-	1.0	Tr.	+
3	1.00	3.1	2.0	-	1.5	5	3	+
4	12.00	1.0	1.0	Tr.	-	2.0	1.0	+

TABLE 2. Chemical Shifts (ppm) of C Atoms in ^{13}C NMR Spectra of RGU and DRGU Polysaccharides

Polysaccharide	C1	C2	C3	C4	C5	C6
RGU						
α-L-Rha*p*	104.7	70.45	71.45	73.26	70.9	17.75
α-D-Gal*p*UAC-1-2α-L-Rha*p*	100.4	70.8	71.45	73.26	73.65	174.97
D-L-Rha*p*-1-2α-L-Rha*p*	99.35	80.0	71.45	72.65	70.04	17.5
DRGU						
α-D-Gal*p*UAC-1-2α-L-Rha*p*	99.9	70.9	71.45	72.45	72.45	173.6
2-α-L-Rha*p*	90.3	80.4	71.6	72.45	71.45	17.7
α-L-Rha*p*1-2α-L-Rha*p*	99.9	80.42	71.45	73.0	69.0	17.7
α-D-Glc*p*	92.7	72.46	73.0	70.1	71.9	61.9
α-D-Gal*p*UAC	100.4	70.6	76.9	74.6	72.65	174.9
α-D-Glc*p*UAC	104.9	74.65	80.4	73.26	76.9	176.7

RGU was oxidized by sodium periodate solution (0.25 M) at room temperature for 20 days to determine the size of the oxide rings [10]. Each mole of anhydrohexose consumed 0.96 mole of oxidant. The amount of formic acid released was 0.03 mole. Smith degradation [11] produced glycerine, glucose, and traces of glucuronic acid according to PC. Traces of glycerine are consistent with a high degree of RGU polymerization and the presence of 1—2 bonds between hexoses in addition to pyranose monosaccharides. The presence of glucose and glucuronic acid indicate that the main chain of RGU may be branched.

Rhamose and glucose in the RGU chain were methylated according to a modified Hakomori method [12] to determine the type of glycoside bonds. The permethylate was obtained in 45% yield, $[\alpha]_D^{24}$ +35° (*c* 1.0, $CHCl_3$) and was subsequently formolyzed and hydrolyzed. TLC and GLC detected 3-O-Me-L-rhamnose, 2,3,4-tri-O-Me-D-glucose, and 3,4-di-O-Me-L-rhamnose in a 3:1:0.5 ratio.

The presence of 3-O-Me-L-rhamnose indicates that the rhamnopyranoses are bonded; of 3,4-di-O-Me-L-rhamnose, that rhamnose may be bonded to uronic acids by 1→4 bond. The formation of 2,3,4-tri-O-Me-D-glucose indicates that the glucoses are 1→4 bonded to the main chain. Demethylation of the methylation products gave rhamnose and glucose [13].

RGU was subjected to partial acid hydrolysis. The products contained rhamnose, glucose, uronic acids, and two oligosaccharides (A and B) according to PC. Preparative PC isolated the pure oligosaccharides. Oligosaccharide A gave only rhamnose upon total acid hydrolysis; oligosaccharide B, rhamnose and glucose.

The results were confirmed by studying the ^{13}C NMR spectrum of RGU, which contains resonances at 104.7 (C_1), 71.45 (C_3), 73.26 (C_4), 70.9 (C_5), and 17.75 (C_6) ppm belonging to C atoms of the L-rhamnopyranoses. A signal at 55.0 ppm belongs to the rhamnose methyl.

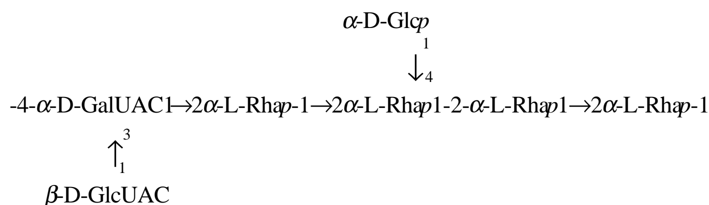
The spectrum also contains weaker signals at 176.05 and 176.76 ppm belonging to C-6 atoms of glucuronic and galacturonic acids. Signals at 100.4 and 80.0 ppm belong to C-1 and C-2 of the α-1→2 bonded rhamnose. The weaker signal at 100.4 ppm is typical of C-1 in β-D-glucuronic acid.

