[Chem. Pharm. Bull. 29(8)2277—2282(1981)]

Plant Mucilages. XXVIII.[1] Isolation and Characterization of a Mucilage, "Althaea-mucilage OL," from the Leaves of *Althaea officinalis*

Masashi Tomoda,* Noriko Shimizu (née Satoh), Hiromi Suzuki, and Tomoko Takasu

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

(Received February 12, 1981)

A representative mucilage, named Althaea-mucilage OL, has been isolated from the leaves of *Althaea officinalis* L. 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 49.0. It was mainly composed of partially acetylated acidic polysaccharide, and its molecular weight was estimated to be about 1800000. The polysaccharide was composed of L-rhamnose: D-galacturonic acid: D-glucuronic acid in the molar ratio of 1.5: 1.1: 1.0. Analysis of component sugars, together with reduction and methylation, and partial degradation studies made it possible to deduce the structural features of the polysaccharide moiety in the mucilage.

Keywords——*Althaea officinalis*; mucilage from leaves; Althaea-mucilage OL; intrinsic viscosity; molecular weight; partially acetylated acidic polysaccharide; analysis of components; reduction and methylation analysis; partial acid hydrolysis; structural features of polysaccharide

In the previous papers of this series,[2,3] the isolation and structural features of a representative mucilage, named Althaea-mucilage O, from the roots of *Althaea officinalis* L. have been reported from this laboratory. The roots of this plant have been used in a well-known crude drug "Althaeae Radix" as an emollient, demulcent, and cough medicine. In addition, the leaves of this plant have been used as a crude drug "Althaeae Folium" for the same purposes. The extract with water from the leaves of this plant contains many mucilages, but no structural study on the mucilages has been reported so far. In the present study we obtained a new representative mucilage from the leaves of *Althaea officinalis*. Its properties and main structural features are described in the present paper.

The fresh leaves were homogenized and extracted with cold water. The crude mucilage was precipitated from the extract by addition of ethanol. The solution of the crude mucilage

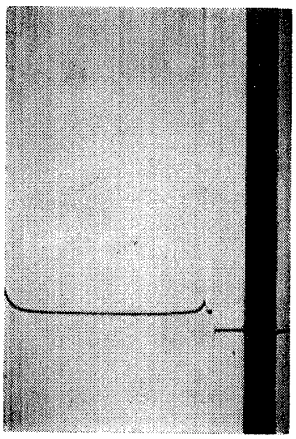

Fig. 1. Ultracentrifugal Pattern of Althaea-mucilage OL

(0.2% in H_2O, 20 °C, 36 min, 60000 rpm, Hitachi UCA-1A ultracentrifuge)

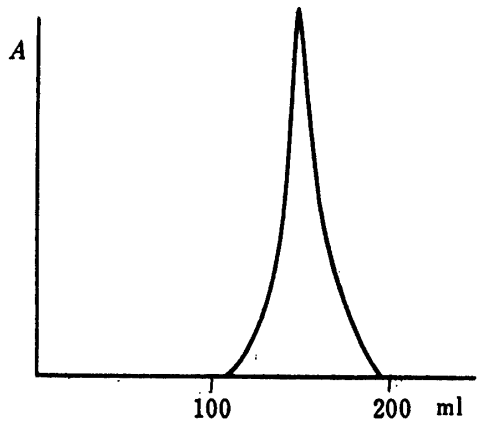

Fig. 2. Chromatogram of Althaea-mucilage OL on Sepharose 4B

was applied to a column of diethylaminoethyl (DEAE)-Sephadex A-25 (carbonate form). After elution with 0.2 M ammonium carbonate, a mucilage was obtained from the eluate with 0.5 M ammonium carbonate solution. The mucilage was homogeneous as determined by ultracentrifugal analysis (Fig. 1), and gave a single spot on cellulose acetate membrane electrophoresis in both a pyridine-acetic acid buffer and an alkaline borate buffer. Furthermore, it gave a single peak on gel chromatography with Sepharose 4B (Fig. 2).

