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Chemical Structures of Main Extracellular Polysaccharides of *Alternaria solani* and *Fusarium solani*.[1] Studies on Fungal Polysaccharides. XVIII[2]

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Major water-soluble extracellular polysaccharides of *A. solani* obtained by DEAE-cellulose column fractionation, ASP-1 and ASP-2 were heteroglycans which were composed of D-galactose, D-glucose and D-mannose. The molar ratios of ASP-1 and ASP-2 were 2.2 : 3.1 : 1.0 and 1.0 : 1.7 : 2.0, respectively. FSP-1 obtained from *F. solani* was a heteroglycan composed of D-galactose, D-glucose, D-mannose, and small amounts of glucuronic acid and rhamnose (approximate molar ratio: 10.5 : 10.6 : 7.6 : 1.8 : 1.0). Optical rotations of these glycans were $[\alpha]_D^{25}$ +66.0° (ASP-1), $[\alpha]_D^{25}$ +38.0° (ASP-2), and $[\alpha]_D^{25}$ +25° (FSP-1), respectively. The results of periodate oxidation, Smith-degradation, methylation studies, and Gas-liquid Chromatography-Mass Spectrum spectra showed that these polysaccharides have highly branched structure and 1,2-, 1,3-, and 1,6-linked glucopyranosyl (ASP-1), 1,2-linked mannopyranosyl and 1,6-linked glucopyranosyl (ASP-1), and 1,2-linked glucopyranosyl and 1,3,6-linked mannopyranosyl residues (FSP-1), and 1,2,6-linked galactofuranosyl residues are present as main linkages of each glycan. The terminal groups are glucopyranosyl and small amount of mannopyranosyl residues. Probable structures are proposed.

In the series of studies on fungal polysaccharides we have examined a relationship between taxonomy and chemical structure. In the previous reports,[4] structural features of the polysaccharides isolated from *Cladosporium herbarum* and *C. tricoides* were discussed. In the present work, extracellular polysaccharides of *Alternaria solani* and *Fusarium solani*, which belong to Imperfect fungi and are common contaminant fungi of foods, were examined. Structural elucidation of extracellular polysaccharide of these fungi has not been carried out. It would be of interest to compare the chemical structure between polysaccharides of *A. solani* and those of *F. solani*.

Methods

Isolation and Purification of Crude Extracellular Polysaccharides——The organisms used in this study, *Alternaria solani* IFO 5924 and *Fusarium solani* IFO 5232, were kindly supplied by the Institute for Fermentation, Osaka. Incubation was carried out at 25° for 14 days and the polysaccharides were extracted by the procedure described in our previous paper.[4] The crude polysaccharides so obtained were purified by DEAE-cellulose column chromatography using stepwise elution with H_2O–$NaHCO_3$–NaOH series, and the main eluates from *A. solani*, ASP, and from *F. solani*, FSP, were re-chromatographed with H_2O–$Na_2B_4O_7$–NaOH series.[4]

Analytical Procedure——Paper chromatography (PPC) was performed by the ascending method on Whatman No. 1 filter paper with the following solvent systems; (A) AcOEt–pyridine–H_2O (10 : 4 : 3), (B) AcOEt–pyridine–AcOH–H_2O (5 : 5 : 1 : 3), and (C) AcOEt–AcOH–H_2O (9 : 2 : 2). Sugars on the paper

1) A part of this work was presented at the 91st Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1971.
2) Part XVII: T. Miyazaki, T. Yadomae, T. Terui, H. Yamada, and T. Kikuchi, *Biochem. Biophys. Acta*, **385**, 345 (1975).
3) Location: *a) 20-1 Kitashinjuku, 3-chome, Shinjuku-ku, Tokyo, 160 Japan*; *b) 24-1 Hyakunin-cho, 3-chome, Shinjuku-ku, Tokyo*, 160 *Japan*.
4) T. Miyazaki and Y. Naoi, *Chem. Pharm. Bull.* (Tokyo), **22**, 1630 (1974); *ibid.*, **23**, 157 (1975).

