Chem. Pharm. Bull.
33(8)3330—3335(1985)

Plant Mucilages. XXXVI.[1] Isolation and Characterization of a Mucilage, "Okra-Mucilage R," from the Roots of *Abelmoschus esculentus*

MASASHI TOMODA,* NORIKO SHIMIZU, and RYŌKO GONDA

Kyoritsu College of Pharmacy, Shibakōen, Minato-ku, Tokyo 105, Japan

(Received December 24, 1984)

A representative mucilage, named Okra-mucilage R, was isolated from the roots of *Abelmoschus esculentus* MOENCH (=*Hibiscus esculentus* L.; Okra). The final preparation was homogeneous as determined by ultracentrifugal analysis, cellulose acetate membrane electrophoresis, and gel chromatography. Its water solution gave an intrinsic viscosity value of 33.4. It was mainly composed of partially acetylated acidic polysaccharide, and its molecular weight was estimated to be about 1700000. The polysaccharide was composed of L-rhamnose : D-galactose : D-galacturonic acid : D-glucuronic acid : *O*-acetyl groups in the molar ratio of 1.1 : 1.9 : 1.0 : 1.0 : 2.0. Methylation and partial acid hydrolysis studies made it possible to deduce the structural features of the polysaccharide in the mucilage.

Keywords——*Abelmoschus esculentus*; root; Okra-mucilage R; intrinsic viscosity; molecular weight; partially acetylated polysaccharide; component analysis; methylation analysis; partial hydrolysis; structural feature

In a previous paper of this series,[2] the isolation and structural features of a representative mucilage, named Okra-mucilage F, from the immature fruits of *Abelmoschus esculentus* MOENCH (=*Hibiscus esculentus* L.; Okra) were reported. In contrast to several mucilages obtained by us from other plants in the Malvaceae family, Okra-mucilage F has a unique structure in having no branch at any of the D-galacturonic acid residues.[2] The root of this plant also contains mucilages, but no structural study on the mucilages has been reported so far. We have now obtained a new representative mucilage from this material. The properties and main structural features of the mucilage are described in the present paper.

The roots were sliced, homogenized and extracted with cold water. The crude mucilage was precipitated from the extract by addition of ethanol, then dissolved in dilute sodium sulfate solution. The precipitate obtained by addition of cetyltrimethyl ammonium bromide was dissolved in sodium chloride solution, then poured into ethanol. The precipitate obtained was dissolved in water, reprecipitated with ethanol, and dialyzed against distilled water. A pure mucilage was obtained by lyophilization of the dialysate.

The mucilage was homogeneous as determined by ultracentrifugal analysis (Fig. 1), and gave a single spot on cellulose acetate membrane electrophoresis. Furthermore, it gave a single peak on gel chromatography with Sephacryl S-500. The mucilage showed a positive specific rotation ($[\alpha]_D^{23}$ +50.0° in H_2O, $c=0.1$), and its solution in water gave the high intrinsic viscosity value of 33.4 at 30 °C. Gel chromatography gave a value of approximately 1700000 for the molecular weight. The name "Okra-mucilage R" is proposed for this substance.

Galactose, rhamnose, galacturonic acid, and glucuronic acid were identified as the component sugars. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of the mucilage showed an acetyl signal at δ 2.16 (s) in addition to a methyl signal of rhamnose at δ 1.33 (d, $J=6$ Hz) and anomeric proton signals of the component sugars. Analysis of the acid hydrolysate of the mucilage by gas-liquid chromatography (GLC)[3] showed the occurrence of

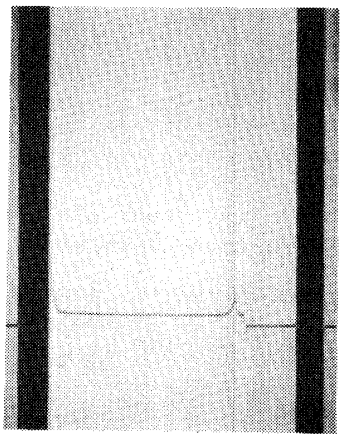

Fig. 1. Ultracentrifugal Pattern of Okra-Mucilage R

0.2% in H_2O, 20 °C, 43 min, 51200 rpm, Hitachi UCA-1A ultracentrifuge.