The mucilage showed a positive specific rotation ($[\alpha]_D^{18}$ +61.6° in 0.1% NH_4OH, c=0.1), and its solution in water gave the high intrinsic viscosity value of 49.0 at 30°C. The relative viscosity of the solution of the pure mucilage was about 9.6 times that of the crude mucilage. In view of this result and the yield, it is reasonable to assume that the pure mucilage is the representative mucous substance in the water extract from the leaves. Gel chromatography gave a value of approximately 1800000 for the molecular weight. The name "Althaea-mucilage OL" is proposed for this substance.

As component sugars of the mucilage, L-rhamnose, D-galacturonic acid, and D-glucuronic acid were identified by cellulose thin-layer chromatography (TLC) of the hydrolysate. These sugars were isolated by preparative paper partition chromatography (PPC) and proved to have the configurations given above.

The carboxyl groups of hexuronic acid residues in the mucilage were reacted with a carbodiimide reagent, then reduced with sodium borohydride to the corresponding neutral sugar units.[4] Quantitative determination showed that the mucilage contained 34.9% rhamnose, 30.6% galacturonic acid, and 27.7% glucuronic acid, and that their molar ratio was 1.5: 1.1: 1.0. As shown in Fig. 3, the infrared (IR) spectrum has absorption bands at 1250 and 1720 cm^{-1}, suggesting the presence of ester linkages. When the acid hydrolysate was analyzed by gas-liquid chromatography (GLC),[5] it gave a single peak, with a retention time equal to that of acetic acid. The acetyl content of the mucilage was determined to be 1.0%. The determination of protein content was carried out by the method of Lowry *et al.*,[6] and a value of 3.3% was obtained. No compound other than carbohydrates and amino acids was detected in the hydrolysate of the mucilage.

The methylation of the carboxyl-reduced mucilage was performed with the methylsulfinyl anion 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] 3,4-di-*O*-methyl-L-rhamnopyranose, 2,3,4,6-tetra-*O*-methyl-D-glucopyranose, 2,3,6-tri-*O*-methyl-

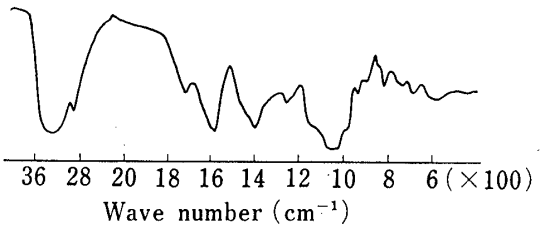

Fig. 3. IR Spectrum of Althaea-mucilage OL

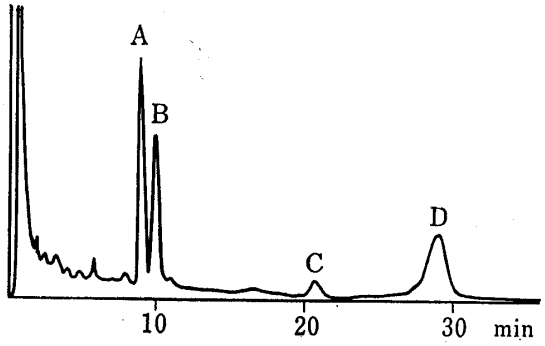

Fig. 4. Gas-Liquid Chromatogram of *O*-Methylated Alditol Acetates obtained from the Methylated Carboxyl-reduced Mucilage

Peak A: 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-L-rhaminitol.
Peak B: 1,5-di-*O*-acetyl-2,3,4-6,tetra-*O*-methyl-D-glucitol.
Peak C: 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol.
Peak D: 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-D-galactitol.

D-galactopyranose, and 2,6-di-*O*-methyl-D-galactopyranose were identified in a molar ratio of 1.5: 1.0: 0.1: 1.0 (Fig. 4).