were detected by spraying solutions of *p*-anisidine–HCl[5] and alkaline $AgNO_3$.[6] Paper electrophoresis (PE) was carried out using 0.026 M borate buffer (pH 10.0) or 1% $Na_2B_4O_7$ solution with Whatman No. 1 filter paper, and sugars were detected with periodate–Schiff reagent[7] or *p*-anisidine–HCl.[5] Quantitative estimation of the sugars was carried out by the procedures of Dubois, *et al.*,[8] and of Bitter–Muir,[9] and polyhydric alcohols were detected by the method of Lambert–Neish.[10] Nitrogen content was determined by elemental analyses, and phosphorus content was determined by the method of Fiske–Subbarow.[11] Gas–liquid chromatography (GLC) of the *O*-methyl-glycosides was carried out with a Shimadzu GC-5A unit, equipped with a flame ionization detector, using a 200 × 0.3 cm glass column packed with 15% polybutane-1,4-diol succinate on Celite 545 (60—80 mesh); column temperature, 175°; N_2 flow rate, 50 ml/min. GLC analyses of *O*-acetyl-*O*-methyl alditols derived from the methylated ASP-1, ASP-2, and FSP-1 were carried out under the conditions of gas flow rate of 40 ml N_2/min on a glass column (200 × 0.3 cm) containing 5% (w/w) of ECNSS-M on Chromosorb W (aw-dmcs, 60—80 mesh), at 180°, and of gas flow rate of 20 ml N_2/min on a glass column (200 × 0.3 cm) containing 3% (w/w) of Silicone OV-225 on Chromosorb W (aw-dmcs, 60—80 mesh), at 170°. Retention values were quoted relative to the mobility of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

For gas–liquid chromatography–mass spectrometry (GLC–MS), alditol acetates derived from each glycan were dissolved in acetone and injected into a Shimadzu Model LKB-9000 gas–mass spectrometer. The mass spectra were recorded at 70 eV, trup current 60 μA, temperature of the ion source, 310°.

Consumption of periodate was determined by the procedure of Malaprade,[12] and formations of HCOOH and HCHO were determined respectively by the methods of Whistler[13] and of O'Dea–Gibbons.[14]

Sedimentation analyses of ASP-1, ASP-2, and FSP-1 were made with a Hitachi Ultracentrifuge Model 282. Measurement was made at 60000 rpm at a concentration of 10 mg/ml of ASP-1, ASP-2, and FSP-1 in 0.1 M NaCl at 22° and photographed at 3, 6, 9, 18, 27, 36, 45, 54, 63, 72, and 81 min after reaching the full speed.

Partial acid hydrolysis of ASP-1, ASP-2, and FSP-1 were carried out as described in the previous paper.[4] After heating with acid, it was dialyzed against distilled water using Visking cellulose tubing, and resultant dialyzable fraction was analyzed by PPC using the solvent systems A and B.

Reduction of Di-O-Methyl Fraction with $NaBD_4$——The hydrolysates of methylated ASP-1, ASP-2, and FSP-1 were separated by PPC using the solvent system C. Each of resultant di-*O*-methyl fractions was reduced with $NaBD_4$ (50 mg) in H_2O (10 ml) for 15 hr. After decationization with Amberlite IR-120 (H^+), the reaction mixture was filtered, H_3BO_3 was removed by codistillation with MeOH, and the product was treated with Ac_2O–pyridine (1 : 1) (10 ml) at 100° for 15 min. The acetylated di-*O*-methyl fraction was diluted with cold water and the residue was dissolved in acetone. GLC–MS spectrometry was carried out as described above.

Result and Discussion

Crude polysaccharides of *A. solani* and *F. solani* isolated from the culture liquid were purified as described above. Yield of aqueous eluate from *A. solani* (ASP) was 64%, and that of aqueous eluate from *F. solani* (FSP) was 43% of the crude material. Further purification of ASP and FSP gave ASP-1 (aqueous eluate, yield 72%) and ASP-2 (0.01 M borate eluate, 16%), and FSP-1 (aqueous eluate, 55%), respectively.

The main fraction of *A. solani*, ASP-1 showed $[\alpha]_D^{25}$ +66.0°(*c*=1, H_2O) and the minor fraction ASP-2, $[\alpha]_D^{25}$ +38.0° (*c*=1, H_2O). The main fraction of *F. solani*, FSP-1, showed $[\alpha]_D^{25}$ +25.0° (*c*=1, H_2O). These glycans were centrifugally (Fig. 1) and paper electro-phoretically pure. Sugar contents of ASP-1, ASP-2, and FSP-1 were 96.2%, 97.3%, and 96.7% as glucose, respectively. They have no N and P, except FSP-1 which contained 0.49% of P. As shown in Table I, their component sugars and molar ratios were different.

