[Chem. Pharm. Bull. 21(12)2667—2671(1973)]

UDC 547.548.02.05 : 581.192 : 615.322.011.5

Plant Mucilages. VIII.[1] Isolation and Characterization of a Mucous Polysaccharide, "Bletilla-glucomannan," from *Bletilla striata* Tubers

Masashi Tomoda, Satomi Nakatsuka, Mieko Tamai and Midori Nagata

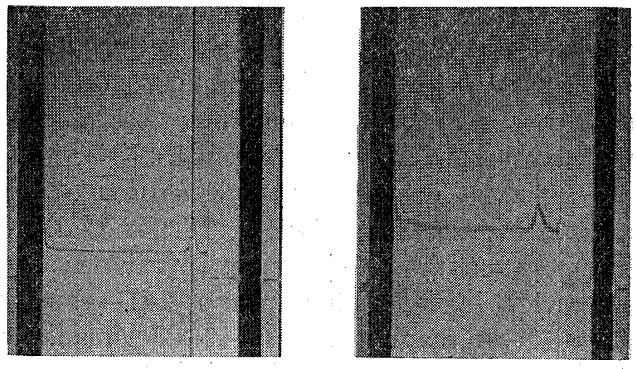
Kyoritsu College of Pharmacy[2]

(Received April 11, 1973)

A mucous polysaccharide, named Bletilla-glucomannan, has been isolated from the tubers of *Bletilla striata* Reichenbach fil. It was homogeneous on glass-fiber paper electrophoresis and by ultracentrifugal analysis. The component sugars of it were D-mannose and D-glucose, and the molar ratio of them was 3 : 1. The molecular weight was estimated at 182000 by the measurement of osmotic pressure. Periodate oxidation and partial acid hydrolysis of it suggested that the polysaccharide is mainly composed of β-1→4 linked aldohexopyranose residues and it contains six aldohexose units per one end group on the average.

The tuber of *Bletilla striata* Reichenbach fil. (Orchidaceae) has been used as a crude drug for the purpose of hemostatic and anti-swelling. On the constituent of this tuber, presence of a mucilage was reported by Ohtsuki.[3] He has concluded that the mucilage is composed of D-mannose and D-glucose in the molar ratio of 4: 1, but its homogeneity was uncertain and the structure has been unknown until now. We have now isolated a pure mucous polysaccharide from the tuber of this plant, and its properties are described in the present paper.

The fresh tubers were extracted with hot methanol, then the residue was extracted with hot water. The crude mucilages were precipitated from the water extract by addition of methanol. The solution of the precipitate was applied to a column of DEAE-cellulose (carbonate form), and a mucous polysaccharide was obtained from the eluate with water. For the purpose of comparison, the material was also extracted with water at room temperature as described in Ohtsuki's report,[3] then the crude mucilage was purified by the use of DEAE-cellulose column chromatography. And it was observed that the mucilage obtained by hot water extraction gives much higher values than that obtained by cold water extraction on both yield and viscosity. So we decided the application of hot water extraction method for the isolation of the mucilage.



(a) (b)

Fig. 1. Ultracentrifugal Pattern of Bletilla-glucomannan

a : 0.5% in H_2O, 25°, 69 min, 60000 rpm
b : 0.1% in H_2O, 25°, 45 min, 60000 rpm
Hitachi model UCA-1A ultracentrifuge

The polysaccharide gave one spot on glass-fiber paper electrophoresis in alkaline borate buffer, and it was found that the polysaccharide is homogeneous by the ultracentrifugal

1) Part VII: M. Tomoda, S. Nakatsuka and N. Satoh, *Chem. Pharm. Bull.* (Tokyo), **21**, 2511 (1973).
2) Location: *1-5-30, Shibakôen, Minato-ku, Tokyo, 105, Japan.*
3) T. Ohtsuki, *Acta Phytochim.*, **10**, 29 (1937).

analysis (Fig. 1) and shows a characteristic sharp sedimentation pattern (Fig. 1-a) of high molecular weight viscous substances.[4-6]