The mucilage was hydrolyzed with 1N sulfuric acid for 2 h, then neutralized and applied to a column of Dowex 50W (H^+). The eluate with water was applied to a column of 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 its chromatographic and electrophoretic properties and the values of specific rotation with those of authentic samples,[3] I to V were identified as the following five oligosaccharides (Chart 1).

The combined yields of I, II, III, IV, and V accounted for over 72% of the total mono- and oligosaccharide fractions obtained from the partial hydrolysate of the mucilage. Consequently, it can be concluded that these oligosaccharides do represent the structural features of the bulk of Althaea-mucilage OL.

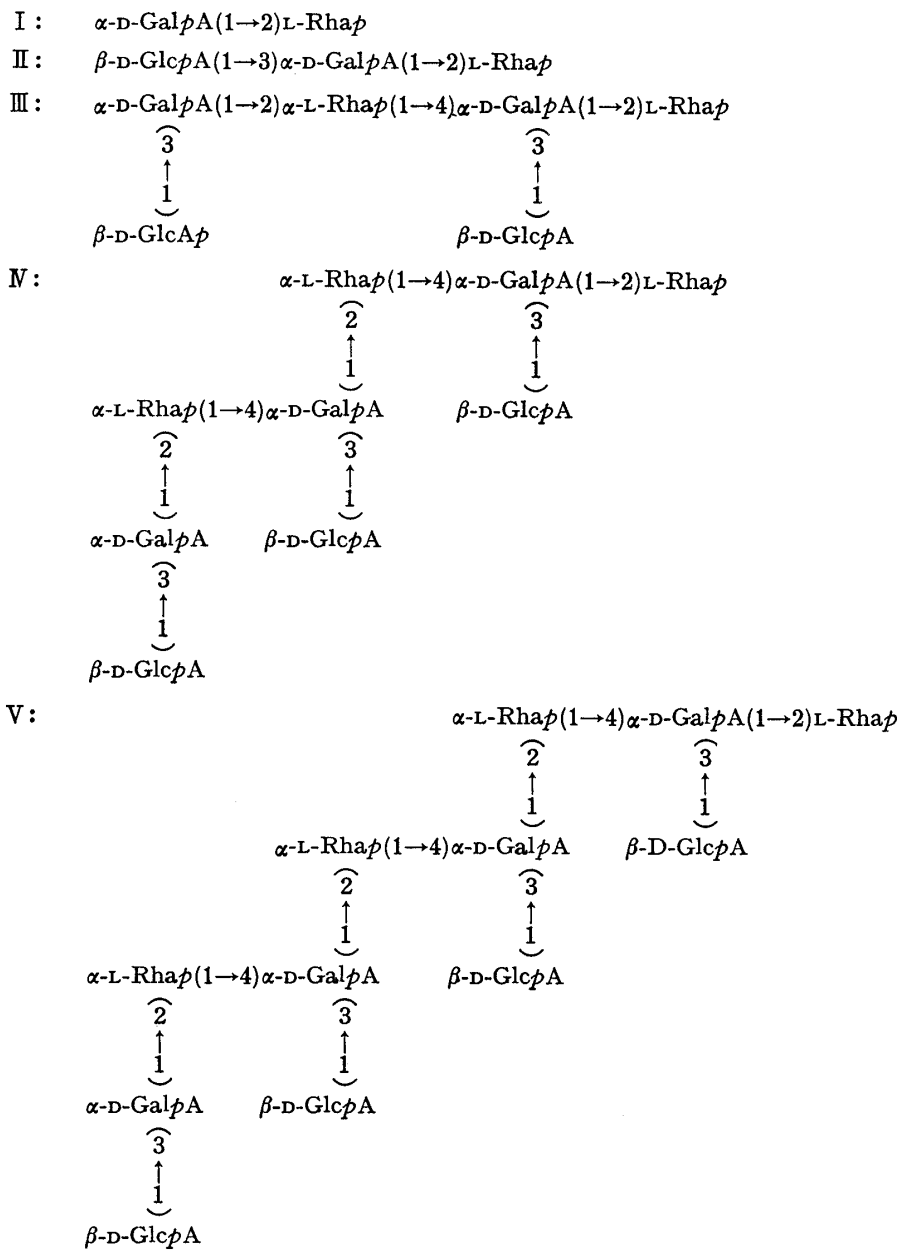

Chart 1. Structural Features of the Oligosaccharides

Based on these results, it can be concluded that the polysaccharide moiety of the mucilage is mainly composed of (1→4)-[*O*-β-(D-glucopyranosyluronic acid)-(1→3)]-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranosyl units. In view of the results of methylation analysis, however, we concluded that one-eleventh of the D-galacturonic acid residues has no branch, and that eleven-fifteenths of the L-rhamnose residues link to position 4 of D-galacturonic acid units but four-fifteenths of the L-rhamnose residues link to each other by 1→2 glycosidic linkages.

The component unit having the repeating 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[3] and the leaves of *Althaea officinalis*. On the other hand, the lack of galactosyl galactose branches at position 4 of the L-rhamnose residues and the partial lack of glucuronic acid branches at position 3 of the D-galacturonic acid residues in the main chain were not found in Althaea-mucilage O. These are common characteristics of structures in both Althaea-mucilage OL and Abelmoschus-mucilage G.[9] In addition, the presence of rhamnosyl rhamnose units in the main chain in Althaea-mucilage OL is unique, compared with the other four mucilages[3,9–11] which have been isolated from Malvacceae plants and reported in the previous papers in this series. 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 rotation was measured with a JASCO DIP-SL automatic polarimeter. Viscosity was determined with an Ubbelohde-type viscosimeter. IR spectra were recorded on a JASCO IRA-2 infrared spectrophotometer. GLC was carried out on a Hitachi 063 gas chromatograph equipped with a hydrogen ionization detector. GLC-MS was performed with a JEOL JGC-20K gas chromatograph and a JEOL JMS-D100 mass spectrometer.

