[Chem. Pharm. Bull. 25(11)3061—3065(1977)]

UDC 547.963.1.02 : 581.192

Plant Mucilages. XVIII.[1] Isolation and Characterization of a Mucilage, "Abelmoschus-mucilage M," from the Roots of *Abelmoschus manihot*

Masashi Tomoda and Yōko Suzuki

Kyoritsu College of Pharmacy[2]

(Received April 9, 1977)

A representative mucilage, named Abelmoschus-mucilage M, has been isolated from the roots of *Abelmoschus manihot* Medicus. It was homogeneous by ultracentrifugal analysis and on cellulose acetate membrane electrophoresis, and its water solution gave the high intrinsic viscosity value of 33.0. It was composed of 82% of polysaccharide and approximately 17% of protein, and molecular weight of its ammonium salt was estimated to be 25300. Its carbohydrate part was composed of L-rhamnose: D-galacturonic acid: D-glucuronic acid in the molar ratio of 1.2: 1.0: 1.0. The mucilage has been subjected to the reduction of carboxyl groups, and the result of methylation analysis of the product revealed the structural feature of the polysaccharide moiety in it.

Keywords——mucilage from *Abelmoschus manihot*; Abelmoschus-mucilage M; ultracentrifugal analysis and electrophoresis; intrinsic viscosity; molar ratio of component sugars; molecular weight; reduction of carboxyl groups; methylation analysis; structure of polysaccharide chain; complex with carbohydrate and peptide

The root of *Abelmoschus manihot* Medicus (=*Hibiscus manihot* L.) contains fairly large amounts of mucilage. It has been used as a crude drug for the purpose of demulcent and cough medicine. Further, the mucous solution extracted from it with water has been used as an important size for the traditional paper manufacture in Japan.

Relatively many studies on the root mucilage of this plant have been published until present time. Machida, *et al.*[3] reported that the polysaccharide purified by a precipitation method with cupric sulfate was composed of galacturonic acid and rhamnose, and Oshibuchi, *et al.*[4] determined the approximate molar ratio of the both component sugars in the polysaccharide as 2: 1. They obtained the polysaccharide by application of pre-heating of the water extract at fairly high temperature for several hours before the addition of the cupric sulfate reagent, but some former investigators observed that the application of heating resulted in a lowering of the viscosity.[4,5] Therefore, it is conceivable that the polysaccharide obtained by them is a kind of degradation products. In addition, Oshibuchi, *et al.*[6] obtained a trisaccharide by partial acid hydrolysis of the mucilage, and Inokawa, *et al.*[7] concluded that its structure was *O*-α-(D-galactopyranosyluronic acid)-(1→4)-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose. Nevertheless, the homogeneity of the mucilages obtained by the former investigators was uncertain, and no further structural study on the mucilage has been reported until now. We have now isolated a pure mucilage having glucuronic acid in addition to galacturonic acid and rhamnose as its component sugars. From the viscosity and the yield, it is probable that the mucilage is the representative substance in the mucosity of water extract from the material. The properties and the main structural features of it are described in the present paper.

1) Part XVII: M. Tomoda and N. Satoh, *Chem. Pharm. Bull.* (Tokyo), **25**, 2910 (1977).
2) Location: *1–5–30, Shibakôen, Minato-ku, Tokyo, 105, Japan.*
3) S. Machida and N. Uchino, *Nippon Kagaku Zasshi*, **72**, 917 (1951).
4) T. Oshibuchi and Y. Ōwada, *Nippon Nogeikagaku Kaishi*, **28**, 556, 558 (1954).
5) S. Inokawa, *Bull. Chem. Soc. Jpn.*, **33**, 1473, 1476 (1960).
6) T. Oshibuchi and H. Kusunose, *Nippon Nogeikagaku Kaishi*, **31**, 481 (1957).
7) S. Inokawa, R. Goto, T. Ban, T. Uchida, and K. Wakaizumi, *Bull. Chem. Soc. Jpn.*, **37**, 1228 (1964).