The polysaccharide showed a negative specific rotation ($[\alpha]_D^{23}$ $-31.6°$ in H_2O, $c=0.9$). Its solution in water gave the intrinsic viscosity value of 8.10 at 31°, while the mucilage obtained by cold water extraction gave the value of 4.75. As the component sugars of it, mannose and glucose were identified by means of cellulose thin-layer chromatography (TLC) of the hydrolysate and gas-liquid chromatography (GLC) of trimethylsilyl derivative of the methanolysate. Quantitative determination of them showed that the molar ratio of mannose: glucose is 3: 1. Thus the pure mucous polysaccharide obtained by us has different properties from those described in the former report,[3] and the name "Bletilla-glucomannan" is proposed for the polysaccharide. The measurement of osmotic pressure gave the value of 182000 as the molecular weight of Bletilla-glucomannan.

As the result of periodate oxidation, 0.98 mole of periodate per one mole of component anhydro sugar unit of the polysaccharide was consumed with 0.17 mole of formic acid liberation. The periodate-oxidized polysaccharide was treated with sodium borohydride,[7] and the reduction product was methanolyzed. Analysis of trimethylsilyl derivative of the methanolysate by GLC revealed the presences of erythritol and mannose as the main products and showed that the yields of erythritol and mannose were 39.0% and 15.1%.

From the value of formic acid liberation after periodate oxidation, it is conceivable that Bletilla-glucomannan contains six aldohexose units per one end group on the average. And on the basis of the yield of mannose by Smith degradation, it is probable that a part of mannose residues occupies branching positions. The formation of a large quantity of erythritol by Smith degradation and the value of periodate consumption suggest that the straight chain parts in Bletilla-glucomannan are composed of 1→4 linked aldohexopyranose residues. These presumptions were supported by the results of partial acid hydrolysis, and the presence of any chain composed of aldohexoses having 1→3 or 1→6 glycosidic linkages was not found.

Bletilla-glucomannan was hydrolyzed with 0.5N sulfuric acid at 90° for 2.5 hr, and the product was fractionated by active charcoal column chromatography. The fractions were applied to paper partition chromatography (PPC), and several oligosaccharides were obtained. From among them three disaccharides (I, II and III), a trisaccharide (IV) and a tetrasaccharide (V) were identified.

Analysis of trimethylsilyl derivatives of their methanolysates by GLC, methylation studies and the comparison by TLC and GLC with authentic samples showed that I is O-β-D-mannopyranosyl-(1→4)-D-mannopyranose, II is O-β-D-mannopyranosyl-(1→4)-D-glucopyranose, III is O-β-D-glucopyranosyl-(1→4)-D-mannopyranose, IV is O-β-D-mannopyranosyl-(1→4)-O-β-D-mannopyranosyl-(1→4)-D-mannopyranose, and V is O-β-D-mannopyranosyl-(1→4)-O-β-D-mannopyranosyl-(1→4)-O-β-D-mannopyranosyl-(1→4)-D-mannopyranose.

These results gave the evidence that most of the D-mannopyranose and D-glucopyranose residues in Bletilla-glucomannan are combined one another by β-1→4 glycosidic linkage. 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, and specific rotation was measured with JASCO model

4) Y. Kawade, "Seibutsu Butsurikagaku Jikkenhô" ed. by I. Watanabe and T. Shimanouchi, Baifûkan Publ. Co., Tokyo, 1962, p. 27.
5) S. Inokawa, *Bull. Chem. Soc. Japan*, **33**, 1476 (1960).
6) T. Satoh, J. Mizuguchi, S. Suzuki and M. Tokura, *Nippon Kagaku Zasshi*, **88**, 216 (1967).
7) M. Abdel-Akher, J.K. Hamilton, R. Montgomery and F. Smith, *J. Am. Chem. Soc.*, **74**, 4970 (1952).

DIP-SL automatic polarimeter. GLC was carried out by the use of Hitachi model 063 gas chromatograph equipped with hydrogen flame ion detector.