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7) E. Köiw and A. Gronwall, *Scand. J. Clin. Invest.*, **4**, 244 (1952).
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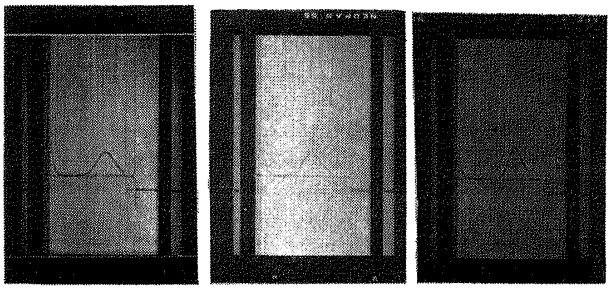
12) L. Malaprade, *Bull. Soc. Chim. France*, [5], **1**, 833 (1934).
13) R.L. Whistler and J.L. Hickson, *J. Am. Chem. Soc.*, **76**, 1971 (1954).
14) J.F. O'Dea and R.T. Gibbons, *Biochem. J.*, **55**, 580 (1953).



ASP-1 ASP-2 FSP-1

Fig. 1. Sedimentation patterns of ASP-1, ASP-2, and FSP-1 conditions: concentration 1% (solvent, 0.1 M NaCl); temperature 22°; speed, 60000 rpm

On periodate oxidation, the amounts of periodate consumed, and formic acid and formaldehyde liberated per sugar unit were 1.10, 0.42, and 0.03 mol for ASP-1, 1.13, 0.42, and 0.03 mol for ASP-2, and 1.02, 0.40, and 0.04 mol for FSP-1, respectively. Results of periodate oxidation are summarized in Table II.

Treatment of periodate-oxidized ASP-1, ASP-2, and FSP-1 by the Smith procedure[15] changed the molar ratios as shown in Table III. Mild acid hydrolysis of these glycans using 0.01 N H_2SO_4 at 100°

Table I. Component Sugars and Molar Ratios of ASP-1, ASP-2 and FSP-1

	Component sugar	Molar ratio
ASP-1	galactose, glucose, mannose, rhamnose (trace)	2.2: 3.1:1.0
ASP-2	galactose, glucose, mannose, rhamnose (trace)	1.0: 1.7:2.0
FSP-1	galactose, glucose, mannose, glucuronic acid, rhamnose	10.5:10.6:7.6: 1.8:1.0

Table II. Results of Periodate Oxidation of ASP-1, ASP-2 and FSP-1

Mole/unit		Time (hr)							
		1	3	6	12	24	48	72	96
ASP-1	IO_4	0.33	0.63	0.67	0.71	0.84	0.98	1.08	1.10
	HCOOH	0.05	0.18	0.24	0.28	0.32	0.36	0.39	0.42
	HCHO	—	—	—	0.01	0.03	0.03	0.03	0.03
ASP-2	IO_4	0.46	0.86	0.88	0.97	1.03	1.10	1.13	1.13
	HCOOH	0.10	0.14	0.19	0.26	0.33	0.38	0.40	0.42
	HCHO	0.02	—	—	0.03	0.03	0.03	0.03	0.03
FSP-1	IO_4	0.51	0.62	0.67	0.91	0.95	1.02	1.02	1.02
	HCOOH	0.09	0.15	0.23	0.29	0.35	0.37	0.40	0.40
	HCHO	0.04	—	—	0.04	0.05	0.05	0.05	0.04

for 4 hr gave oligosaccharides and a small amount of galactose, glucose, and mannose. These oligosaccharides appearing near the spotted line on the paper chromatogram were re-chromatographed, and resultant oligosaccharides were hydrolyzed with 2 N H_2SO_4, at 100° for 6 hr, and these results are shown in Table IV. Non-dialyzable fragments of ASP-1, ASP-2, and FSP-1 obtained by hydrolysis with 0.5 N H_2SO_4, at 100° for 2 hr gave mannose and less amount of glucose by further acid hydrolysis.