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). Negligibly small amounts of the substances adsorbed were eluted with water and 0.1 M ammonium carbonate solution, and the main mucilage was obtained from the eluate with 0.5 M ammonium carbonate solution. A minor mucilage was obtained from the eluate with 1 M ammonium carbonate solution. The same component sugars as those of the main mucilage were found in it, but no further structural study on this substance was carried out.

The main mucilage was homogeneous by the ultracentrifugal analysis (Fig. 1), and gave a single spot on cellulose acetate membrane electrophoresis. Its solution in water gave the high intrinsic viscosity value of 33.0 at 31°, and it showed a positive specific rotation ($[\alpha]_D^{22}$ +51.7° in 0.1% NH_4OH, c=0.1). The relative viscosity of the solution of the main mucilage was about 3.7 times as high as the value of the crude mucilage. The water solution of the minor mucilage gave the intrinsic viscosity value of 23.0 at the same condition.

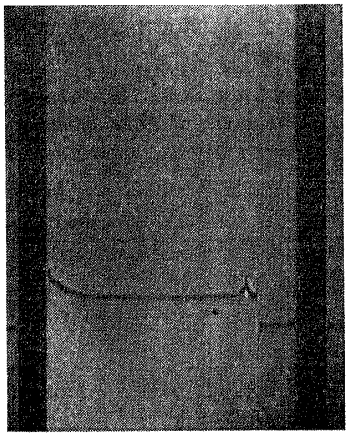

Fig. 1. Ultracentrifugal Pattern of Abelmoschus-mucilage M

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

Table I. Amino Acid Composition (%) after Hydrolysis with 6 N Hydrochloric Acid at 110° for 24 hr

Lysine	0.50
Histidine	0.09
Arginine	0.22
Aspartic acid	0.70
Threonine	0.23
Serine	0.27
Glutamic acid	0.72
Proline	0.18
Glycine	0.48
Alanine	0.27
Valine	0.27
Methionine	0.09
Isoleucine	0.19
Leucine	0.24
Tyrosine	0.17
Phenylalanine	0.15

As the component sugars of the main mucilage, L-rhamnose, 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. Quantitative determination of the component sugars showed that the molar ratio of rhamnose: galacturonic acid: glucuronic acid is 1.2: 1.0: 1.0, and that the total content of carbohydrates is 82.0%.

The mucilage contained 2.69% of nitrogen. The amino acid composition of it after hydrolysis with 6 N hydrochloric acid is shown in Table I. Any nitrogen-containing compound, other than amino acids, was not detected in the hydrolyzate. Thus the total value of the amino acids was less than one third of the value expected from nitrogen content. The cause for such a discrepancy may be attributed to unavoidable decomposition of amino acids arising from the presence of a large quantity of carbohydrates. The attempts of uses of methanesulfonic acid or mercaptoethanesulfonic acid for the hydrolysis reagents were unsuccessful and gave much less values.

The measurement of osmotic pressure gave the value of 25300 as the molecular weight of the ammonium salt of the mucilage. The name "Abelmoschus-mucilage M" is proposed for it.

The carboxyl groups of hexuronic acid residues in the mucilage were reacted with a

carbodiimied reagent, then reduced with sodium borohydride to the corresponding neutral sugar units.[8] The methylation of the carboxyl-reduced mucilage was performed with methylsulfinylmethyl sodium and methyl iodide in dimethyl sulfoxide.[9] The fully methylated product was successively hydrolyzed with formic acid and dilute sulfuric acid. The products derived from component sugars were analyzed by gas–liquid chromatography–mass spectrometry (GLC–MS) after conversion to alditol acetates,[10] and identified as 3,4-di-*O*-methyl-L-rhamnopyranose, 2,3,4,6-tetra-*O*-methyl-D-glucopyranose, and 2,6-di-*O*-methyl-D-galactopyranose. They were obtained in the molar ratio of 1.1: 1.0: 1.0.