TABLE I. Amino Acid Compositions (Molar Percent)

	Okra-mucilage R	Okra-mucilage F		Okra-mucilage R	Okra-mucilage F
Lysine	4.35	5.56	Glycine	11.17	10.35
Histidine	1.73	2.49	Alanine	11.04	8.20
Arginine	4.63	5.15	Valine	6.72	7.37
Aspartic acid	10.77	9.84	Methionine	2.69	2.08
Threonine	5.41	5.77	Isoleucine	4.81	5.75
Serine	5.74	6.37	Leucine	8.15	9.01
Glutamic acid	13.26	9.56	Tyrosine	2.16	3.06
Proline	3.90	4.60	Phenylalanine	3.47	4.84

acetic acid. Quantitative determination showed that the mucilage contained 26.4% galactose, 13.9% rhamnose, 15.2% galacturonic acid, 15.2% glucuronic acid, and 7.4% acetyl group, and that their molar ratio was 1.9 : 1.1 : 1.0 : 1.0 : 2.0. Determination of protein content was carried out by the method of Lowry *et al.*,[4] and a value of 19.3% was obtained. The amino acid composition after hydrolysis with 6 N hydrochloric acid is listed in Table I, together with that of Okra-mucilage F.[2] No nitrogen-containing compound other than amino acids was detected in the hydrolysate. There is no significant difference in amino acid composition between Okra-mucilages R and F, except for the values of glutamic acid and alanine.

The carboxyl groups of hexuronic acid residues in the mucilage were reacted with a carbodiimide reagent, then reduced with sodium borohydride to give the corresponding neutral sugar units.[5] Methylations of the original and the carboxyl-reduced mucilages were performed with methylsulfinyl carbánion and methyl iodide in dimethyl sulfoxide.[6] The fully methylated products were hydrolyzed, reduced, and acetylated. The final products obtained were analyzed by GLC-mass spectrometry (MS). Methyl ethers of the hexuronic acids were removed from the hydrolysis products of the methylated original mucilage by treatment with an anion-exchange resin, and the residual products were identified as alditol acetates of 3,4-di-*O*-methyl-L-rhamnopyranose, 3-*O*-methyl-L-rhamnopyranose, 2,3,4,6-tetra-*O*-methyl-D-galactopyranose, and 2,3,6-tri-*O*-methyl-D-galactopyranose in a molar ratio of 1.0 : 2.1 : 2.2 : 3.9, while alditol acetates of 3,4-di-*O*-methyl-L-rhamnopyranose, 3-*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 in a molar ratio of 1.0 : 1.9 : 3.0 : 2.3 : 3.6 : 3.1 from the carboxyl-reduced product.

These results suggested that the minimal repeating unit of the polysaccharide moiety of Okra-mucilage R is composed of six kinds of component sugar units as shown in Chart 1.

(one)[a]	→2 L-Rha*p* 1→	(three)[a]	D-Glc*p*A 1→
(two)[a]	→4 L-Rha*p* 1→	(three)[a]	→4 D-Gal*p*A 1→
	2		3
	↑		↑
(two)[a]	D-Gal*p* 1→		
(four)[a]	→4 D-Gal*p* 1→		

Rha*p*, rhamnopyranose;
Gal*p*, galactopyranose;
Glc*p*A, glucopyranosyluronic acid;
Gal*p*A, galactopyranosyluronic acid

Chart 1. Component Sugar Residues in the Minimal Repeating Unit in the Structure of Okra-Mucilage R

a) Number of residues.

