Chem. Pharm. Bull.
25(6)1357—1362(1977)

UDC 547.458.02 : 581.192

Plant Mucilages. XVI.[1] Isolation and Characterization of a Mucous Polysaccharide, "Althaea-mucilage O," from the Roots of *Althaea officinalis*

Masashi Tomoda, Sachiko Kaneko, Mineko Ebashi,
and Teruyo Nagakura

Kyoritsu College of Pharmacy[2]

(Received September 24, 1976)

A representative mucous polysaccharide, named Althaea-mucilage O, has been isolated from the roots of *Althaea officinalis* L. It was homogeneous on glass-fiber paper electrophoresis and by ultracentrifugal analysis, and its water solution gave the high intrinsic viscosity value of 50.0. It was composed of L-rhamnose: D-galactose: D-galacturonic acid: D-glucuronic acid in the molar ratio of 3: 2: 3: 3, and molecular weight of its ammonium salt was estimated to be 34000. The O-acetyl groups in it were identified and the content was 0.7%. The mucilage has been subjected to the reduction of carboxyl groups, and the results of methylation analysis of the original and the carboxyl-reduced polysaccharides revealed the structural features of the backbone and side chains in the mucilage.

Keywords——mucous polysaccharide from *Althaea officinalis*; Althaea-mucilage O; ultracentrifugal analysis and electrophoresis; intrinsic viscosity; acetyl groups; molecular weight; reduction of carboxyl groups; molar ratios of components; methylation analysis; backbone chain having branches

The roots of *Althaea officinalis* L. have been used as a crude drug for the purpose of emollient, demulcent, and cough medicine. It is well known that the roots contain relatively large amounts of mucilage. On the chemical property of the mucilage, Friedrichs[3] found glucose and xylose as its components, and Beauquesne[4] reported that it was made up of an uronic acid, methylpentoses, and hexoses. More recently, Franz[5] reported that the crude mucilage of the roots contained a glucan, an arabinogalactan, and an acidic polysaccharide. He obtained these polysaccharides by diethylaminoethyl (DEAE)-cellulose (acetate form) column chromatography, and reported that the neutral fraction was composed of 21% of glucose, 52% of galactose, and 27% of arabinose. On the acidic polysaccharide, he reported that it was composed of 58% of galacturonic acid, 39% of rhamnose, 3% of galactose, and trace amount of glucose. He isolated α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnose as a partial hydrolyzate of the acidic polysaccharide. But no further structural study on its whole molecule has been reported until present time. We have now isolated a new mucous polysaccharide having glucuronic acid in addition to galacturonic acid, rhamnose, and galactose as its component sugars. In this study, the stationary phase and the mobile phases of the chromatography for the purification of the mucilage differ from those utilized by the former investigator. Any neutral polysaccharide was not obtained by our procedure. There was no description on the values of viscosity of the polysaccharides in the report[5] of the former investigator. The relative viscosity of the solution of the new mucous polysaccharide was about 2.3 times as high as the value of the crude mucilage. From this result and the yield, it is conceivable that the polysaccharide is the representative

---

1) Part XV: M. Tomoda, S. Satoh, and Y. Suzuki, *Chem. Pharm. Bull.* (Tokyo), **25**, 968 (1977).
2) Location: *1-5-30, Shibakōen, Minato-ku, Tokyo, 105, Japan.*
3) O. von Friedrichs, *Arch. Pharm.*, **257**, 288 (1919).
4) L. Beauquesne, *Ann. Pharm. Franc.*, **4**, 271 (1946).
5) G. Franz, *Planta Med.*, **14**, 90 (1966).

substance in the mucosity of water extract from the material. The properties and the structural features of it are described in the present paper.

The fresh roots were crushed 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 DEAE-Sephadex A–25 (carbonate form). None of the substances adsorbed was eluted with water and 0.1 M ammonium carbonate solution, and a mucous polysaccharide was obtained from the eluate with 0.5 M ammonium carbonate solution.

The polysaccharide was homogeneous by the ultracentrifugal analysis (Fig. 1), and gave a single spot on glass-fiber paper electrophoresis in alkaline borate buffer. It contained no nitrogen, and showed a positive specific rotation ($[\alpha]_D^{26}+50.5°$ in H_2O, $c=0.1$). Its solution in water gave the high intrinsic viscosity value of 50.0 at 23°.

