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Plant Mucilages. XI.[1] Isolation and Characterization of a Mucous Polysaccharide, "Paniculatan," from the Barks of *Hydrangea paniculata*

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A mucous polysaccharide, named paniculatan, has been isolated from the inner barks of *Hydrangea paniculata* SIEB. It was homogeneous by ultracentrifugal analysis and on glass-fiber paper electrophoresis. It was composed of L-rhamnose: D-galactose: 4-O-methyl-D-glucuronic acid: D-galacturonic acid in the molar ratio of 8: 16: 10: 23. The O-acetyl groups in it were identified and the content was 2.0%. Molecular weight of its ammonium salt was estimated at 109000. Periodate oxidation study suggested that the polysaccharide has the high branching structure, whose terminals are occupied by all of 4-O-methyl-D-glucuronic acid units and by about three fourth of D-galacturonic acid units.

The inner bark of *Hydrangea paniculata* SIEB. (Saxifragaceae) contains large amounts of mucilage. The mucous solution extracted from it with water has been used as a good size for the traditional paper manufacture in Japan.

On the chemical property of the mucilage, Hara[3] reported that it is a galactoarabinan, and Komatsu, *et al.*[4] found arabinose, galactose, glucose, rhamnose and galacturonic acid in the hydrolysate of it. More recently, Machida, *et al.*[5] obtained a polyuronide composed of galactose, rhamnose and galacturonic acid in the molar ratio of 10: 7: 3, and they presumed that it is the essential part of the mucilage. But the homogeneity of the polysaccharides obtained by the former investigators was uncertain, and it must be the cause of the disagreement of their conclusions. We have now isolated a pure mucous polysaccharide from the inner barks of *Hydrangea paniculata*, and its properties are described in the present paper.

The material barks were ground and extracted with cold water. The crude mucilage was precipitated from the extract by addition of ethanol. The solution of the precipitate was applied to a column of diethylaminoethyl (DEAE)-cellulose (carbonate form). None of the substances adsorbed was eluted with water, and a mucous polysaccharide was obtained from the eluate with 1M ammonium carbonate solution.

The polysaccharide was homogeneous by the ultracentrifugal analysis (Fig. 1), and gave one spot on glass-fiber paper electrophoresis in alkaline borate buffer. No nitrogen was found in it and it showed a positive specific rotation ($[\alpha]_D^{23}$ +80.0° in 0.05% NH_4OH, c=0.1). Its solution in water gave the high intrinsic viscosity value of 54.0 at 28°. And the relative viscosity of the solution of the polysaccharide is about two times as high as the value of the crude mucilage in the same condition. From this result and the yield, it is conceivable that the pure polysaccharide is the representative substance in the mucosity of water extract from the inner bark.

The complete hydrolysis of the polysaccharide was accomplished by heating with 2N sulfuric acid, and after neutralization, the hydrolysate was applied to preparative paper

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partition chromatography (PPC). L-Rhamnose, D-galactose, 4-O-methyl-D-glucuronic acid and D-galacturonic acid were obtained as the component sugars, and they were respectively identified by the synthesis of each derivative. They were also analyzed by means of cellulose thin-layer chromatography (TLC). A part of the neutral sugars in the hydrolysate were derived to alditol acetates by reduction and acetylation.[6] On the other hand, trimethylsilylated aldonic acids were synthesized[7] from a part of the acidic sugars in the hydrolysate. Then they were separately analyzed by gas-liquid chromatography (GLC).

