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Plant Mucilages. IV.[1] Main Structural Features of the Mucous Polysaccharide isolated from *Digenea simplex*

Masashi Tomoda, Satomi Nakatsuka and Eiko Minami

Kyôritsu College of Pharmacy[2]

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The mucilage of *Digenea simplex* Agardh has been isolated and subjected to partial methanolysis. Agarobiose dimethylacetal was obtained in high yield in addition to 3,6-anhydro-L-galactose dimethylacetal, methyl D-galactoside, methyl D-xyloside and the other oligosaccharides. By acetylation of the mucilage followed by extraction with chloroform and alkali treatment, a pure polysaccharide was obtained. Partial methanolysis and methylation studies provided the evidence that the polysaccharide is mainly composed of alternately repeated units of 1,3-linked D-galactose and 1,4-linked 3,6-anhydro-L-galactose, but the presences of 1,4-linked D-xylose and sulfate ester were also found in part of it.

As the constituents of *Digenea simplex* Agardh, α-kainic acid,[3] succinic acid,[3] sodium α-D-mannosido-D-glycerate,[4a,b] α-allokainic acid,[5] fifteen amino acids and several volatile organic acids[6] have been reported until the present time. Although it has been known that the seaweed contains a mucilage, the structure of the polysaccharide in the seaweed was still unknown. We can see only a few reports[6,7] on the component sugars of the mucilage, and they showed the presences of D-galactose, D-xylose and a Seliwanoff's reaction positive substance.

The structural study described in this paper gives the evidence that the mucilage isolated from *Digenea simplex* belongs to agar-type polysaccharides, and this seaweed should be added to the agar-producing group in Rhodophyceae, in addition to species of *Gelidium, Gracilaria, Acanthopeltis, Ahnfeltia, Ceramium, Campylaephora, Phyllophora,* and *Pterocladia* spp.[8]

After repeated washing with water to the dried seaweed, the residue was extracted with hot water. The extract was treated with freezing and thawing method to yield grayish white fibrous flakes. The yield of the mucilage was 5.5%.

The complete methanolysis of the mucilage was accomplished by heating with 3% methanolic hydrogen chloride for thirty hours. The methanolysate was subjected to saponification with barium hydroxide followed by treatment with ion-exchange resins for the removal of sulfuric acid residue. The acidic substance adsorbed by the anion-exchange resin was eluted with excess of acid and isolated as a barium salt. The salt was proved to be barium methylsulfate derived from sulfate ester in the mucilage.

When neutral methanolysate was analyzed by thin-layer chromatography (TLC), 3,6-anhydro-L-galactose dimethylacetal, methyl D-galactopyranoside and methyl D-xylopyranoside were detected. Further, the methanolysate was converted into trimethylsilyl deriva-

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[1] Part III: M. Tomoda and M. Uno, *Chem. Pharm. Bull.* (Tokyo), **20**, 778 (1972).

[2] Location: *1-5-30, Shibakôen, Minato-ku, Tokyo.*

[3] S. Murakami, T. Takemoto and Z. Shimizu, *Yakugaku Zasshi*, **73**, 1026 (1953).

[4] *a*) K. Kawaguchi, S. Yamada and S. Miyama, *Nippon Suisangaku Kaishi*, **19**, 481 (1953); *b*) S. Murakami, T. Takemoto and Z. Shimizu, *Yakugaku Zasshi*, **73**, 1028 (1953).

[5] S. Murakami, T. Takemoto, Z. Tei, K. Daigo and N. Takagi, *Yakugaku Zasshi*, **75**, 766, 1252 (1955).

[6] Y. Takao, *Yakugaku Zasshi*, **38**, 507 (1918).

[7] H. Watanabe and T. Takano, *Yakugaku Zasshi*, **73**, 529 (1953).

[8] E. Percival and R.H. McDowell, "Chemistry and Enzymology of Marine Algal Polysaccharides," p. 127 (Academic Press, London and New York, 1967).

tive and analyzed by gas liquid chromatography (GLC). The result also showed the presences of the same three component sugar derivatives. Determination of components showed that the mucilage is consisted of 43.8% of 3,6-anhydro-L-galactose, 43.6% of D-galactose, 3.0% of D-xylose and 3.3% of sulfuric acid residue. We observed small amounts of insoluble residue after methanolysis of the mucilage. This may be cause some lowering of the measured values of component sugars.

