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Plant Mucilages. XXX.[1] Isolation and Characterization of a Mucilage, "Dioscorea-mucilage B," from the Rhizophors of *Dioscorea batatas*

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A representative mucilage, named Dioscorea-mucilage B, was isolated from the rhizophors of *Dioscorea batatas* DECAISNE. The final preparation was homogeneous as determined by ultracentrifugal analysis, polyacrylamide gel electrophoresis, and gel chromatography. Its water solution gave the intrinsic viscosity value of 21.0, and its molecular weight was estimated to be about 2000000. It was composed of partially acetylated mannan and of protein containing small amounts of phosphorus in a ratio of approximately 1.0: 2.2. The polysaccharide moiety was isolated by treatment with Pronase followed by gel chromatography. Its molecular weight was estimated to be about 99000. Methylation and partial acetolysis studies showed that the polysaccharide is mainly composed of β-1→4 linked D-mannopyranose residues having about one fourth degree of branching at the C-3 positions. The *O*-acetyl groups were located at positions 6 and 2, 3, 6 of some of the mannose units.

Keywords——Dioscorea-mucilage B; mucilage from rhizophors; *Dioscorea batatas*; intrinsic viscosity; molecular weight; amino acid composition; isolation of polysaccharide; structural features of polysaccharide moiety; location of *O*-acetyl groups

The tuberous rhizophors of *Dioscorea batatas* DECAISNE are used as a crude drug for the purpose of antidiarrheic, antidipticum, tonic, and cough medicine, and are also widely used as a food. It is well known that the rhizophors contain a relatively large amount of mucilage. In 1928, Takahashi[2] showed that the mucilage was a kind of glycoprotein having large amounts of arginine, lysine, histidine, tryptophan, and mannose in addition to small amounts of cystine and glucose. However, the mucilage obtained by him was not homogeneous and was insoluble in water. More recently, Satoh *et al.*[3] obtained a homogeneous mucilage from the rhizophors of *Dioscorea batatas* forma *Icho*, and they reported that it contained 48% mannan, 10% protein, and 3.8% phosphorus. Satoh[4] suggested that the mannan fraction was a linear chain consisting of β-1→4 glycosidic partially acetylated D-mannose residues. Misaki *et al.*[5] extracted a mucilage from the tubers of *Dioscorea batatas* forma *Tsukune*. It contained 71.5% protein and 13.8% carbohydrate, although its homogeneity was uncertain. They isolated a homogeneous mannan from the mucilage, and suggested that it was mainly composed of partially acetylated D-mannose units having β-1→4 glycosidic linkages and that it had a small degree of branching at the C-3 positions. No further structural study on the mucilage of the rhizophors of *Dioscorea batatas* and its form has yet been reported. We have now isolated a pure mucilage from the rhizophors of this plant. From the relative viscosity of the solution of the mucilage and its yield, we consider it probable that the pure mucilage obtained by us is the major component accounting for the mucosity of the water extract from the material. This paper is concerned with the constitution of the mucilage, particularly the structural features of a polysaccharide moiety in it.

The fresh rhizophors were homogenized and extracted with cold water. The crude mucilage was precipitated from the extract by addition of ethanol, then dissolved again in water. The solution was treated with sodium lauryl sulfate and sodium chloride, and after centrifugation, the supernatant obtained was poured into ethanol. The precipitate obtained was dissolved in water and reprecipitated twice with ethanol. A pure mucilage was obtained by lyophilization.

The mucilage was homogeneous as determined by ultracentrifugal analysis (Fig. 1), and gave a single band on polyacrylamide gel electrophoresis (Fig. 2). Furthermore, it gave a single peak on gel chromatography with Sepharose 4B. It showed a negative specific rotation ($[\alpha]_D^{20}$ $-47.3°$ in H_2O, $c=0.05$), and its solution in water gave the intrinsic viscosity value of 21.0 at 30°. The relative viscosity of the solution of the pure mucilage was about 2.5 times that of the crude mucilage. Gel chromatography gave a value of approximately 2000000 for the molecular weight. The name "Dioscorea-mucilage B" is proposed for this substance.