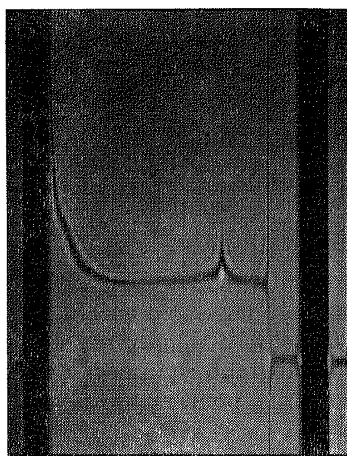

Fig. 1. Ultracentrifugal Pattern of Paniculatan

0.1% in H_2O, 20°, 60 min, 60000 rpm
Hitachi model UCA-1A ultracentrifuge

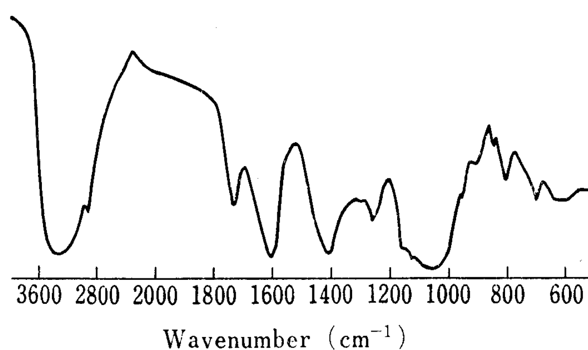

Fig. 2. IR Spectrum of Paniculatan

Quantitative determination of the component sugars showed that the molar ratio of rhamnose: galactose: 4-O-methyl-glucuronic acid: galacturonic acid is 8:16:10:23. The measurement of osmotic pressure gave the value of 109000 as the molecular weight of the ammonium salt of the polysaccharide and this value was also supported by the result of gel chromatography on Sephadex G-200.

As shown in Fig. 2, the infrared (IR) spectrum of it has the absorption bands of 1730 and 1250 cm^{-1} suggesting the presence of ester linkages. The acid hydrolysate of the polysaccharide was analyzed by GLC,[8] and it gave one peak, whose retention time was precisely equal to that of authentic sample of acetic acid. The possibility of presence of other volatile acids was eliminated. The acetyl content of the polysaccharide was determined to be 2.0% by GLC. The name "paniculatan" is proposed for the polysaccharide.

As the result of periodate oxidation, 1.08 mole of periodate per one mole of the average component anhydro sugar unit of the polysaccharide was consumed with 0.45 mole of formic acid liberation. The periodate-oxidized polysaccharide was treated with sodium borohydride,[9] and the reduction product was obtained after purification with gel chromatography on Sephadex G-15. Analysis of the components of the reduction product revealed the presences of 13.3% of rhamnose, 8.9% of galactose and 11.1% of galacturonic acid in it, but no 4-O-methyl-glucuronic acid was found in the product. This result shows the fact that all rhamnose units, five sixteenth of galactose units and about one fourth of galacturonic acid units in the polysaccharide are not decomposed with periodate.

The complete degradation of 4-O-methyl-glucuronic acid by periodate oxidation suggests that all residues of this component uronic acid are located on the terminals of the polysac-

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charide. This result and the value of formic acid liberation show a high branching structure for the polysaccharide.

In addition to two aldohexoses and a hexuronic acid, threitol was found in 3.9% yield in the hydrolysate of the reduction product. This value means that at least a half of galactose units in the polysaccharide has not produced formic acid with periodate. Therefore, it is conceivable that formic acid produced by periodate oxidation has been derived from both about a half of galactose units and about three fourth of galacturonic acid units in the polysac charide. Consequently, the latter galacturonic acid residues must be located on the terminals of the molecule.

On the other hand, it can be presumed that the majority of neutral component sugars, namely all rhamnose and nearly a half of galactose units, form backbone structure of the polysaccharide. Detailed elucidation of the structure will be reported in following papers.

Experimental

Solutions were concentrated at or below 40° with rotary evaporators under reduced pressure. Viscosity was measured with an Ubbelohde-type viscosimeter. Optical rotations were measured with JASCO model DIP-SL automatic polarimeter. IR spectra were recorded on Hitachi model EPI-G3 infrared spectrophotometer. GLC was performed by the use of Hitachi model 063 gas chromatograph equipped with hydrogen flame ionization detector. Melting points were determined on a micro melting point apparatus (an air-bath type) and are uncorrected.

Isolation of Polysaccharide——The material was obtained in May of 1973 from the plants grew in Hokkaido. The barks (100 g) were ground, then extracted with water (3000 ml) under stirring overnight at room temperature. After filtration through sheets of gauze and absorbent cotton, the extraction was similarly repeated with water (2000 ml). The residue was centrifuged at 10000 rpm for 20 min at 5°. The filtrate and the supernatant were combined and poured into two volumes of ethanol, then filtered. The precipitate was repeatedly washed with ethanol, and dried *in vacuo* in a desiccator. Yield, 8.5%. This crude mucilage (1 g) was dissolved in water and applied to a column (5×80 cm) of DEAE-cellulose (Brown Co.). DEAE-cellulose was used as carbonate form by previous successive treatments with 0.5 N sodium hydroxide, water, 1 M ammonium carbonate and water. After elution with water (1400 ml), the column was eluted with 1 M ammonium carbonate. Fractions of 50 ml were collected and analyzed by phenol-sulfuric acid method.[10] The eluates obtained from tubes 21 to 80 were combined, concentrated and poured into ethanol. The precipitate was repeatedly washed with aqueous ethanol, then lyophilized. Paniculatan (0.45 g) was obtained as white powder.