To clarify the nature of linkage in the main part of mucilage molecule, the partial methanolysis of the material was carried out by heating with 0.5% methanolic hydrogen chloride for two hours. The partial methanolysate was treated with barium hydroxide and ion-exchange resins as described above, and the neutral part was applied to an active charcoal column chromatography. In addition to 3,6-anhydro-L-galactose dimethylacetal, methyl D-galactoside and methyl D-xyloside, agarobiose dimethylacetal was obtained in a good yield (45.3%) from the starting material. Crystalline agarobiose dimethylacetal was identified by comparison with the authentic sample prepared from agar.[9] These results gave the evidence that agarobiose represents the chief repeating unit of the mucilage molecule like the structure of agarose.[10]

The mucilage was acetylated by heating with pyridine and acetic anhydride, then the acetate was extracted with chloroform. Chloroform-soluble product was precipitated by addition of petroleum ether, and the dried precipitate was deacetylated by successive treatment with 1N ethanolic potassium hydroxide and 0.5N potassium hydroxide. After neutralization, the alkali-insoluble part was treated with freezing and thawing method to yield white fibrous mass. The product occupied the main part of the mucilage, and it was found to be a homogeneous polysaccharide by the ultracentrifugal analysis (Fig. 1).

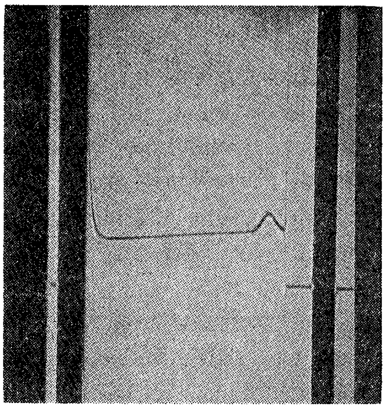

Fig. 1. Ultracentrifugal Pattern of Polysaccharide (Digenea-agarose)

0.5% dimethyl sulfoxide solution, 24°, 180 min, Hitachi model UCA-1A ultracentrifuge (60000 rpm)

Determination of components showed that the polysaccharide is consisted of 49.6% of D-galactose, 48.8% of 3,6-anhydro-L-galactose, 1.2% of D-xylose and 0.5% of sulfuric acid residue.

The polysaccharide yielded 86.9% of agarobiose dimethylacetal by partial methanolysis under the condition of heating at 70° with 0.5% methanolic hydrogen chloride.

After methylation with sodium hydride and methyl iodide in dimethyl sulfoxide,[11] methylated polysaccharide was methanolyzed and the methanolysate was analyzed by GLC and TLC. Methyl glycosides of 2,3,4,6-tetra-O-methyl-D-galactose, 2,4,6-tri-O-methyl-D-galactose, 2,3,4-tri-O-methyl-D-xylose, 2,3-di-O-methyl-D-xylose, and 2-O-methyl-3,6-anhydro-L-galactose were identified in addition to a few unknown minor products.

From the results of partial methanolysis and methylation studies, it is able to conclude that the main structure of the mucilage consists of alternately repeated units of 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose.

Thus the main part of the mucilage has agarose-type structure like agar, but 6-methyl D-galactose, D-glucuronic acid and pyruvic acid, which have been reported as components

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[9] C. Araki and S. Hirase, *Bull. Chem. Soc. Japan*, **27**, 109 (1954).
[10] C. Araki, *Bull. Chem. Soc. Japan*, **29**, 543 (1956).
[11] S. Hakomori, *J. Biochem.*, **55**, 205 (1964).
[12] *a*) S. Hirase and C. Araki, *Bull. Chem. Soc. Japan*, 34, 1048 (1961); *b*) S. Hirase, *Bull. Chem. Soc. Japan*, 30, 68 (1957).

of agar,[12] were not found. We also obtained the evidences of the presences of 1,4-linked D-xylose and sulfate ester in part of the polysaccharide. The name "Digenea-agarose" is proposed for the homogeneous polysaccharide isolated from the mucilage. Methylation study showed that the non-reducing terminal of agarose type chain of this polysaccharide is D-galactose. This is a different structural conclusion from the other normal agarose.