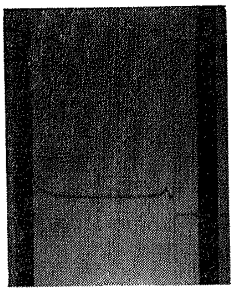

Fig. 1. Ultracentrifugal Pattern of Althaea-mucilage O

0.16% in H_2O, 20°, 60 min, 60000 rpm
Hitachi model UCA–1A ultracentrifuge

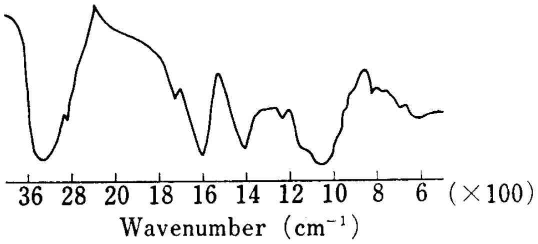

Fig. 2. IR Spectrum of Althaea-mucilage O

As the component sugars of it, L-rhamnose, D-galactose, D-galacturonic acid, and D-glucuronic acid were identified by means of cellulose thin–layer chromatography (TLC) of the hydrolyzate. These sugars were isolated by preparative paper partition chromatography (PPC) and proved to have the configurations given above. As shown in Fig. 2, the infrared (IR) spectrum has the weak absorption bands of 1235 and 1730 cm^{-1} suggesting the presence of ester linkages. When the acid hydrolyzate of the polysaccharide was analyzed by gas–liquid chromatography (GLC),[6] it gave one peak, whose retention time was equal to that of authentic sample of acetic acid. The acetyl content of the polysaccharide was determined to be 0.7% by GLC. The measurement of osmotic pressure gave the value of 34000 as the molecular weight of the ammonium salt of the polysaccharide. The name "Althaea-mucilage O" is proposed for the polysaccharide.

The carboxyl groups of hexuronic acid residues in the polysaccharide were reacted with a carbodiimide reagent, then reduced with sodium borohydride to the corresponding neutral sugar units.[7] Quantitative determinations of the neutral component sugars of the original and the carboxyl-reduced polysaccharides were carried out by GLC of alditol acetates derived from the hydrolyzates, and hexuronic acids in the original polysaccharide were estimated by a colorimetric method. The results showed that the original polysaccharide contained rhamnose, galactose, and hexuronic acids in the molar ratio of 3.3: 2.0: 6.0, and showed that the carboxyl-reduced polysaccharide was composed of rhamnose, galactose, and glucose in the molar ratio of 2.3: 3.1: 2.0.

The methylations of the original and the carboxyl-reduced polysaccharides were performed with methylsulfinylmethyl sodium and methyl iodide in dimethyl sulfoxide.[8] The fully methylated products were successively hydrolyzed with formic acid and dilute sulfuric

---

6) M. Tomoda, S. Nakatsuka, and N. Satoh, *Chem. Pharm. Bull.* (Tokyo), **22**, 2710 (1974).

7) R.L. Taylor and H.E. Conrad, *Biochemistry*, **11**, 1383 (1972).

8) S. Hakomori, *J. Biochem.*, **55**, 205 (1964).

acid. The products were analyzed by gas–liquid chromatography–mass spectrometry (GLC–MS) after conversion to alditol acetates.[9,10] Methyl ethers of the hexuronic acids were removed from the hydrolysis products of the methylated original polysaccharide by treatment with an anion-exchange resin, and the residual products were identified as 3,4-di-O-methyl-L-rhamnopyranose, 3-mono-O-methyl-L-rhamnopyranose, 2,3,4,6-tetra-O-methyl-D-galactopyranose, and 2,3,6-tri-O-methyl-D-galactopyranose. They were obtained in the molar ratio of 2.0: 1.0: 1.1: 1.0. In the case of the hydrolysis products of the methylated carboxyl-reduced polysaccharide, 3,4-di-O-methyl-L-rhamnopyranose, 3-mono-O-methyl-L-rhamnopyranose, 2,3,4,6-tetra-O-methyl-D-glucopyranose, 2,3,4,6-tetra-O-methyl-D-galactopyranose, 2,3,6-tri-O-methyl-D-galactopyranose and 2,6-di-O-methyl-D-galactopyranose were identified and obtained in the molar ratio of 4.1: 2.0: 5.9: 1.8: 1.0: 6.0.

These results suggested that the minimal repeating unit of Althaea-mucilage O was composed of six kinds of the component sugar units as shown in Chart 1.