Isolation of the Mucilage——The material was obtained at the beginning of September 1979 from plants cultivated in Saitama prefecture. The fresh leaves (400 g), which contained 80.9% water, were homogenized and extracted with water (4000 ml) under stirring for 1.5 h at room temperature. After centrifugation, the extraction was repeated with water (3000 ml). The extracts were combined and poured into two volumes of ethanol, then centrifuged. The precipitate was lyophilized, and a light brown powder (4.83 g) was obtained. A part of the crude mucilage (2.04 g) was dissolved in water and applied to a column (5.0×40 cm) of DEAE-Sephadex A-25 (Pharmacia Co.). DEAE-Sephadex was pretreated as described in a previous report.[2] After elution with 0.2 M ammonium carbonate (1500 ml), the column was eluted with 0.5 M ammonium carbonate. Fractions of 50 ml were collected and analyzed by the phenol-sulfuric acid method.[12] The eluates obtained from tubes 12 to 34 were combined, concentrated and poured into ethanol. The precipitate was dissolved in water, then dialyzed against running water overnight and lyophilized. Althaea-mucilage OL (0.22 g) was obtained as a white powder.

Cellulose Acetate Membrane Electrophoresis——Electrophoresis was carried out as described in a previous report[11] of this series. The sample gave a single spot at distances of 5.3 cm in the pyridine-acetic acid buffer and 4.9 cm in the alkaline borate buffer from the origin towards the anode.

Gel Chromatography——The sample (3 mg) was dissolved in 0.1 M Tris-HCl buffer (pH 7.0) and applied to a column (2.6×92 cm) of Sepharose 4B. Elution was carried out by the ascending method with the same buffer. Fractions of 5 ml were collected and analyzed by the phenol-sulfuric acid method.[12] The calibration curve for the measurement of molecular weight was obtained by the use of standard dextrans as described in a previous report[11] of this series.