Experimental

All melting points are uncorrected. Specific rotations were measured by the use of JASCO model DIP-SL automatic polarimeter. GLC was carried out by the use of Hitachi model 063 gas chromatograph equipped with hydrogen flame ion detector. Solutions were evaporated at 40° or below with rotary evaporators under reduced pressure.

Isolation of Polysaccharide——The dried seaweed *Digenea simplex* (100 g, dry weight 89.7 g), collected in Okinawa, was extracted with water (4000 ml) by stirring at room temperature for 30 min, followed by suction filtration and repetition of the process twice. The residue was extracted with hot water (1500 ml) at 100° for 1 hr, followed by filtration and the process was repeated twice. The filtrates were collected and frozen solid overnight and allowed to thaw at room temperature. The remaining solid part was dissolved in hot water (1000 ml), and the filtrate was submitted to freezing and thawing method. The residue was washed with water twice, then stirred in acetone twice and dried *in vacuo*. Grayish white fibrous mass (4.93 g) was obtained.

Qualitative Analyses of Methanolysate——The mucilage (0.5 g) was heated with 3% methanolic HCl (12 ml) in a sealed tube at 70° for 30 hr. After cooling to room temperature and removal of small amounts of insoluble part by filtration, the filtrate was neutralized with silver carbonate and treated with active charcoal, followed by refiltration. The solution was evaporated to syrup *in vacuo*, then treated with 0.2N barium hydroxide (20 ml) at 60° for 2 hr. The solution was neutralized by treatment with carbon dioxide and filtered, then evaporated *in vacuo*. The methanol solution of the residue was analyzed by TLC.

TLC: plate, Wakô-gel B5; solvent, benzene: chloroform: methanol (2:2:1); detection, spraying of 2N sulfuric acid and heating at 150°.

On the other hand, the methanolysate was converted into trimethylsilyl derivatives by the method of Sweeley, *et al.*,[13] and applied to a gas chromatograph.

GLC: column, 5% SE 30 on Chromosorb G (80 to 100 mesh) (0.3 cm × 2 m long stainless steel); column temperature, 200°; carrier gas, N_2 (20 ml per min).

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

TABLE I. *Rf* Values and Retention Times of Components in the Methanolysate

	TLC (*Rf*)	GLC (t_R)
3,6-Anhydro-L-galactose dimethylacetal	0.78	11.0
Methyl D-xylopyranoside	0.69	8.3, 8.9
Methyl D-galactofuranoside	0.58	13.2
Methyl D-galactopyranoside	0.41	15.2, 17.1

Determination of Components——3,6-Anhydro-L-galactose was determined by resorcinol method.[14] D-Xylose and D-galactose were determined by GLC in the conditions described above using trimethyllopropane (t_R, 3.9) as an internal standard. Sulfuric acid residue was estimated by benzidine method[15] after hydrolysis with 25% formic acid at 100° for 24 hr.

Partial Methanolysis——The mucilage (5 g) was soaked in 0.5% methanolic HCl (50 ml) overnight at room temperature, then heated under reflux at 70° for 2 hr. After cooling, undissolved material was filtered off and washed with methanol. The filtrate and washings were combined, neutralized with silver carbonate, followed by filtration and subsequent evaporation *in vacuo*. The resulting syrup was treated with 0.2N barium hydroxide (40 ml) at 60° for 2 hr. The solution was neutralized with carbon dioxide and filtered, then concentrated to syrup. The syrup (4 g) was dissolved in water (20 ml) and the solution was applied to a column (2 × 20 cm) of Dowex 50W—X8 (H^+, 50 to 100 mesh) and a column (2 × 20 cm) of Dowex 44 (OH^-, 50 to 100 mesh) in succession, and the columns were washed with water until no Molisch reaction was shown. The effluent and washings were combined and evaporated to syrup; yield, 3.4 g.

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13) C.C. Sweeley, R. Bentley, M. Makita and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).
14) W. Yaphe, *Anal. Chem.*, **32**, 1327 (1960).
15) C.A. Antonopoulos, *Acta Chem. Scand.*, **16**, 1521 (1962).