Isolation of Polysaccharide——The material was obtained in July of 1972 from the plants cultivated in Saitama prefecture. The fresh tubers (50 g), which contain 82.7% of water, were crushed, then extracted with hot methanol (200 ml) for 30 min. After suction filtration, the extraction was similarly repeated. The extracts were combined, concentrated and lyophilized. Yellow powder (1.82 g) was obtained. After extraction with methanol, the residue was extracted with hot water (200 ml) for 30 min twice. After suction filtration, the extracts were combined and poured into two volumes of methanol, then filtered. The filtrate was concentrated and lyophilized. Pale yellow powder (0.1 g) was obtained. The precipitate was treated with absolute methanol, then dried *in vacuo*. White powder (2.07 g) was obtained. This crude mucilage was dissolved in water and applied to a column (4×80 cm) of DEAE-cellulose (Brown Co.). DEAE-cellulose was used as carbonate form by previous successive treatments with 0.5N sodium hydroxide, water, 1M ammonium carbonate and water. The column was eluted with water, and fractions of 20 ml were collected and analyzed by phenol-sulfuric acid method.[8] The eluates obtained from tubes 31 to 60 were combined, concentrated and lyophilized. Bletilla-glucomannan (1.09 g) was obtained as white powder.

On the other hand, the crushed fresh tubers (50 g) were extracted with water (200 ml) under stirring at room temperature for 1 hr twice. After suction filtration, the extracts were combined and poured into two volumes of methanol, then filtered. The precipitate was treated with absolute methanol and dried (Yield, 1.87 g), then dissolved in water and applied to a column of DEAE-cellulose (carbonate form) as described above. After elution with water, a mucilage (0.45 g) was obtained by lyophilization.

Glass-Fiber Paper Electrophoresis——Electrophoresis was carried out with Whatman GF 83 glass-fiber and alkaline borate buffer of pH 12 in the same manner as a preceding report[9] of this series. The condition of 380 volt for 1 hr was used. Bletilla-glucomannan gave one spot at a distance of 8.7 cm from the origin toward the cathod. Distance moved by standard glucose was 10.6 cm.

Qualitative Analyses of Component Sugars——The sample was hydrolyzed with 2N sulfuric acid in a sealed tube at 100° for 8 hr followed by neutralization with barium carbonate. The hydrolysate was applied to TLC using Avicel SF cellulose and the following two solvent systems: A, AcOEt: pyridine: AcOH: H_2O (5: 5: 1: 3); B, C_6H_5OH: 1% NH_4OH (2: 1). Component sugars were revealed with silver nitrate reagent,[10] benzidine reagent[11] and naphthoresorcinol-phosphoric acid reagent.[12]

On the other hand, the sample was methanolyzed with 4% methanolic HCl in a sealed tube at 75° for 16 hr. After removal of HCl by the repeated addition and evaporation of methanol, the methanolysate was trimethylsilylated[13] and applied to GLC.

GLC: condition A, column, 3% SE 52 on Chromosorb W (80 to 100 mesh) (0.3 cm×2 m long spiral stainless steel); programmed column temperature, increase in 3° per min from 130 to 280°; carrier gas, N_2 (20 ml per min); condition B, column, 2% OV 17 on Chromosorb W (80 to 100 mesh) (0.3 cm×2 m long spiral stainless steel); programmed column temperature, increase in 3° per min from 120 to 280°; carrier gas is the same as condition A.

Table I shows *Rf* values in TLC and retention times in GLC of components.

Table I. *Rf* Values and Retention Times of Component Sugars

	Cellulose TLC (*Rf*)		GLC (t_R)	
	Solvent A	Solvent B	Condition A	Condition B
Hydrolysate	0.54, 0.50	0.39, 0.33		
Methanolysate			16.0, 19.1	15.6, 19.6
Mannose	0.54	0.39		
Glucose	0.50	0.33		
Methylmannoside			16.0	15.6
Methylglucoside			19.1	19.6
Myoinositol (internal standard)				22.8

Determination of Component Sugars——The sample was hydrolyzed with 2N sulfuric acid at 100° for 8 hr, then the hydrolysate was methanolyzed with 4% methanolic HCl at 75° for 16 hr as described above.

8) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
9) M. Tomoda, Y. Yoshida, H. Tanaka and M. Uno, *Chem. Pharm. Bull.* (Tokyo), **19**, 2173 (1971).
10) W.E. Trevelyan, D.P. Procter and J.S. Harrison, *Nature*, **166**, 444 (1950).
11) J.S.D. Bacon and J. Edelman, *Biochem. J.*, **48**, 114 (1951).
12) V. Prey, H. Berbalk and M. Kausz, *Mikrochim. Acta*, **1961**, 968.
13) C.C. Sweeley, R. Bentley, M. Makita and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).

The methanolysate was trimethylsilylated and applied to GLC under condition B. Myoinositol was used as an internal standard. The result revealed that the sample was composed of 74.6% of mannose and 24.4% of glucose.

Measurement of Osmotic Pressure——This was carried out by the use of Knauer Electronic Membrane Osmometer in the same manner as described in a former report[14] of this series.

Periodate Oxidation and Smith Degradation——The sample (10 mg) was oxidized with 0.05M sodium metaperiodate (5 ml) at 22° in a dark place. The periodate consumption was measured by a spectrophotometric method[15] and an arsenite method.[16] The oxidation was completed after two weeks, then the half of the solution was used for the measurement of formic acid liberation by a titration with 0.01N sodium hydroxide after addition of one drop of ethyleneglycol. The residuary one fifth of the reaction mixture was reduced with sodium borohydride (10 mg) at 5° for 16 hr, then acetic acid was added up to pH 5. The solution was treated with saturated aqueous lead acetate (1 ml) at 4° and the precipitate was filtered off. The filtrate was passed through a column (0.8×10 cm) of Dowex 50W-X8 (H^+) and the effluent was evaporated to dryness. The residue was treated with repeating addition of ethanol followed by evaporation.

Analysis of Smith Degradation Products——The reduction product was methanolyzed with 4% methanolic HCl in a sealed tube at 70° for 16 hr. After removal of HCl, the methanolysate was trimethylsilylated and applied to GLC. For the determination of mannose, GLC was carried out under condition B and myoinositol was used as an internal standard. For the determination of erythritol, trimethylolpropane was used as internal standard and GLC was carried out under condition C: column, 5% SE 30 on Chromosorb G (80 to 100 mesh) (0.3 cm×2 m long spiral stainless steel); programmed column temperature increase in 5° per min from 60 to 260°; carrier gas, N_2 (30 ml per min). Table II shows retention times of trimethylsilyl derivatives of Smith degradation products on GLC.

Table II. Retention Times of Smith Degradation Products

	Condition B	Condition C
Erythritol	6.6	26.0
Methylmannoside	15.6	32.4
Trimethylolpropane (internal standard)	4.1	23.0
Myoinositol (internal standard)	22.8	39.0

Partial Acid Hydrolysis——The polysaccharide (1 g) was dissolved in 0.5N sulfuric acid (200 ml) and heated under reflux at 90° for 2.5 hr. After neutralization with barium carbonate, the filtrate and washings were combined and concentrated, then applied to a column (2×15 cm) of active charcoal (for chromatographic use, Wako-Junyaku Co.). The charcoal was treated before use with hot 15% acetic acid followed by washing with hot water. The column was eluted successively with water (250 ml), 5% ethanol (400 ml), 10% ethanol (300 ml), 15% ethanol (300 ml) and 20% ethanol (300 ml). Fractions were collected at 50 ml and carbohydrates in eluates were measured by phenol-sulfuric acid method. The eluates obtained from the column were divided into six groups: Frac. 1, tubes 1 to 5; Frac. 2, tubes 6 to 9; Frac. 3, tubes 10 to 13; Frac. 4, tubes

Table III. *Rf* Values of Partial Hydrolysates and Retention Times of Their Trimethylsilyl Derivatives