Hydrolysis, Isolation and TLC of Component Sugars——These were carried out as described in a previous report[2] 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.[10] Rhamnose was also determined by the thioglycolic acid method,[13] and hexuronic acids in the original mucilage were estimated by a modification of the carbazole method.[14]

Determination of O-Acetyl Groups——The IR spectrum of the mucilage showed ester absorption bands. IR ν_{max}^{KBr} cm^{-1}: 1250, 1720 (ester). 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[5] of this series.

Reduction of the Mucilage——The mucilage (60 mg) was dissolved in water (30 ml), then 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (0.6 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 (6 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 running water overnight, then the non-dialyzable fraction was concentrated to 30 ml. The product was reduced twice more under the same conditions. The final non-dialyzable fraction was applied to a column (5 × 82 cm) of Sephadex G-15. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 28 to 32 were combined and lyophilized. Yield, 44 mg.

Methylation——The carboxyl-reduced mucilage (10 mg) was dissolved in dimethyl sulfoxide (2 ml). The solution (4 ml) of methylsulfinyl anion was prepared as described in a previous report[11] and 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 running water for two days. After addition of water (30 ml), the non-dialyzable fraction was extracted five times with chloroform (30 ml each). The combined extract was washed five times with water (150 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, 1 ml), then applied to a column (2 × 10 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 15 to 20 were combined and concentrated to dryness. The final product (11 mg) was a yellow powder, and its IR spectrum showed no absorption band of hydroxyl groups.

Analysis of the Methylated Product——The product was hydrolyzed with dilute sulfuric acid in acetic acid under the same conditions as in a previous report.[11] After neutralization with Dowex 2 (OH^-), the hydrolysate was reduced with sodium borohydride and then acetylated with acetic anhydride-pyridine mixture. GLC and GLC–MS of the partially methylated alditol acetates obtained were carried out under the same conditions as in a previous report.[11] 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 I.

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

	Relative retention times[a]	Main fragments (*m/e*)
1,2,5-Ac-3,4-Me-L-Rhamnitol	0.88	43, 89, 129, 131, 189
1,5-Ac-2,3,4,6-Me-D-Glucitol	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,4,5-Ac-2,3,6-Me-D-Galactitol	2.04	43, 45, 87, 99, 101, 113, 117, 233
1,3,4,5-Ac-2,6-Me-D-Galactitol	2.90	43, 45, 87, 117, 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.*, 1,2,5-Ac-3,4-Me-=1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-).

Partial Hydrolysis and Isolation of Oligosaccharides——The mucilage (95.8 mg) was dissolved in 1 N sulfuric acid (10 ml) and heated under reflux at 100°C for 2 h. The solution was neutralized with barium carbonate, and after filtration, the filtrate was passed through a column (0.7 × 5 cm) of Dowex 50W-X8 (H^+). The eluate with water was concentrated and applied to a column (1 × 7.5 cm) of DEAE-Sephadex A-25 (formate form). The column was eluted successively with water (25 ml), 0.1 M formic acid (65 ml), 0.2 M formic acid (35 ml), 0.3 M formic acid (100 ml), 0.4 M formic acid (25 ml), 0.5 M formic acid (30 ml), 0.6 M formic acid (85 ml), 0.8 M formic acid (45 ml), 1 M formic acid (70 ml), and 1.5 M formic acid (90 ml). Fractions of 5 ml were collected and analyzed by the phenol-sulfuric acid method. The eluates obtained from the column were divided into eleven groups: Frac. 1, tubes 1 to 5; Frac. 2, tubes 8 to 12; Frac. 3, tubes 13 to 19; Frac. 4, tubes 20 to 25; Frac. 5, tubes 28 to 36; Frac. 6, tubes 37 to 46; Frac. 7, tubes 47 to 55; Frac. 8, tubes 59 to 70; Frac. 9, tubes 75 to 81; Frac. 10, tubes 84 to 92; Frac. 11, tubes 98 to 110. The yields were 8.2 mg in Frac. 1, 4.3 mg in Frac. 2, 2.4 mg in Frac. 3, 1.5 mg in Frac. 4, 11.4 mg in Frac. 5, 2.5 mg in Frac. 6, 0.9 mg in Frac. 7, 11.0 mg in Frac. 8, 2.0 mg in Frac. 9, 8.2 mg in Frac. 10, and 7.8 mg in Frac. 11. I, II, III, IV, and V were obtained from Fracs. 2, 5, 8, 10, and 11, respectively, after rechromatography under the same conditions. The yield of I was 3.7 mg. In the cases of II, III, IV, and V, almost quantitative yields were obtained in the rechromatography.