The mucilage contained 64.0% protein, 26.2% mannan, 2.7% acetyl group, and 1.2% phosphorus. Its amino acid composition after hydrolysis with 6 N hydrochloric acid is listed in Table I. There is no significant difference in amino acid composition between Dioscorea-mucilage B and the mucilage from *Dioscorea batatas* forma *Tsukune*,[5] except for the values of cysteine, methionine, and tryptophan. On the other hand, a pronounced difference in composition was found as compared with the mucilage[3] from *Dioscorea batatas* forma *Icho*.

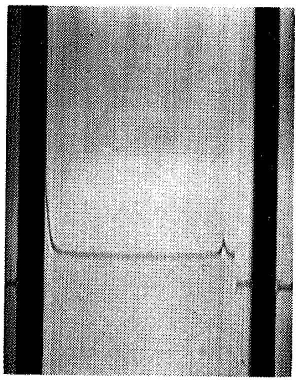

Fig. 1. Ultracentrifugal Pattern of Dioscorea-mucilage B

0.41% in H_2O, 23°C, 36 min, 60000 rpm, Hitachi UCA-1A ultracentrifuge.

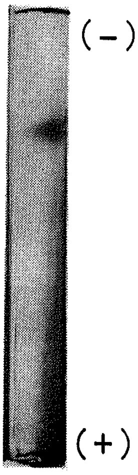

Fig. 2. Polyacrylamide Gel Electrophoresis of Dioscorea-mucilage B

Table I. Amino Acid Compositions (Molar Percent)

	Dioscorea-mucilage B	the Mucilage[3] from *D. batatas* f. *Icho*	the Mucilage[5] from *D. batatas* f. *Tsukune*
Lysine	3.93	9.69	4.04
Histidine	1.56	1.36	1.67
Arginine	5.10	6.40	5.42
Cysteine	0.94	—	—
Aspartic acid	12.52	13.95	12.45
Threonine	4.12	11.43	3.65
Serine	5.46	13.95	6.66
Glutamic acid	13.25	9.30	11.16
Proline	4.70	5.43	4.61
Glycine	5.81	13.76	6.81
Alanine	5.81	1.74	5.89
Valine	5.46	1.16	4.13
Methionine	—	0.39	2.34
Isoleucine	3.39	1.16	3.54
Leucine	7.26	2.52	7.23
Tyrosine	2.34	0.97	2.95
Phenylalanine	4.86	1.16	4.52
Tryptophan	0.47	0.39	—
NH_3	13.01	5.23	12.93

The carbohydrate moiety in the mucilage was isolated by treatment with Pronase followed by gel chromatography with Sephadex G-50. The polysaccharide fraction obtained was homogeneous as determined by ultracentrifugal analysis (Fig. 3), and gave a single spot on glass-fiber paper electrophoresis. Furthermore, it gave a single peak on gel chromatography with Sepharose 4B. Gel chromatography gave a value of approximately 99000 for its molecular weight. It showed a negative specific rotation ($[\alpha]_D^{19}$ $-29.3°$ in 0.1% NH_4OH, $c=0.2$).

Its infrared (IR) spectrum has absorption bands at 1250 and 1735 cm^{-1}, suggesting the presence of ester linkages, in addition to the band of 890 cm^{-1} due to the presence of *β*-glycosidic linkages (Fig. 4). When its acid hydrolysate was analyzed by gas-liquid chromatography (GLC),[6] it gave a single peak, with a retention time equal to that of acetic acid.

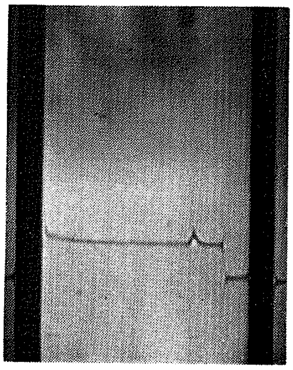

Fig. 3. Ultracentrifugal Pattern of the Polysaccharide Moiety

0.2% in H_2O, 26°C, 15 min, 60000 rpm, Hitachi UCA-1A ultracentrifuge.