Partial hydrolysis of the mucilage was carried out with 1 N sulfuric acid for 2 h, then the reaction mixture was neutralized and applied to a column of Dowex 50W (H^+). The eluate with water was applied to a column of diethylaminoethyl (DEAE)-Sephadex A-25 (formate form). Five oligosaccharides (I to V) were obtained by stepwise elution with dilute formic acid, then purified by rechromatography. Based on the results of component sugar analysis, and by comparing their chromatographic properties, the ^{1}H-NMR spectra and the values of specific rotation with those of authentic samples,[7,8] I to V were identified as the following five oligosaccharides (Chart 2).

I: α-D-Gal*p*A 1→2 L-Rha*p*
II: β-D-Glc*p*A 1→3 α-D-Gal*p*A 1→2 L-Rha*p*
III: ($n=0$)
IV: ($n=1$)
V: ($n=2$)

α-D-Gal*p*A 1→2 α-L-Rha*p* 1→[4 α-D-Gal*p*A 1→2 α-L-Rha*p* 1→]$_n$4 α-D-Gal*p*A 1→2 α-L-Rha*p*
3 3 3
↑ ↑ ↑
1 1 1
β-D-Glc*p*A β-D-Glc*p*A β-D-Glc*p*A

Chart 2. Structural Features of Oligosaccharides I—V

All galactose residues were liberated from the mucilage under the conditions of the partial hydrolysis. In addition to the results of methylation analysis, this fact suggests the presence of galactosyl-(1→4)-galactosyl-(1→4)-galactose as the average length side chain or longer galactosyl side chains, having the same glycosidic linkages, linking to position 4 of two-thirds of the rhamnose residues in the backbone chain. The value of specific rotation of the galactose fraction was consistent with the D configuration.

The ^{1}H-NMR spectrum of the mucilage showed four anomeric proton signals at δ 4.48 (d, $J=8$ Hz), δ 4.71 (d, $J=7$ Hz), δ 5.00 (d, $J=2$ Hz), and δ 5.29 (d, $J=3$ Hz), and their integral ratio was 2 : 1 : 1 : 1. The signals at δ 4.71, 5.00, and 5.29 are due to β-D-glucuronic acid, α-L-rhamnose, and α-D-galacturonic acid residues, respectively.[7] The signal at δ 4.48 suggests that the D-galactose residues in the mucilage are β-linked.

Based on the accumulated evidence described above, it can be concluded that the polysaccharide moiety of the mucilage has the following repeating unit (Chart 3).

The presence of the component unit having the structure (1→4)-[*O*-β-(D-glucopyranosyluronic acid)-(1→3)]-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranose is common in the mucilages from the roots of *Althaea officinalis*,[8] *Abelmoschus manihot*,[7] *Abelmoschus glutinotextilis*,[9] and *Althaea rosea*,[10] the leaf of *Althaea officinalis*,[11] and the inner bark of *Hydrangea paniculata*.[12] The main part of Okra-mucilage R is also made up of this component unit, whereas Okra-mucilage F has no branch at the D-galac-

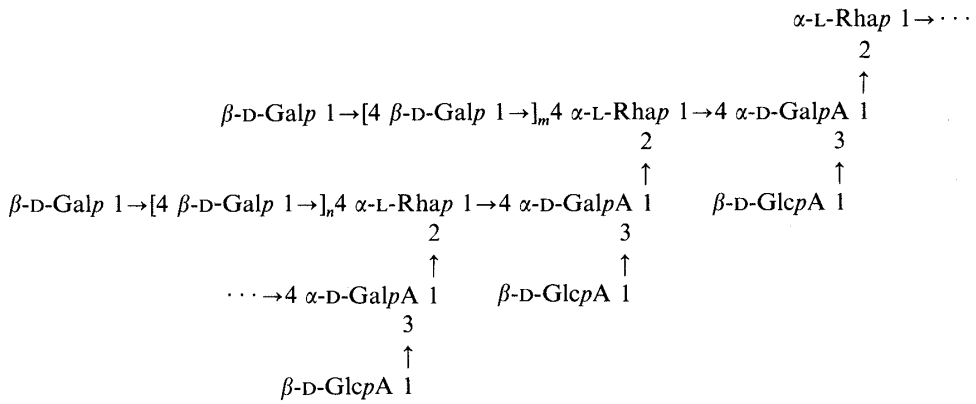

Chart 3. A Possible Structural Fragment of the Polysaccharide Moiety of Okra-mucilage R