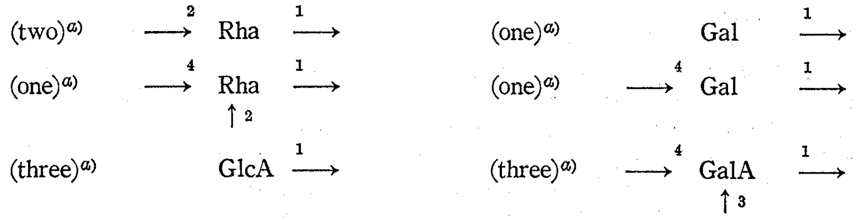

Chart 1. Component Sugar Residues in the Minimal Repeating Unit in the Structure of Althaea-mucilage O

a) number of residues
Rha: L-rhamnopyranose
Gal: D-galactopyranose
GalA: D-galactopyranosyluronic acid
GlcA: D-glucopyranosyluronic acid

Owing to these results, it can be concluded that the backbone chain in the mucilage is composed of rhamnose and galacturonic acid in the approximate molar ratio of 1: 1. The value of molar ratio of galacturonic acid corresponded to that of glucuronic acid, and the value of molar ratio of the terminal galactose was approximately equal to that of the rhamnose unit being situated in a branching point. Consequently, it can be presumed that each galacturonic acid residue forms a branch having a terminal glucuronic acid, and about one third of rhamnose residues form branching points having the side chains which are composed of two 1→4 linked galactose units.

As described above, the results of the component sugar determination and of the methylation analysis revealed that the approximate molar ratio of rhamnose: galactose: hexuronic acids in the original mucilage was 6: 4: 12 and that of rhamnose: original galactose: the hexoses derived from hexuronic acids in the carboxyl-reduced polysaccharide was 6: 3: 12. So it is conceivable that the carboxyl-reduced polysaccharide has lost about one fourth of galactose units in the original polysaccharide in the process of reduction. As the result of methylation analysis, the molar ratio of 2,3,4,6-tetra-O-methyl-D-galactopyranose: 2,3,6-tri-O-methyl-D-galactopyranose was 1.1: 1.0 in the case of the original polysaccharide and 1.8: 1.0 in the case of the carboxyl-reduced polysaccharide. From this result, it seems likely that about a half of galactose-galactose linkages in the mucilage were hydrolyzed during the reaction. The glycosidic linkages of the terminal galactose residues in the mucilage are much more easily cleaved than those of the other component sugars. For example, one third of galactose units in the polysaccharide were hydrolyzed and liberated after treatment with 0.5 N sulfuric acid at 100° for 30 min. None of rhamnose and hexuronic

---

9) H. Björndal, B. Lindberg, and S. Svensson, *Acta Chem. Scand.*, **21**, 1801 (1967).
10) H. Björndal, B. Lindberg, and S. Svensson, *Carbohyd. Res.*, **5**, 433 (1967).

acids, on the contrary, was liberated from the polysaccharide in the same condition. Therefore, the loss of a part of galactose residues may be attributed to the property of their glycosidic linkages. Another example describing the loss of some part of component sugars during the reduction of an acidic polysaccharide by the method of Taylor and Conrad has recently reported by the other investigators.[11)]

Detailed elucidation of the structure of Althaea-mucilage O will be reported in a following paper.

Experimental

Solutions were concentrated at or below 40° with rotary evaporators under reduced pressure. Optical rotations were measured with JASCO model DIP-SL automatic polarimeter. Viscosity was determined with an Ubbelohde-type viscosimeter. IR spectra were recorded on Hitachi model EPI-G3 infrared spectrophotometer. GLC was carried out by the use of Hitachi model 063 gas chromatograph equipped with hydrogen flame ionization detector. GLC–MS was performed by the use of JEOL model JGC-20K gas chromatograph and JEOL model JMS-D100 mass spectrometer.

Isolation of Polysaccharide——The material was obtained in October of 1975 from the plants cultivated in Saitama prefecture. The fresh roots (300 g), which contain 63.7% of water, were crushed, then extracted with water (3000 ml) under stirring for 3 hr at room temperature. After suction filtration, the extraction was similarly repeated with water (2000 ml). The extracts were combined and poured into two volumes of ethanol, then filtered. The precipitate was treated with ethanol, then dried *in vacuo*. Light brown powder (3.51 g) was obtained. A part of crude mucilage (2.5 g) was dissolved in water and applied to a column (4.7×60 cm) of DEAE-Sephadex A-25 (Pharmacia Co.). DEAE-Sephadex was used as carbonate form by previous successive treatment with 0.5 N sodium hydroxide, water, 1 M ammonium carbonate, and water. After elution with water and 0.1 M ammonium carbonate (1400 ml each), the column was eluted with 0.5 M ammonium carbonate. Fractions of 50 ml were collected and analyzed by phenol–sulfuric acid method.[12)] The eluates obtained from tubes 21 to 48 were combined, concentrated and poured into ethanol. The precipitate was repeatedly washed with aqueous ethanol, then dried *in vacuo*. Althaea-mucilage O (0.65 g) was obtained as white powder.