Barium Methylsulfate——The Dowex 44 resin, which adsorbed an acidic substance, was transfered into a beaker from the column and stirred with 2N sulfuric acid (50 ml) under ice cooling. Then the resin was retransfered into a galss tube to form a column. Excess of the liquid was allowed to drain, and the column was washed with additional 2N sulfuric acid (50 ml) and water in succession until the washing became neutral. The effluents and washings were combined, neutralized with barium hydroxide, then filtered and evaporated. The residue was obtained as a colorless solid; yield, 0.160 g. No carbohydrate was detected in this sample by TLC and the determination of the ash content supported that the solid obtained above was barium methulsylfate.

Anal. Calcd. for $(CH_3SO_4)_2Ba \cdot H_2O$: $BaSO_4$, 61.8%. Found: Ash (as $BaSO_4$), 61.6%.

Chromatography of Partial Methanolysate——The syrup (3.4 g) of the partial methanolysate was dissolved in water (50 ml) and applied to the column (2×25 cm) of charcoal (for chromatographic use, Wakô-Junyaku Co., pretreated with hot 15% acetic acid,[16]) followed by successive elution with water (1500 ml), 2% ethanol (700 ml), 5% ethanol (900 ml), 7.5% ethanol (300 ml), 15% ethanol (500 ml) and 30% ethanol (400 ml). Fractions were collected at 50 ml and carbohydrates in eluates were measured by phenol-sulfuric acid method.[17] The eluates obtained from the column were divided into seven groups: Frac. 1, tubes 1 to 23; Frac. 2, tubes 24 to 30; Frac. 3, tubes 31 to 44; Frac. 4, tubes 45 to 62; Frac. 5, tubes 63 to 68; Frac. 6, tubes 69 to 78; Frac. 7, tubes 79 to 86. The yields were 1.39 g in Frac. 1, 0.12 g in Frac. 2, 0.32 g in Frac. 3, 0.29 g in Frac. 4, 0.05 g in Frac. 5, 0.04 g in Frac. 6 and 0.26 g in Frac. 7. Frac. 1 was dissolved in hot ethanol and the solution was left in a refrigerator for a week. Deposited crystals of agarobiose dimethylacetal were filtered and washed with ethanol; yield, 0.71 g. Each fraction was analyzed by TLC as described above, and agarobiose dimethylacetal (*Rf* 0.26) was also obtained from Frac. 2, 3 and 4. From the ethanol-soluble part of Frac. 1, 3,6-anhydro-L-galactose dimethylacetal, methyl D-xyloside, methyl D-galactofuranoside, methyl D-galactopyranoside and remaining agarobiose dimethylacetal were found. Frac. 5,6 and 7 were probably mixture of higher oligosaccharide derivatives, but these fractions were not studied.

Agarobiose Dimethylacetal——Pure agarobiose dimethylacetal was obtained as colorless needles by recrystallization from ethanol, mp 165—166°, $[\alpha]_D^{20}$ −29.5° (c=1.00, H_2O), $[\alpha]_D^{20}$ −37.5° (c=1.00, MeOH). It was identified by comparing with the authentic sample prepared from agar[9] of *Gelidium amansii* LAMOUROUX by mixing mp, $[\alpha]_D$, TLC and IR spectra.

Isolation of Homogeneous Polysaccharide——The mucilage (16 g) was soaked in hot water (32 ml), then pyridine (320 ml) was added slowly under agitation. After 6 hr stirring at 60°, acetic anhydride (320 ml) was dropped into the mixture and stirring was continued for 10 hr at 60°. After cooling, the reaction mixture was poured into ice water and pale brown fibrous precipitate was filtered and washed repeatedly with water. The precipitate was dried and added into pyridine (200 ml) and the mixture was stirred for 5 hr at 70°, then acetic anhydride (200 ml) was added and the reaction mixture was stirred for 15 hr at 70°. The acetylated product was obtained as described above and dried *in vacuo*. Yield, 20.6 g. The acetate was extracted with chloroform (400 ml) and centrifuged at 10000 rpm for 10 min. The insoluble matter was separated and washed with chloroform. The extract and washing were combined and poured into twofold volumes of petroleum ether under stirring. The precipitate was filtered off and dried *in vacuo*. Yields were 83.4% in chloroform-soluble part and 16.6% in chloroform-insoluble part.