Analysis of the Oligosaccharides——Analysis of component sugars was carried out as described above. PPC by the descending method and paper electrophoresis with an alkaline borate buffer were carried out as described in a previous report.[3] I and II were converted into the corresponding alditols of carboxyl-reduced products, then acetylated and applied to GLC in the manner described in a previous report.[10] The results are listed in Table II.

TABLE II. Specific Rotations, Sugar Compositions, R_{Rha} and M_{GalA} Values of Oligosaccharides, and Retention Times of Alditol Acetates of the Carboxyl-reduced Oligosaccharides

Oligosaccharide	$[\alpha]_D^{18}$ in H_2O	Sugar composition	PPC (R_{Rha})	Paper electrophoresis (M_{GalA})	GLC (t_R) (min)
I	+93.9°	GalA: Rha=1: 1	0.79	0.73	18.3
II	+86.4°	GlcA: GalA: Rha=1: 1: 1	0.66	1.10	29.3
III	+82.3°	GlcA: GalA: Rha=1: 1: 1	0.39	1.32	—
IV	+78.4°	GlcA: GalA: Rha=1: 1: 1	0.22	1.38	—
V	+72.5°	GlcA: GalA: Rha=1: 1: 1	0.13	1.42	—

Abbreviations: GalA=galacturonic acid, GlcA=glucuronic acid; Rha=rhamnose.

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

References and Notes

1) Part XXVII: M. Tomoda, M. Yokoi, A. Torigoe, and K. Maru, *Chem. Pharm. Bull.*, **28**, 3251 (1980).
2) M. Tomoda, S. Kaneko, M. Ebashi, and T. Nagakura, *Chem. Pharm. Bull.*, **25**, 1357 (1977).
3) M. Tomoda, N. Satoh, and K. Shimada, *Chem. Pharm. Bull.*, **28**, 824 (1980).
4) R.L. Taylor and H.E. Conrad, *Biochemistry*, **11**, 1383 (1972).
5) M. Tomoda, N. Satoh, and C. Ohmori, *Chem. Pharm. Bull.*, **26**, 2768 (1978).
6) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
7) S. Hakomori, *J. Biochem.*, **55**, 205 (1964).
8) H. Björndal, B. Lindberg, and S. Svensson, *Carbohydr. Res.*, **5**, 433 (1967).
9) M. Tomoda, M. Arai, Y. Suzuki, M. Ohmura, and H. Takayama, *Chem. Pharm. Bull.*, **28**, 1546 (1980).
10) M. Tomoda, Y. Suzuki, and N. Satoh, *Chem. Pharm. Bull.*, **27**, 1651 (1979).
11) M. Tomoda, K. Shimada, Y. Saito, and M. Sugi, *Chem. Pharm. Bull.*, **28**, 2933 (1980).
12) J.E. Hodge and B.T. Hofreiter, "Methods in Carbohydrate Chemistry," Vol. I, ed. by R.L. Whistler and M.L. Wolfrom, Academic Press, New York and London, 1962, p. 388.
13) M.N. Gibbons, *Analyst* (London), **80**, 268 (1955).
14) T. Bitter and H.M. Muir, *Anal. Biochem.*, **4**, 330 (1962).