The very characteristic chemical shifts of 99.3 and 100.44 ppm for C-1 of the rhamnopyranose indicate that rhamnose is located in the middle of the chain. The chemical shift of 76.9 ppm (C-4) indicates that there is a α-1→4 bond between

rhamnose and galacturonic acid. There are no signals at 77-79 ppm. Therefore, rhamnopyranose has the α-configuration and is bonded to galacturonic acid by an α-1→2 bond. The remaining chemical shifts are listed in Table 2.

The attachment sites of uronic acids to the RGU chain were established using also ^{13}C NMR. For this, RGU was destroyed, i.e., hydrolyzed by H_2SO_4 (1 N) for 2 h at 90ºC, to give destroyed rhamnoglucuronan (DRGU) with MW 24,300, $[\alpha]_D^{22}$ +75º (*c* 1.0, H_2O), glucose:rhamose = 1:4, and uronic acid content 50%. The spectrum exhibited signals typical of α-1→2 bound rhamnopyranose. The integration values are given in Table 2.

Based on the results, the following sequence is proposed for the repeating unit of RGU:



EXPERIMENTAL

Solutions were evaporated in a rotary evaporator at 40±5ºC. Paper chromatography was carried out on FN 11, 13, and 16 paper in descending mode using 1-butanol—pyridine—water (6:4:3, by volume).

Thin-layer chromatography was performed on Silufol UV-254 plates in $CHCl_3$—CH_3OH (9:1). Free and methylated monosaccharides were identified using acidic anilinium phthalate (10 min at 105-110°C); polyols, bromophenol blue.

GLC was performed on a Chrom-5 apparatus with a flame-ionization detector under the following conditions: steel column (0.3×200 cm), Chromaton NAW 0.200-0.230 mm impregnated with 5% silicone XE-60, He carrier gas, 60 mL/min, 210-270ºC.

IR spectra were recorded on a Perkin—Elmer 2000 Fourier spectrometer. ^{13}C NMR spectra were taken on a Unity 400 Plus spectrometer (Varian) at working frequency 100 MHz for D_2O solutions. Methanol was used as an internal standard.

The specific rotation was measured on a Zeiss polarimeter (1 dm tube length, 10 mL volume; and 0.5 dm, 1 mL, at 20-30ºC).

Ultracentrifugation was carried out on a MOM-3170 apparatus (5,000 rpm, 20ºC) for 30 min.

Isolation of Polysaccharides. Air-dried and ground raw material (300 g) was boiled in 96º alcohol (500 mL) for 1 h and filtered. The remainder of the raw material was dried and extracted thrice with water (1:10) at room temperature for 1 h. The extracts were combined and treated with chloroform—butanol (5:1). The precipitate of proteins was isolated by centrifugation. The solution was evaporated to half the volume. Polysaccharides were precipitated by ethanol (1:2 ratio). The precipitate was separated, washed with ethanol, and dried in vacua over P_2O_5. Yield 10 g.

Hydrolysis of Polysaccharides. The substance (100 mg) was hydrolyzed by H_2SO_4 (5 mL, 2 N) on a boiling-water bath for 8 h, neutralized by barium carbonate, deionized by KU-2 cation exchanger (H^+), and evaporated. PC revealed galacturonic and glucuronic acids and neutral monosaccharides.

Fractional Precipitation of Polysaccharides. Polysaccharide (6 g) was dissolved in water (600 mL) and treated dropwise with ethanol (150 mL) while vigorously stirring (on a magnetic stirrer). The precipitate was separated by centrifugation, washed with ethanol and acetone, and dried to constant weight. Yield 0.5 g (fraction 1). The supernatant was treated analogously with ethanol (300 mL). The precipitate was worked up. Yield 4.2 g (fraction 2). The solution from isolation of fraction 2 was again treated with ethanol (600 mL). The precipitate was separated and dried as described above. Yield 0.06 g (fraction 3). The solution was treated again with ethanol (600 mL) to give fraction 4 (0.7 g).

The resulting fractions (10 mg each) were hydrolyzed by H_2SO_4 (1 mL, 1 N) for 8 h. Their monosaccharide compositions were determined using PC and GLC as acetates of the aldonitriles (Table 1).