Glass-Fiber Paper Electrophoresis——Electrophoresis was carried out with Whatman GF 81 glass-fiber and alkaline borate buffer of pH 9.2 (0.1 N NaOH: 0.025 M borax, 1: 10) at the condition of 380 volt for 2 hr in the same manner as a preceding report[11] of this series. Paniculatan gave one spot at a distance of 1.7 cm from the origin toward the cathod. Distance moved by standard glucose was 16.2 cm.

Hydrolysis and Isolation of Component Sugars——The sample was hydrolyzed with 2 N sulfuric acid in a sealed tube at 100° for 6 hr followed by neutralization with barium carbonate. The filtrate was concentrated and applied to a small column of Dowex 50W-X8 (H^+). After elution with water, the eluate was concentrated and applied to PPC with Tôyô-Roshi No. 50 and solvent system A, AcOEt: pyridine: AcOH: H_2O (5: 5: 1: 3). L-Rhamnose, D-galactose, 4-O-methyl-D-glucuronic acid and D-galacturonic acid were respectively extracted with water from the parts showing *Rf* values of 0.67, 0.43, 0.31, and 0.18. Specific rotations of them in water at 21° were +7.2°, +62.7°, +33.3°, and +67.7°.

Identification of Component Sugars——Following derivatives were synthesized from each fraction.

L-Rhamnose: The sample was dissolved in conc. hydrochloric acid and the solution was stirred after addition of ethyl mercaptan, then the product was acetylated with acetic anhydride-pyridine mixture.[12] The final product was recrystallized from aqueous methanol, and tetra-O-acetyl-L-rhamnose diethyldithioacetal was obtained as colorless rods, mp 55—56°.

D-Galactose: The sample was acetylated with acetic anhydride and sodium acetate.[13] The product was recrystallized from ethanol, and β-D-galactose pentaacetate was obtained as colorless plates, mp 142—143°.

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D-Galacturonic Acid: The sample was oxidized with bromine saturated water.[14] The product was successively washed with water, ethanol and ether, and D-galactaric acid was obtained as colorless prisms, mp 220—221° (decomp.).

These three derivatives were respectively identified by comparing with the authentic samples by mixing mp and IR spectra.

4-O-Methyl-D-glucuronic Acid: The sample was methanolyzed with methanolic HCl and reduced with lithium aluminum hydride in tetrahydrofuran, then the product was heated with phenylhydrazine and acetic acid.[15] The final product was recrystallized from benzene, and 4-O-methyl-D-glucose phenylosazone was obtained as yellow needles, mp 157°. *Anal.* Calcd. for $C_{19}H_{24}O_4N_4$: C, 61.27; H, 6.49; N, 15.04. Found: C, 61.22; H, 6.29; N, 14.96.

TLC of Component Sugars——The hydrolysate of the sample was applied to TLC using Avicel SF cellulose and the solvent systems A and B, C_6H_5OH: 1% NH_4OH (2: 1). Component sugars were revealed with benzidine reagent[16] and silver nitrate reagent.[17] *Rf* values on TLC are shown in Table I.

Table I. *Rf* Values of Components on TLC and Retention Times of their Derivatives on GLC

	Cellulose TLC (*Rf*)		GLC (t_R)	
	Solvent A	Solvent B	Condition A	Condition B
Rhamnose	0.80	0.58		
Galactose	0.62	0.38		
4-O-Methyl-glucuronic acid	0.52	0.13		
Galacturonic acid	0.32	0.09		
Rhamnitol acetate			4.7	
Galactitol acetate			21.5	
TMS-aldonic acid from 4-O-methyl-glucuronic acid				9.8
TMS-aldonic acid from galacturonic acid				16.0

GLC of Component Sugar Derivatives——The sample was successively hydrolyzed with 90% formic acid at 90° for 16 hr and with 0.5 N sulfuric acid at 100° for 6 hr, then a part of the hydrolysate was neutralized with Dowex 2 (OH^-). The filtrate was reduced with sodium borohydride for 1 hr. After neutralization with Dowex 50W (H^+), the filtrate was evaporated and boric acid was removed by repeated addition and evaporation of methanol. Then the products were acetylated with acetic anhydride–pyridine mixture (1: 1) at 100° for 20 min. After evaporation of the solution, the residue was dissolved in chloroform–methanol mixture (1: 1) and applied to GLC. GLC was carried out under condition A, a column (0.3 cm × 2 m long spiral glass) packed with 3% ECNSS-M on Gaschrom Q (100 to 200 mesh) at 195° with a flow of 30 ml per min of N_2. Retention times of alditol acetates on GLC are given in Table I.