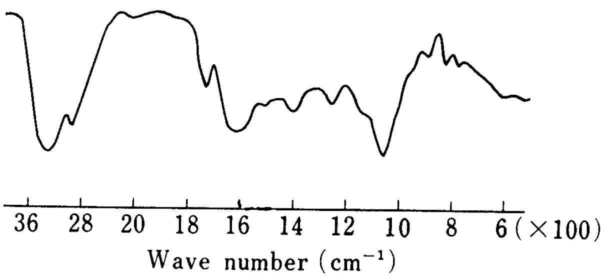

Fig. 4. IR Spectrum of the Polysaccharide Moiety

Quantitative determination of the components showed that the polysaccharide moiety contained 84.8% mannose, 6.8% acetyl group, and 1.4% of a peptide composed of serine, threonine, glycine, alanine, valine, aspartic acid, glutamic acid, and lysine.

Methylation of the polysaccharide was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide.[7] The fully methylated product was hydrolyzed with dilute sulfuric acid in acetic acid. The products were analyzed by gas-liquid chromatography–mass spectrometry (GLC–MS) after conversion into alditol acetates,[8] and identified as 2,3,4,6-tetra-*O*-methyl-D-mannopyranose, 2,3,6-tri-*O*-methyl-D-mannopyranose, and 2,6-di-*O*-methyl-D-mannopyranose. They were obtained in a molar ratio of 1.0: 1.9: 1.0.

The polysaccharide was peracetylated with acetic anhydride and pyridine in formamide, then partially degraded with sulfuric acid in acetic anhydride. After deacetylation, the products were analyzed by thin-layer chromatography (TLC) and by GLC of trimethylsilylated derivatives. Comparison with authentic samples[9] showed the presence of D-mannose, *O*-*β*-D-mannopyranosyl-(1→4)-D-mannopyranose, and *O*-*β*-D-mannopyranosyl-(1→4)-*O*-*β*-D-mannopyranosyl-(1→4)-D-mannopyranose.

The polysaccharide was treated with a *β*-D-mannanase obtained from Driselase in order to improve the low solubility in organic solvents. Then the product was exhaustively treated with methyl vinyl ether in the presence of *p*-toluenesulfonic acid in dimethyl sulfoxide.[10] After conversion of the free hydroxyl groups into 1-methoxyethyl ethers, the derivative was deacetylated, then methylated with methyl iodide and silver oxide in *N*,*N*-dimethylformamide.[11] The resulting product was hydrolyzed and analyzed by GLC and GLC-MS after conversion into alditol acetates as described above. Two hexose methyl ethers were detected and identified as 6-*O*-methyl-D-mannose and 2,3,6-tri-*O*-methyl-D-mannose in a molar ratio of 1.0: 1.2. The results indicate that some residues of 6-*O*-acetyl-D-mannose and 2,3,6-tri-*O*-acetyl-D-mannose are present in the polysaccharide.

Based on these results, it can be concluded that the polysaccharide moiety is mainly composed of partially acetylated β-1→4 linked D-mannopyranose units having about one fourth degree of branching at the C-3 positions.

Misaki *et al.* isolated a partially acetylated mannan from the mucilage of the tubers of *Dioscorea batatas* forma *Tsukune.*[5] They suggested that the acetyl groups were located at positions 2 and/or 3, but definitive elucidation of the location has not been achieved. The polysaccharide moiety obtained by us possesses higher values of both molecular weight and degree of branching than the mannan moiety obtained by them, though the manner of branching is similar in both. The location of acetyl groups in Dioscorea-mucilage B is also different from that in the mucilage obtained from *Dioscorea batatas* forma *Tsukune.*

The mode of polysaccharide-protein linkage or the possibility of polysaccharide-protein complex formation by some type of molecular association in Dioscorea-mucilage B remains to be investigated. Further work is in progress.