	Cellulose TLC (*Rf*)		GLC (t_R)	
	Solvent A	Solvent C[a]	Condition A	Condition B'[b]
Disaccharide I	0.42	0.30	41.8, 43.7	36.6, 38.5
Disaccharide II	0.33	0.23	42.2, 43.6	37.9, 39.1
Disaccharide III	0.53	0.38	39.9, 42.1	35.0, 36.9
Trisaccharide IV	0.22	0.16	62.1	53.4, 55.6
Tetrasaccharide V	0.09	0.05	—	—

a) solvent C, BuOH:pyridine:H_2O (6:4:3)
b) condition B', 2% OV 17 on Chromosorb W, from 130° to 280° (3°/min)

14) M. Tomoda and S. Nakatsuka, *Chem. Pharm. Bull.* (Tokyo), **20**, 2491 (1972).
15) *a*) J.S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954); *b*) G.O. Aspinall and R.J. Ferrier, *Chem. Ind.*, **1957**, 1216.
16) P. Fleury and J. Lange, *J. Pharm. Chim.*, **17**, 107 (1933).

14 to 19; Frac. 5, tubes 20 to 25; Frac. 6, tubes 26 to 31. Fractions 2 to 6 were respectively applied to PPC by ascending method using Toyo-Roshi No. 50. Solvent A in Table I was used for Frac. 2 and I was obtained from a part showing *Rf* value of 0.35 by means of the extraction with water, and II was obtained from a part showing *Rf* value of 0.28. Similarly, the same solvent was used for Frac. 3 and IV was obtained from a part showing *Rf* value of 0.18. In the case of Frac. 4, III was obtained from a part showing *Rf* value of 0.55, and V was obtained from a part showing *Rf* value of 0.14 with the same solvent. Each oligosaccharide fraction obtained by PPC was separately applied to a column (1.6×40 cm) of Sephadex G-15 and carbohydrate eluted with water was lyophilized. Yields, 45.3 mg in I; 2.8 mg in II; 24.1 mg in III; 23.8 mg in IV; 6.6 mg in V.

TLC and GLC of Partial Acid Hydrolysates——These were carried out as described in the preceding paper[1] of this series. Table III gives *Rf* values of partial acid hydrolysates on TLC and retention times of their trimethylsilyl derivatives on GLC in several conditions.

Methylation and Analysis of Methylation Products——Methylation was performed with sodium hydride and methyl iodide in dimethyl sulfoxide,[17] then methanolyzed and analyzed by GLC using columns packed with 15% Polybutane 1,4-diol succinate on Chromosorb W and with 5% Neopentylglycol succinate on Chromosorb G in the same manners as described in the preceding paper.[1] Table IV shows relative retention times of the methanolysates to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside in the two conditions.

Table IV. Relative Retention Times[a] of Methylation Products

	Condition D[b]	Condition E[c]
Methanolysate of methylated I	1.33, 1.86, 3.62, 4.24	1.42, 2.23, 3.39, 4.53
Methanolysate of methylated II	1.33, 1.86, 3.67, 4.07	1.42, 2.23, 3.36, 4.28
Methanolysate of methylated III	1.00, 1.37, 3.62, 4.24	1.00, 1.43, 3.38, 4.52
Methanolysate of methylated IV	1.33, 1.86, 3.62, 4.24	1.42, 2.23, 3.39, 4.53
Methanolysate of methylated V	1.33, 1.86, 3.62, 4.24	1.42, 2.23, 3.39, 4.53
Methyl 2,3,4,6-tetra-O-methyl-D-glucoside	1.00, 1.37	1.00, 1.43
Methyl 2,3,6-tri-O-methyl-D-glucoside	3.67, 4.07	3.36, 4.28
Methyl 2,3,4,6-tetra-O-methyl-D-mannoside	1.33, 1.86	1.42, 2.23
Methyl 2,3,6-tri-O-methyl-D-mannoside	3.62, 4.24	3.39, 4.53

a) methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside=1.00
b) condition D, 15% poly-butane 1,4-diol succinate on Chromosorb W at 175°
c) condition E, 5% neopentylglycol succinate on Chromosorb G at 150°

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

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