Owing to those results, it can be concluded that Abelmoschus-mucilage M is composed of 82% of acidic polysaccharide and approximately 17% of protein (from N, 2.69%). The polysaccharide chain in it must be composed of 1→2 linked L-rhamnopyranose units and 1→4 linked D-galactopyranosyluronic acid units having D-glucopyranosyluronic acid residues at position 3.

The presence of the component unit composed of 1→2 linked L-rhamnose and 1→4 linked D-galacturonic acid having D-glucuronic acid moiety has been reported in the cases of the gum from *Sterculia urens*,[11] and of the mucilages from inner barks of *Hydrangea paniculata*,[1] and roots of *Althaea officinalis*.[12] In these examples, D-galactose,[11,12] or D-galactose and 4-*O*-methyl-D-glucuronic acid[1] were also found as the other component sugars. Thus the manner of the carbohydrate linkage in Abelmoschus-mucilage M is not so complicated as these three polysaccharides, but it belongs to one of the complexes with carbohydrate and peptide. 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. Optical rotation was measured with JASCO model DIP-SL automatic polarimeter. Viscosity was determined with an Ubbelohde-type viscosimeter. Amino acids were determined by the use of Hitachi mdoel KLA-5 amino acid analyzer. Infrared (IR) spectrum was 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 the Mucilage——The material was obtained in November of 1975 from the plants cultivated in Saitama prefecture. The fresh roots (417 g), which contain 77.1% of water, were crushed, then extracted with water (4200 ml) under stirring for 3 hr at room temperature. After suction filtration, the extraction was similarly repeated with water (3000 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 (5.29 g) was obtained. A part of crude mucilage (1.9 g) was dissolved in water and applied to a column (4.5×45 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 (1150 ml) and 0.1 M ammonium carbonate (900 ml), the column was eluted with 0.5 M ammonium carbonate (3750 ml) and 1 M ammonium carbonate (1050 ml). Fractions of 50 ml were collected and analyzed by phenol–sulfuric acid method.[13] The eluates obtained from tubes 53 to 72 were combined, concentrated and poured into ethanol. The precipitate was repeatedly washed with aqueous ethanol, then dried *in vacuo*. Abelmoschus-mucilage M (0.37 g) was obtained as white powder. The eluates obtained from tubes 119 to 136 gave the minor mucilage (0.08 g) in the similar way.

Cellulose Acetate Membrane Electrophoresis——Electrophoresis was carried out with Separax (Fuji Film Co., 6×21 cm long) and the following two buffers: A, 0.025 M borax: 0.1 N sodium hydroxide (10: 1, pH 9.2); B, 0.08 M pyridine–0.046 M acetic acid (pH 5.3). The condition of 420 volts for 1 hr was used. Samples were applied in line at the center. The inside of the apparatus was cooled with dry ice. The sample was revealed with 0.5% toluidine blue in 3% acetic acid (for acidic polysaccharides) and 0.1% amidoblack 10B

8) R.L. Taylor and H.E. Conrad, *Biochemistry*, **11**, 1383 (1972).
9) S. Hakomori, *J. Biochem.*, **55**, 205 (1964).
10) H. Björndal, B. Lindberg, and S. Svensson, *Carbohyd. Res.*, **5**, 433 (1967).
11) G.O. Aspinall and Nasir-ud-din, *J. Chem. Soc.*, **1965**, 2710.
12) M. Tomoda, S. Kaneko, M. Ebashi, and T. Nagakura, *Chem. Pharm. Bull.* (Tokyo), **25**, 1357 (1977).
13) J.E. Hodge and B.T. Hofreiter, *Meth. Carbohyd. Chem.*, **1**, 388 (1962).

in acetate buffer[14] (for proteins). The coloration with the former reagent was very clear, but that with the latter reagent was faint. Nevertheless, there was no difference in the positions of the spots detected with the both reagents. The sample gave a single spot at distances of 5.0 cm in buffer A and 6.6 cm in buffer B from the origin toward the anode.