$m+n=4$.

turonic acid residues in the main chain.[2] Thus, this characteristic difference in the structure of the backbone chain between Okra-mucilages R and F appears to be a result of the difference in the part of the plant material, *i.e.*, roots and immature fruits, used as a source. The mucilages from the roots of *Althaea officinalis*[8] and the immature fruits of *Abelmoschus esculentus*[2] have branches composed of 4-*O*-β-D-galactosyl D-galactose at position 4 of a part of the L-rhamnose units in the main chain. On the other hand, the presence of side chains composed of (on average) three β-1→4 linked D-galactopyranosyl residues is a characteristic of the structure of Okra-mucilage R. The locations of acetyl groups remain to be determined. The results of detailed analysis of the structure will be reported in subsequent papers.

Experimental

Solutions were concentrated at or below 40 °C with rotary evaporators under reduced pressure. Optical rotations were measured with a JASCO DIP-140 automatic polarimeter. Infrared (IR) spectra were recorded on a JASCO IRA-2 infrared spectrophotometer. ^{1}H-NMR spectra were recorded on a JEOL JNM-GX 270 FT NMR spectrophotometer in heavy water containing sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard at 70 °C. GLC was carried out on a Shimadzu GC-7AG gas chromatograph equipped with a hydrogen flame ionization detector. GLC-MS was performed with a JEOL JGC-20K gas chromatograph and a JEOL JMS-D100 mass spectrophotometer. Amino acids were determined with a Hitachi KLA-5 amino acid analyzer. Viscosity was determined with an Ubbelohde-type viscosimeter.

Isolation of the Mucilage——The material was obtained in October 1982 from plants cultivated in Saitama prefecture. The fresh roots (380 g), which contained 73.9% water, were sliced and homogenized, then extracted with water (2500 ml) under stirring for 3 h at room temperature. After centrifugation, the supernatant was poured into two volumes of ethanol, and the whole was filtered. The precipitate was dissolved in water and lyophilized, and a light brown powder (2.1 g) was obtained. A part of the crude mucilage (780 mg) was dissolved in water (87 ml) and 0.02% sodium sulfate (87 ml) was added, followed by 10% cetyltrimethyl ammonium bromide (10 ml). The precipitate was separated by centrifugation, and dissolved in 2 M sodium chloride (82 ml). The solution was centrifuged to remove small amounts of impurities. The supernatant thus obtained was poured into two volumes of ethanol. The resulting precipitate was dissolved in water, and treated again with ethanol, followed by dialysis against distilled water. Okra-mucilage R (280 mg) was obtained as a grayish-white powder after lyophilization.

Cellulose Acetate Membrane Electrophoresis——This was carried out as described in a previous report[2] of this series at 420 volts for 1 h with the following two buffers: A, 0.08 M pyridine–0.04 M acetic acid (pH 5.4); B, 0.025 M borax : 0.1 N sodium hydroxide (10 : 1, pH 9.3). The sample gave a single spot at distances of 7.3 cm in buffer A and 5.5 cm in buffer B from the center toward the anode.

Gel Chromatography——The sample (3 mg) was dissolved in water and applied to a column (2.6 × 96 cm) of Sephacryl S-500. Elution was carried out with 0.1 M Tris–HCl buffer (pH 7) containing 0.1 M sodium chloride; fractions of 5 ml were collected, and analyzed by the phenol–sulfuric acid method.[13]

Hydrolysis, Isolation and Thin-Layer Chromatography (TLC) of Component Sugars——These were carried out as

described in a previous report[14] of this series.