Experimental

Solutions were concentrated at or below 40°C with rotary evaporators under reduced pressure. Optical rotations were measured with a JASCO DIP-SL automatic polarimeter. Viscosity was determined with an Ubbelohde-type viscosimeter. Amino acids were determined with a Hitachi KLA-5 amino acid analyzer. GLC was carried out on a Hitachi 063 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 spectrometer. IR spectra were recorded on a JASCO IRA-2 infrared spectrophotometer.

Isolation of the Mucilage——The material was obtained in April 1979 from plants cultivated in Saitama prefecture. The fresh rhizophors (498 g), which contain 81.8% water, were crushed, then extracted with water (2500 ml) under stirring for 1 h at room temperature. After centrifugation (10000 rpm, 20 min), the supernatant was poured into four volumes of ethanol. The precipitate was treated with aqueous ethanol, then dried *in vacuo*. A grayish-white powder (4.58 g) was obtained. The crude mucilage was dissolved in water (300 ml) and 5% sodium lauryl sulfate (30 ml) was added, followed by sodium chloride (17.53 g) at 5°C. After centrifugation, the supernatant was poured into five volumes of ethanol. The precipitate was washed with aqueous ethanol, then lyophilized. Dioscorea-mucilage B (1.74 g) was obtained as a white powder.

Polyacrylamide Gel Electrophoresis——Electrophoresis was carried out on a column (0.5 × 10 cm) of polyacrylamide gel[12] with 0.05 M Tris-glycine buffer (pH 8.6) at 48 mA for 90 min. The sample was visualized with 1% amidoblack 10B in 7% acetic acid.

Gel Chromatography with Sepharose 4B——This was carried out in the manner described in a previous report[13] of this series.

Qualitative Analysis of Component Sugars——Samples were hydrolyzed[14] and analyzed[15] as described in previous reports of this series.

Determination of Components——Mannose was determined by the chromotropic acid method.[16] The determination of protein content was carried out by the method of Lowry *et al.*[17] The amino acid composition was estimated after hydrolysis with 6 N hydrochloric acid at 108°C for 22 h. Tryptophan was determined by the method of Goodwin *et al.*[18]

Isolation of the Polysaccharide Moiety——The mucilage (207 mg) was dissolved in water (10 ml) and heated at 100°C for 10 min, then the solution was cooled to room temperature and 0.2 M Tris-HCl buffer containing 0.004 M calcium chloride (pH 7.9, 10 ml) was added. Pronase E (Kaken Kagaku Co., 21.5 mg) was added to the resulting solution, which was then incubated at 40°C with a few drops of toluene for 72 h. After heating at 80°C for 10 min followed by addition of Pronase E (20.4 mg), the incubation was continued for 48 h under the same conditions. The solution was heated at 80°C for 10 min, then centrifuged at room temperature. The supernatant was applied to a column (5 × 82 cm) of Sephadex G-50. The column was eluted with water, and fractions of 50 ml were collected and analyzed by the phenol-sulfuric acid method.[19] The eluates obtained from tubes 14 to 27 were combined, concentrated and applied to columns (2 × 3 cm) of Dowex 50W-X8 (H^+) and Dowex WGR (OH^-) successively. The eluate with water was concentrated and lyophilized. Yield, 57 mg.

Glass-Fiber Paper Electrophoresis——Electrophoresis was carried out with Whatman GF 83 glass-fiber in the manner described in a previous report[20] of this series, with the following buffers and conditions: A, 0.08 M pyridine–0.04 M acetic acid (pH 5.4) at 570 volts for 90 min; B, 0.025 M borax: 0.1 N sodium hydroxide (10 :1, pH 9.3) at 380 volts for 60 min. The polysaccharide moiety was applied in a line at 13 cm from the surface of the liquid at the anode, and gave a single spot at a distance of 2.7 cm (A) or 3.2 cm (B) from the origin toward the cathode.

Determination of *O*-Acetyl Groups——The IR spectrum of the polysaccharide moiety showed ester absorption bands. IR ν_{max}^{KBr} cm^{-1}: 1735, 1250 (ester), 890 (β-glycosidic linkage).