Gel Filtration of RGU. Acidic polysaccharide (30 mg) was dissolved in water (3 mL), placed on a Sephadex G-75 column (2×55 cm), and eluted with the same solvent. The eluates were collected (3 mL, 15 min) and analyzed by phenol—H_2SO_4. The column was calibrated using dextrans with molecular weights 110,000, 80,000, and 40,000. The void volume of the column was V_e 275.5 mL; V_e 110,000, 324 mL; V_e 80,000, 385 mL; V_e 40,000, 453 mL.

Oxidation by Chromic Anhydride. RGU (0.12 g) was dissolved in formamide (3 mL), treated with pyridine (2 mL)

and acetic anhydride (5 mL), and stirred for 5 d. The acetate was precipitated in cold distilled water. The precipitate was filtered off and washed with methanol and acetone. Yield 0.08 g of RGU peracetate.

The peracetate (0.08 g) was treated with chromic anhydride (0.16 g) dissolved in glacial acetic acid (6 mL) and heated for 3 h at 50ºC. The mixture was diluted with water and extracted with $CHCl_3$. The extract was dried over anhydrous sodium sulfate and evaporated to dryness. The solid was dissolved in H_2SO_4 (1 mL, 1 N) and hydrolyzed for 16 h on a boiling-water bath. The hydrolysis products did not contain monosaccharides according to PC (in butanol—pyridine—water, 6:4:3, visualization using acidic anilinium phthalate).

Periodate Oxidation of RGU. Fraction 2 (0.011 g) was dissolved in water (24.9 mL), treated with sodium periodate solution (5.1 mL, 0.25 M), and left at 5ºC. A control was run in parallel. Aliquots (1 mL) were collected every day and titrated with sodium thiosulfate solution (0.01 N).

After 10 d, 0.97 moles of sodium periodate were consumed. This quantity has not changed further. The formic acid released during the reaction was titrated with NaOH (0.01 N). The quantity was 0.03 mole.

Oxidized polysaccharide was reduced overnight by $NaBH_4$ (0.12 g). Then, the mixture was treated with KU-2 ion exchanger (H^+), evaporated, and treated with methanol to remove boric acid. The solid was hydrolyzed by H_2SO_4 (5 mL, 0.5 N) for 8 h on a boiling-water bath. Glycerine, rhamnose, glucose, and uronic acids were identified by PC.

Methylation of RGU. RGU (0.1 g) was dissolved in DMSO (10 mL), treated with NaOH (0.25 g), stirred for 1 h, treated with pure methyl iodide (2 mL), stirred for 2 h, extracted with $CHCl_3$, evaporated, and precipitated with ethanol. Yield 0.07 g of RGU permethylate.

Formolysis and Hydrolysis of the Permethylate. The permethylate (0.1 g) was boiled with formic acid (4 mL, 85%) for 1 h, cooled, and evaporated. The solid was dissolved in H_2SO_4 (5 mL, 0.5 N) and hydrolyzed for 16 h at 100ºC. The hydrolysate was treated as usual. The products were studied by TLC and GLC.

Demethylation of Di-O-Me-Rhamnose. Di-O-Me-rhamnose (0.05 g) was dissolved in HBr (2 mL, 45%) in a 2-mL ampule. The solution was saturated at 10ºC with gaseous HBr formed by reaction of Br_2 with tetraline using iron filings as catalyst.

The ampule was sealed and placed in a refrigerator for 4 d. Then the reaction mixture was transferred into a flask and diluted with water. The HBr was removed in vacua. PC and TLC detected free rhamnose.

Polysaccharides were reduced by an excess of $NaBH_4$ for 5-6 h. The solution was neutralized by KU-2 cation exchanger (H^+), filtered, and evaporated to dryness. The dry solid was treated several times with methanol, evaporating it in vacua each time. The solid was hydrolyzed by H_2SO_4 (1 N) and neutralized. The content of free sugars was determined using PC and GLC.

Partial Hydrolysis. RGU (0.5 g) was dissolved in H_2SO_4 (25 mL, 1 N) and held at 90ºC for 2 h. The hydrolysate was neutralized with $BaCO_3$, treated with KU-2 cation exchanger (H^+), and evaporated to a syrup. PC detected oligosaccharides A and B, uronic acids, rhamnose, and glucose.

Separation of Oligosaccharides. The hydrolysate was separated by preparative PC. Bands corresponding to oligosaccharides A and B were isolated, eluted with water, evaporated, and hydrolyzed with H_2SO_4 (2 mL, 1 N) for 2 h at 100ºC.

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