On the other hand, a part of the acid hydrolysate was neutralized with barium carbonate. The filtrate was passed through a small column of Dowex 50W-X8 (H^+), then uronic acids in the eluate were reduced to their corresponding aldonic acids by treatment with triethanolamine and sodium borohydride as described by Matsunaga, *et al.*[7] After trimethylsilylation with pyridine, hexamethyldisilazane and trimethylchlorosilane,[18] the reaction mixture was applied to GLC. GLC was carried out under condition B, a column (0.3 cm × 2 m long spiral stainless steel) packed with 2% XF 1105 on Gaschrom P (80 to 100 mesh) at 170° with a flow of 40 ml per min of N_2. Retention times of trimethylsilyl derivatives on GLC are also shown in Table I.

Determination of Component Sugars——Rhamnose and galactose were determined by GLC after conversion to corresponding alditol acetates as described above. Xylose was used as an internal standard. The amounts of 4-O-methyl-glucuronic acid and galacturonic acid were calculated from the difference in coloration degree between results by modified carbazole method[19] and orcinol method.[20] The sample was heated at 100° for 50 min in orcinol method. The results revealed that the polysaccharide was composed of 12.0% of rhamnose, 26.6% of galactose, 19.9% of 4-O-methyl-glucuronic acid and 41.7% of galacturonic acid.

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Determination of Molecular Weight——The measurement of osmotic pressure was carried out by the use of Knauer Electronic Membrane Osmometer at 60°. The polysaccharide was dissolved in 0.05% NH_4OH followed by evaporation and lyophilization. The sample obtained was dissolved in water, and 0.30, 0.21, 0.16, and 0.10% solutions were used.

For the gel chromatography, a column (2.6×96 cm) of Sephadex G-200 (Pharmacia Co.) was prepared and the elution was carried out as described in the first report[21] of this series.

Determination of O-Acetyl Groups——The IR spectrum of the polysaccharide showed the absorption bands of ester. IR ν_{max}^{KBr} cm^{-1}: 1730, 1250 (ester).

The sample was hydrolyzed with 1 N hydrochloric acid containing propionic acid as an internal standard in a sealed tube at 100° for 2 hr. The hydrolysate was directly applied to GLC. GLC was carried out under condition C, a column (0.3 cm×2 m long spiral stainless steel) packed with 20% tetramethyl cyclobutanediol adipate-4% phosphoric acid on Chromosorb W (80 to 100 mesh) at 120° with a flow of 20 ml per min of N_2; t_R, acetic acid 6.2; propionic acid (internal standard) 9.7.

Periodate Oxidation followed by Reduction——The sample (100 mg) was oxidized with 0.05 M sodium metaperiodate (50 ml) at 5° in a dark place. The periodate consumption was measured by a spectrophotometric method.[22] The oxidation was completed after 24 days, then a part of the solution was used for the measurement of formic acid liberation by thiobarbituric acid method.[23] The residuary nine tenth of the reaction mixture was neutralized with 0.1 N sodium hydroxide after addition of ethylene glycol (1 ml), then reduced with sodium borohydride (400 mg) at 5° for 16 hr followed by addition of acetic acid up to pH 5. The solution was concentrated to one tenth volume, then poured into ethanol. The precipitate was dissolved in water and applied to a column (5×83 cm) of Sephadex G-15 (Pharmacia Co.). Fractions of 50 ml were collected and analyzed by phenol-sulfuric acid method. The eluates obtained from tubes 11 to 14 were combined, concentrated and lyophilized. Yield, 75.2 mg.

Analysis of Reduction Product——The reduction product was hydrolyzed with 2 N sulfuric acid in a sealed tube at 100° for 6 hr. After neutralization with barium carbonate, the components were qualitatively analyzed by TLC and GLC as described above.

For the determination of rhamnose and galactose, the hydrolysate was neutralized with Dowex 2 (OH^-). The components in the filtrate were converted to corresponding alditol acetates, then determined by GLC under condition A; t_R, threitol 3.1; xylitol (internal standard) 10.7. For the accurate estimation of threitol, a part of the hydrolysate was trimethylsilylated and applied to GLC. Trimethylolpropane was used as an internal standard and GLC was carried out under condition D, a column (0.3 cm×2 m long spiral stainless steel) packed with 5% SE 30 on Chromosorb G (80 to 100 mesh); programmed column temperature increase in 5° per min from 60 to 260° with a flow of 30 ml per min of N_2; t_R, threitol 25.8; trimethylolpropane (internal standard) 23.0.

Galacturonic acid was directly determined by modified carbazole method.

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