Chloroform-soluble acetate (10 g) was treated with 1N ethanolic KOH (200 ml) for 24 hr at room temperature and filtered. The residue was treated with 0.5N KOH (200 ml) under stirring for 1 hr at room temperature and the procedure was repeated once more. The insoluble matter was neutralized by washing with 3% acetic acid and filtered. The residue was dissolved in hot water, and the filtrate was submitted to freezing and thawing method. The remaining polysaccharide was washed with water twice and dried *in vacuo*. White fibrous mass (4.66 g) was obtained.

Determination of Agarobiose Dimethylacetal in Partial Methanolysate——The polysaccharide (20 mg) was soaked in 0.5% methanolic HCl (2 ml) overnight at room temperature in a sealed tube, then heated at 70° for 2 hr. After cooling, methanol solution containing trehalose (4 mg) was added as an internal standard. The solution was evaporated repeatedly to remove HCl, and the residue was trimethylsilylated by the method of Sweely, *et al.*,[13] then applied to a gas chromatograph.

GLC: column, 3% SE 52 on Chromosorb W (80 to 100 mesh) (0.3×2 m long stainless steel); column temperature, 230°; carrier gas, N_2 (30 ml per min); t_R, agarobiose dimethylacetal 11.7; trhalose 16.2.

Methylation and Methanolysis——Sodium hydride (200 mg) was mixed with $(CH_3)_2SO$ (10 ml) and the mixture was stirred at 70° for 1 hr. Polysaccharide (100 mg) in $(CH_3)_2SO$ (20 ml) was added into this mixture. After 20 min stirring at 70°, CH_3I (10 ml) was added and the reaction mixture was stirred overnight at room temperature. The procedure were carried out in nitrogen atmosphere. After dilution with water, the mixture was extracted with $CHCl_3$ thrice. The extract was dried and the solvent was evaporated *in vacuo*. The residue was methylated again under the same condition. The infrared spectra of the final

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[16] M. Tomoda, S. Katô and M. Ōnuma, *Chem. Pharm. Bull.* (Tokyo), **19**, 1455 (1971).

[17] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

product had no absorption near 3400 cm^{-1}. The fully methylated product was heated with 3% methanolic HCl (10 ml) in a sealed tube at 70° for 30 hr. After cooling, the solution was treated with Amberlite IR4B (OH$^-$) to remove HCl, then evaporated *in vacuo*.

Analysis of Methanolysate——Chloroform solution of the methanolysate was applied to a gas chromatograph. Following two conditions were used; A, a column (0.3 cm × 2 m long stainless steel) packed with 15% Poly-butane 1,4-diol succinate on Chromosorb W (80 to 100 mesh) at 175° with a flow of 20 ml per min of N_2; B, a column (0.3 cm × 2 m long stainless steel) packed with 5% Neopentylglycol succinate on Chromosorb G (60 to 80 mesh) at 150° with a flow of 20 ml per min of N_2. Table II shows relative retention times of the products obtained by methanolysis to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside in the two gas chromatographic conditions. On the other hand, TLC using Wakô-gel B5 was carried out with the solvent system of benzene: acetone (4:1) and 10% sulfuric acid was used for detection at 150°. R_G values of the products to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside are also shown in Table II.

Table II. Relative Retention Times and R_G Values of Methylation Products

	GLC		TLC
	Condition A (15% BDS)	Condition B (5% NPGS)	
Methyl 2,3,4-tri-O-methyl-D-xylopyranoside	0.64	0.60	0.93
Methyl 2,3,4,6-tetra-O-methyl-D-galactopyranoside	1.61	1.74	0.86, 0.75
Methyl 2,3-di-O-methyl-D-xylopyrnoside	1.80	3.36	0.67, 0.58
Methyl 2,4,6-tri-O-methyl-D-galactopyranoside	3.50, 4.00	3.64, 4.30	0.54, 0.42
Methyl 2-O-methyl-3,6-anhydro-L-galactopyranoside	6.30	6.14	0.64

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