The sample was hydrolyzed with 1 N hydrochloric acid containing propionic acid as an internal standard in a sealed tube at 100°C for 2 h. The hydrolysate was directly subjected to GLC under the same conditions as in a previous report[6] of this series.

Methylation of Polysaccharide and Analysis of the Methylated Product——These were carried out in the manner and under the conditions described in a previous report[21] of this series. The methylation reaction was repeated ten times. 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 mass spectra, are listed in Table II.

Partial Acetolysis and Analysis of Degradation Products——These were also carried out in the manner and under the conditions described in a previous report[21] of this series. *Rf* values of products and retention times of their trimethylsilylated derivatives are shown in Table III.

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

	Relative retention times[a]	Main fragments (*m/e*)
1,5-Ac-2,3,4,6-Me-D-Mannitol	0.98	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,4,5-Ac-2,3,6-Me-D-Mannitol	1.91	43, 45, 87, 99, 101, 113, 117, 233
1,3,4,5-Ac-2,6-Me-D-Mannitol	2.76	43, 45, 87, 117, 129
1,2,3,4,5-Ac-6-Me-D-Mannitol	3.59	43, 45, 87, 115, 129

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

TABLE III. *Rf* Values and Retention Times (min) of Trimethylsilyl Derivatives of Standard Sugars and Partial Acetolysis Products

Standard and products	Cellulose TLC (*Rf*)		GLC (t_R) under condition B
	Solvent A	Solvent B	
Man	0.69	0.57	3.5,[a] 4.7
β-Man-(1→4)-Man	0.55	0.36	22.3,[a] 24.2
β-Man-(1→4)-β-Man-(1→4)-Man	0.28	0.17	39.1,[a] 40.7
Partial acetolysis products	0.69	0.57	3.5
	0.55	0.36	4.7
	0.28	0.17	22.3
			24.2
			39.1
			40.7

Man=D-mannopyranose.
Solvent A, AcOEt: pyridine: AcOH: H_2O (5: 5: 1: 3) at 23°C.
Solvent B, BuOH: pyridine: H_2O (6: 4: 3) at 28°C.
Condition B: a column (0.3 cm × 2 m spiral glass) packed with 2% OV 101 on Uniport HP (80 to 100 mesh) and with a programmed temperature increase of 3°C per min from 180°C to 300°C at a nitrogen flow rate of 30 ml per min.
a) Main peaks.

Treatment of Methyl Vinyl Ether——A Tris-HCl–AcOH buffer (pH 6) solution of Driselase was prepared in the manner described in a previous report[21] of this series. The sample (45 mg) was dissolved in water (3 ml) and the enzyme solution (9 ml) was added, then the solution was incubated at 37°C for 70 h. After centrifugation, the supernatant was applied to columns (1 × 2 cm) of Dowex 50W-X8 (H+) and Dowex 2 (OH$^-$) successively. The eluate with water was concentrated and lyophilized. The product (37 mg) was suspended in dimethyl sulfoxide (4 ml) and *p*-toluenesulfonic acid (7 mg) was added. The mixture was stirred at 15°C, then methyl vinyl ether (1.8 ml), condensed at −10°C, was added in portions under stirring. The reaction mixture was stirred at 15°C for 4 h, then dialyzed against running water overnight. The non-dialyzable fraction was concentrated to dryness, and the reaction procedure was repeated eight times. The final solution was applied to a column (2 × 35 cm) of Sephadex LH-20. The column was eluted with acetone, and fractions of 10 ml were collected. The eluates in tubes 4 to 8 were combined and concentrated. The IR spectrum of the final product had no absorption of hydroxyl groups.

Deacetylation of the Products followed by Methylation——These procedures were carried out in the manner described in a previous report[21] of this series. Methylation of the deacetylated product was repeated thirteen times.

Analysis of the O-Methyl Derivative——The product was hydrolyzed with dilute sulfuric acid in acetic acid and derivatized into partially methylated alditol acetates, then analyzed by GLC and GLC-MS as described above. 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 mass spectra, are also listed in Table II.

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