Determination of Components——Neutral sugars in the original and the carboxyl-reduced mucilages were analyzed by GLC after conversion into alditol acetates as described in a previous report[7] of this series. Rhamnose was also determined by the thioglycolic acid method,[15] and hexuronic acids in the original mucilage were estimated by a modification of the carbazole method.[16]

Determination of *O*-Acetyl Groups——The sample was hydrolyzed with 1 N hydrochloric acid at 100 °C for 2 h. Propionic acid was used as an internal standard, and the hydrolysate was directly subjected to GLC under the same conditions as in a previous report[3] of this series.

Reduction of the Mucilage——The mucilage (100 mg) was suspended in water (30 ml), then 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (1 g) was added. The pH of the reaction mixture was maintained at 4.75 by titration with 0.1 N hydrochloric acid under stirring for 2 h, then 2 M sodium borohydride (10 ml) was added gradually to the reaction mixture during 4 h while the pH was maintained at 7.0 by titration with 4 N hydrochloric acid under stirring at room temperature. The solution was dialyzed against distilled water overnight, then the non-dialyzable fraction was concentrated to 30 ml. The product was reduced four times more under the same conditions. The final non-dialyzable fraction was centrifuged, then the supernatant was concentrated and applied to a column (5 × 78.5 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 34 to 50 were combined and lyophilized. Yield, 23.3 mg.

Methylation——The sample (5 mg) was dissolved in dimethyl sulfoxide (1 ml). Sodium hydride (20 mg) was mixed with dimethyl sulfoxide (2 ml) in an ultrasonic bath for 30 min, followed by stirring at 70 °C for 1 h, then the mixture was added to the sample solution. The reaction mixture was stirred at room temperature for 4 h, then methyl iodide (2 ml) was added and the whole was stirred overnight at room temperature. All procedures were carried out under nitrogen. The reaction mixture was then dialyzed against distilled water. The non-dialyzable fraction was concentrated to dryness. The product was methylated once more under the same conditions. After addition of water (20 ml), the reaction mixture was extracted five times with chloroform (20 ml each). The combined extract was washed five times with water (100 ml each), then dried over sodium sulfate, and the filtrate was concentrated to dryness. The residue was methylated twice more under the same conditions. The final residue was dissolved in chloroform–methanol mixture (2 : 1), and applied to a column (1 × 16.5 cm) of Sephadex LH-20. The column was eluted with the same solvent, and fractions of 1 ml were collected. The eluates obtained from tubes 6 to 8 were combined and concentrated. The IR spectrum of the final residue showed no absorption of hydroxyl groups. Yield, 3 mg.

Analysis of the Methylated Products——Each product was hydrolyzed with 90% formic acid followed by 0.5 N sulfuric acid, then reduced and acetylated in the manner described in a previous report[17] of this series. GLC and GLC-MS were carried out under condition B described in the previous report.[17] The relative retention times of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and their main fragments in the MS are listed in Table II.

Partial Hydrolysis and Isolation of Oligosaccharides——The mucilage (200 mg) was suspended in 1 N sulfuric acid (40 ml) and heated under reflux at 100 °C for 2 h. The resulting water-insoluble fraction (104 mg) was separated by centrifugation, then the supernatant was neutralized with barium carbonate, and, after filtration, the filtrate was passed through a column (1 × 6 cm) of Dowex 50W × 8 (H^+). The eluate with water was concentrated and applied to a column (1 × 9 cm) of DEAE-Sephadex A-25 (formate form). The column was eluted successively with water (30 ml), 0.1 M formic acid (30 ml), 0.2 M formic acid (30 ml), 0.4 M formic acid (55 ml), 0.8 M formic acid (85 ml), 1 M formic acid (50 ml), and 1.5 M formic acid (50 ml). Fractions of 5 ml were collected and analyzed by the phenol–sulfuric acid

Table II. Relative Retention Times on GLC and Main Fragments in MS of Partially Methylated Alditol Acetates