Glass-Fiber Paper Electrophoresis——Electrophoresis was carried out with Whatman GF 83 glass-fiber and alkaline borate buffer of pH 9.2 (0.1 N sodium hydroxide: 0.025 M borax, 1: 10) at the condition of 380 volt for 2 hr in the same manner as a preceding report[13)] of this series. The sample gave one spot at a distance of 2.5 cm from the origin toward the cathod. Standard glucose showed a spot at a distance of 16.4 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 50 W-X8 (H^+). After elution with water, the eluate was heated at 100° for 30 min, then concentrated and applied to PPC with Toyo-Roshi No. 50 and solvent system A, AcOEt: pyridine: AcOH: H_2O (5: 5: 1: 3). D-Glucuronolactone, L-rhamnose, D-galactose, and D-galacturonic acid

Table I. *Rf* Values of Components on TLC and Retention Times (min) of Their Derivatives on GLC

	Cellulose TLC (*Rf*)		GLC (t_R) Condition B
	Solvent A	Solvent B	
Rhamnose	0.75	0.60	
Glucose	0.56	0.36	
Galactose	0.49	0.31	
Glucuronolactone	0.86	0.68	
Glucuronic acid	0.23	0.11	
Galacturonic acid	0.18	0.09	
Rhamnitol acetate			5.2
Galactitol acetate			19.0
Glucitol acetate			21.2

---

11) I.R. Siddiqui and P.J. Wood, *Carbohyd. Res.*, **50**, 97 (1976).

12) J.E. Hodge and B.T. Hofreiter, *Meth. Carbohyd. Chem.*, **1**, 388 (1962).

13) M. Tomoda, Y. Yoshida, H. Tanaka, and M. Uno, *Chem. Pharm. Bull.* (Tokyo), **19**, 2173 (1971).

were respectively extracted with water from the parts showing *Rf* values of 0.78, 0.67, 0.43, and 0.16. Specific rotations of them in water at 20° were +19.2°, +8.2°, +80.2°, and +56.7°.

TLC of Component Sugars——The hydrolyzate of the sample was applied to TLC using Avicel SF cellulose and the solvent systems A and B, BuOH: pyridine: H_2O (6: 4: 3). Component sugars were revealed with silver nitrate reagent[14] and *p*-anisidine hydrochloride reagent.[15] *Rf* values on TLC are shown in Table I.

Determination of O-Acetyl Groups——The IR spectrum of the polysaccharide showed the absorption bands of ester. IR $\nu_{\max}^{\text{KBr}}$ cm^{-1}: 1730, 1235 (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 hydrolyzate was directly applied to GLC. GLC was carried out under condition A, 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 nitrogen; t_R (min), acetic acid 7.4; propionic acid (internal standard) 11.5.

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% ammonium hydroxide followed by evaporation and lyophilization. The sample was dissolved in water, and 0.33, 0.25, 0.21, 0.17, 0.12, and 0.08% solutions were used.

The value of the molecular weight was also supported by the result of the gel chromatography. A column (2.6 × 86 cm) of Sephadex G-200 (Pharmacia Co.) was prepared and the elution was carried out as described in the first report[16] of this series.

Reduction of Polysaccharide——Ammonium salt of Althaea-mucilage O (100 mg) was dissolved in water (30 ml), then 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-*p*-toluenesulfonate (1 g) was added into this solution. The pH of the reaction mixture was maintained at 4.75 by titration with 0.1 N hydrochloric acid under stirring for 2 hr. Then 2 M sodium borohydride (10 ml) was added gradually into the reaction mixture during 4 hr and the pH was maintained at 7.0 by titration with 4 N hydrochloric acid under stirring at room temperature. The solution was dialyzed against running water overnight, then the non-dialyzable fraction was concentrated up to 30 ml. The product was reduced four more times under the same condition. The final non-dialyzable fraction was applied to a column (5.5 × 76 cm) of Sephadex G-15 (Pharmacia Co.). The column was eluted with water, and fractions were collected at 50 ml. The eluates obtained from tubes 11 to 14 were combined, concentrated and lyophilized. Yield, 65.3%.

Determination of Component Sugars——The sample was hydrolyzed with 2 N sulfuric acid at 100° for 6 hr followed by neutralization with Dowex 2 (OH^-). The filtrate was reduced with sodium borohydride for 1 hr. After neutralization with Dowex 50 W-X8 (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 B, a column (0.3 cm × 2 m long spiral glass) packed with 3% OV 225 on Gaschrom Q (100 to 120 mesh) at 210° with a flow of 30 ml per min of nitrogen. Xylose was used as an internal standard. Retention times of alditol acetates are given in Table I. Hexuronic acids were determined by modified carbazole method[17] and the value was calculated on the assumption that the molar ratio of galacturonic acid: glucuronic acid is 1: 1. The results of determination are shown in Table II.