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-glucuronic acid, and D-galacturonic acid were respectively extracted with water from the parts showing *Rf* values of 0.67, 0.21, and 0.16. Specific rotations of rhamnose and ammonium salts of the two hexuronic acids in water at 20° were +7.8°, +11.7°, and +37.1°.

TLC of Component Sugars——The hydrolyzate of the sample was applied to TLC using Avicel SF cellulose and the solvent systems A and B, AcOEt: AcOH: HCOOH: H_2O (18: 3: 1: 4). Component sugars were revealed with *p*-anisidine hydrochloride reagent[15] and silver nitrate reagent.[16] *Rf* values on TLC are shown in Table II.

Table II. *Rf* Values of Component Sugars on TLC

	Solvent A	Solvent B
Rhamnose	0.75	0.54
(Glucuronolactone)	0.86	0.64
Glucuronic acid	0.23	0.24
Galacturonic acid	0.18	0.17

Determination of Component Sugars——Rhamnose was determined by thioglycolic acid method.[17] Hexuronic acids were estimated by modified carbazole method[18] and the values were calculated on the assumption of the presences of equimolar galacturonic acid and glucuronic acid based on the result of methylation analysis. The results indicated that the mucilage contained 27.0% of rhamnose, 27.5% of galacturonic acid, and 27.5% of glucuronic acid.

Determination of Molecular Weight——The measurement of osmotic pressure was carried out by the use of Knauer Electronic Membrane Osmometer at 60°. The mucilage was dissolved in 0.05% ammonium hydroxide followed by evaporation and lyophilization. The sample was dissolved in water, and 0.39, 0.30, 0.19, and 0.10% solutions were used.

Reduction of the Mucilage——The mucilage (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 × 78 cm) of Sephadex G-15 (Pharmacia Co.). The column was eluted with water, and fractions were collected at 20 ml. The eluates obtained from tubes 26 to 36 were combined, concentrated and lyophilized, Yield, 56.9%.

Methylation of the Reduction Product——Sodium hydride (40 mg) was mixed with dimethyl sulfoxide (5 ml) and the mixture was stirred at 70° for 1 hr. The sample (20 mg) was dissolved in dimethyl sulfoxide (7 ml) and the solution of methylsulfinylmethyl sodium was added into this mixture. After stirring at room temperature for 4 hr, methyl iodide (4 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 to dryness. The product was methylated four more times under the same condition. The final non-dialyzable fraction was extracted with chloroform (20 ml each) five times. The extracts were combined and washed with water (100 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 Product——A part of the product was successively treated with 90% formic acid at 90° for 16 hr and 0.5 N sulfuric acid at 100° for 2 hr. After neutralization with Dowex 2 (OH^-), the

14) J. Uriel, *Bull. Soc. Chim. Biol.*, **40**, 277 (1958).
15) L. Hough and J.K.N. Jones, *Meth. Carbohyd. Chem.*, **1**, 28 (1962).
16) W.E. Trevelyan, D.P. Procter, and J.S. Harrison, *Nature*, **166**, 444 (1962).
17) M.N. Gibbons, *Analyst* (London), **80**, 268 (1955).
18) T. Bitter and H. Muir, *Anal. Biochem.*, **4**, 330 (1962).

hydrolyzate was reduced with sodium borohydride for 2 hr. After neutralization with Dowex 50W-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 40 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% OV 225 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 A 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 main 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)]	Main fragments (*m/e*)
3,4-Me-1,2,5-Ac-L-Rhamnitol	0.87	43, 89, 129, 131, 189
2,3,4,6-Me-1,5-Ac-D-Glucitol	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
2,6-Me-1,3,4,5-Ac-D-Galactitol	2.97	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)

Acknowledgement The authors are grateful to Prof. G. Matsumura, School of Pharmaceutical Sciences, Showa University, for ultracentrifugal analysis, Dr. N. Aimi, Faculty of Pharmaceutical Sciences, Chiba University, for elemental analysis, and Dr. S. Kobayashi, Division of Physiology, this College, for the determination of amino acids. They also thank Misses M. Tanaka and Y. Yano for their technical assistance.