	Relative retention times[a]	Main fragments (*m/z*)
1,2,5-Ac-3,4-Me-L-Rhamnitol	0.89	43, 89, 129, 131, 189
1,2,4,5-Ac-3-Me-L-Rhamnitol	1.59	43, 87, 101, 129, 143, 189, 203
1,5-Ac-2,3,4,6-Me-D-Glucitol	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,5-Ac-2,3,4,6-Me-D-Galactitol	1.16	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,4,5-Ac-2,3,6-Me-D-Galactitol	2.03	43, 45, 87, 99, 101, 113, 117, 233
1,3,4,5-Ac-2,6-Me-D-Galactitol	2.78	43, 45, 87, 117, 129

Abbreviations: Ac = acetyl; Me = methyl (*e.g.*, 1,2,5-Ac-3,4-Me = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-). *a*) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

method. The eluates obtained were divided into seven groups: Frac. 1, tubes 1 to 6; Frac. 2, tubes 10 to 13; Frac. 3, tubes 14 to 19; Frac. 4, tubes 23 to 30; Frac. 5, tubes 31 to 48; Frac. 6, tubes 49 to 52; Frac. 7, tubes 58 to 67. Galactose and rhamnose were obtained from Frac. 1, and their yields were 26.4% and 0.5%, respectively. Glucuronic acid was obtained from Frac. 3 and the yield was 1.6%. Oligosaccharides I, II, III, IV, and V were obtained from Fracs. 2, 4, 5, 6, and 7, respectively, after rechromatography under the same conditions. The yields were 2.3%, 3.7%, 5.8%, 2.1%, and 1.5%, respectively.

Analysis of the Oligosaccharides——Analysis of component sugars was carried out as described above. The values of specific rotation and the results of paper partition chromatography (PPC) by the descending method were in agreement with the data described in a previous report[8] of this series.

Acknowledgement We are grateful to Prof. G. Matsumura, School of Pharmaceutical Sciences, Showa University, for ultracentrifugal analysis, and to Dr. S. Kobayashi, this College, for the determination of amino acids.

References and Notes

1) Part XXXV: M. Tomoda, N. Shimizu, K. Shimada, and R. Gonda, *Chem. Pharm. Bull.*, **33**, 48 (1985).
2) M. Tomoda, K. Shimada, Y. Saito, and M. Sugi, *Chem. Pharm. Bull.*, **28**, 2933 (1980).
3) M. Tomoda, N. Satoh, and C. Ohmori, *Chem. Pharm. Bull.*, **26**, 2768 (1978).
4) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
5) R. L. Taylor and H. E. Conrad, *Biochemistry*, **11**, 1383 (1972).
6) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
7) M. Tomoda, Y. Suzuki, and N. Satoh, *Chem. Pharm. Bull.*, **27**, 1651 (1979).
8) M. Tomoda, N. Satoh, and K. Shimada, *Chem. Pharm. Bull.*, **28**, 824 (1980).
9) M. Tomoda, M. Arai, Y. Suzuki, M. Ohmura, and H. Takayama, *Chem. Pharm. Bull.*, **28**, 1546 (1980).
10) M. Tomoda, K. Shimada, and N. Shimizu, *Chem. Pharm. Bull.*, **31**, 2677 (1983).
11) M. Tomoda, N. Shimizu, H. Suzuki, and T. Takasu, *Chem. Pharm. Bull.*, **29**, 2277 (1981).
12) M. Tomoda and N. Satoh, *Chem. Pharm. Bull.*, **25**, 2910 (1977).
13) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1952).
14) M. Tomoda, S. Kaneko, M. Ebashi, and T. Nagakura, *Chem. Pharm. Bull.*, **25**, 1357 (1977).
15) M. N. Gibbons, *Analyst* (London), **80**, 268 (1955).
16) T. Bitter and H. M. Muir, *Anal. Biochem.*, **4**, 330 (1962).
17) M. Tomoda, N. Shimizu, K. Shimada, R. Gonda, and H. Sakabe, *Chem. Pharm. Bull.*, **32**, 2182 (1984).