Methylation——Sodium hydride (100 mg) was mixed with dimethyl sulfoxide (5 ml) and the mixture was stirred at 70° for 1 hr. The sample (50 mg) was dissolved in dimethyl sulfoxide (10 ml) and the solution of methylsulfinylmethyl sodium was added into this mixture. After stirring at room temperature for 5 hr,

TABLE II. Sugar Compositions (%) of the Polysaccharide and Its Derivative

	Original polysaccharide	Carboxyl-reduced polysaccharide
Rhamnose	26.0	28.4
Galactose	17.2	43.7
Glucose	—	27.9
Galacturonic acid	56.8[a]	—
Glucuronic acid		—

a) total hexuronic acid value

---

[14] W.E. Trevelyan, D.P. Procter, and J.S. Harrison, *Nature*, **166**, 444 (1950).

[15] L. Hough and J.K.N. Jones, *Meth. Carbohyd. Chem.*, **1**, 28 (1962).

[16] M. Tomoda and M. Uno, *Chem. Pharm. Bull.* (Tokyo), **19**, 1214 (1971).

[17] T. Bitter and H. Muir, *Anal. Biochem.*, **4**, 330 (1962).

methyl iodide (5 ml) was added and the mixture was stirred overnight at room temperature. All procedures were carried out in nitrogen atmosphere. Then the reaction mixture was dialyzed against running water overnight. The non-dialyzable fraction was concentrated and lyophilized. The product was methylated four more times under the same condition. The final non-dialyzable fraction was extracted with chloroform (50 ml each) five times. The extracts were combined and washed with water (250 ml each) five times, then dried over sodium sulfate and the filtrate was evaporated to dryness. The IR spectrum of the final product had no absorption near 3400 cm^{-1}.

Analysis of the Methylated Products——A part of the product was successively treated with 90% formic acid at 100° for 5 hr and 0.5 N sulfuric acid at 100° for 18 hr. After neutralization with Dowex 2 (OH$^-$), the hydrolyzate was reduced with sodium borohydride, then acetylated with acetic anhydride-pyridine mixture as described above. GLC of partially methylated alditol acetates was carried out under following two conditions: B′, the same column as condition B but at 180° with a flow of 30 ml per min of nitrogen; C, a column (0.3 cm × 2 m long spiral glass) packed with 3% ECNSS-M on Gaschrom Q (100 to 120 mesh) at 180° with a flow of 30 ml per min of nitrogen. GLC–MS was carried out under condition B′ using helium as carrier gas. Relative retention times of the products to 2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl-D-glucitol and fragments of them in the mass spectra are shown in Table III.

TABLE III. Relative Retention Times on GLC and Fragments in MS of Partially Methylated Alditol Acetates

	Relative retention times[a]		Fragments (m/e)
	Condition B′	Condition C	
3,4-me-1,2,5-ac-L-Rhamnitol	0.87	0.92	43, 89, 129, 131, 189
2,3,4,6-me-1,5-ac-D-Glucitol	1.00	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
2,3,4,6-me-1,5-ac-D-Galactitol	1.17	1.23	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
3-me-1,2,4,5-ac-L-Rhamnitol	1.62	1.83	43, 87, 101, 129, 143, 189, 203
2,3,6-me-1,4,5-ac-D-Galactitol	2.09	2.45	43, 45, 87, 99, 101, 113, 117, 233
2,6-me-1,3,4,5-ac-D-Galactitol	2.97	3.67	43, 45, 87, 117, 129

a) relative to 2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl-D-glucitol
abbreviations: me=methyl: ac=acetyl
(*e.g.*, 3,4-me-1,2,5-ac-=3,4-di-O-methyl-1,2,5-tri-O-acetyl)

Partial Hydrolysis of Galactose Linkages——The mucilage was heated with 0.5 N sulfuric acid in a sealed tube at 100° for 30 min followed by neutralization with barium carbonate and treatment with a small column of Dowex 50 W-X8 (H$^+$). TLC of the hydrolyzate showed only one spot of galactose. Determination of the sugar liberated was carried out by GLC as described above, and the result revealed that the rate of galactose liberation was 33.7%.

Acknowledgement The authors are grateful to Prof. G. Matsumura, School of Pharmaceutical Sciences, Showa University, for ultracentrifugal analysis.