After each glycan was methylated by the methods of Hakomori[16] and then of Purdie[17] methanolysis and hydrolysis were carried out. Resultant *O*-methyl-monosaccharides were examined by PPC, thin-layer chromatography (TLC), PE, and GLC. In the PPC using

15) J.K. Hamilton and F. Smith, *J. Am. Chem. Soc.*, **73**, 5907 (1956).
16) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
17) T. Purdie and T.C. Irvine, *J. Chem. Soc.*, **83**, 1021 (1903).

Table III. Results of Smith-type Degradation of ASP-1, ASP-2 and FSP-1

	Main degradation product	Molar ratio
ASP-1	galactose, glucose, mannose, arabinose, threitol, glycerol	4.6:1.1:1.0:1.1:1.0:10.7
ASP-2	galactose, glucose, mannose, arabinose, threitol, glycerol	4.5:1.0:1.2:0.5:0.2:16.5
FSP-1	galactose, glucose, mannose, glucuronic acid, arabinose, threitol, erythrytol, glycerol, rhamnose	7.4:1.0:1.6:1.6:2.3:0.5:0.6:16.3:1.0

Table IV. Component Sugar and Molar Ratio of Main Oligosaccharides Obtained by Mild Acid Hydrolysis (0.01N H_2SO_4, 100°, 4 hr) of ASP-1, ASP-2, and FSP-1

	Oligosaccharide		
	I	II	III
ASP-1	Gal: Glc: Man=1:1:1	Gal: Glc=1:1	Gal: Glc=1:2
ASP-2	Man: Man=1:1	Gal: Glc=1:1	Gal: Glc=1:2
FSP-1	Gal: Glc: Man=1:1:2	Gal: Glc=1:1	Gal: Glc=1:2

Table V. Relative Retention Times and Molar Ratios of Acetyl Alditol derived from Methylated Glycans. Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol

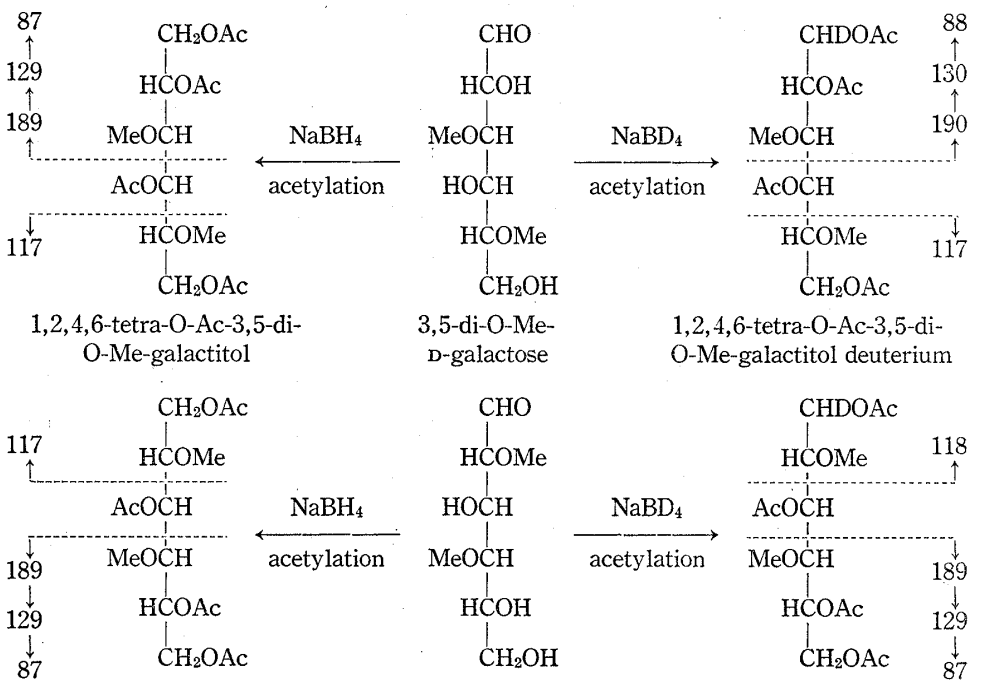
Component	ECNSS-M column	Authentic *O*-Me-alditol	OV-225 column	Authentic *O*-Me-alditol	Molar ratio in ASP-1	ASP-2	FSP-1
1,5-Di-*O*-acetyl-2,3,4-tri-*O*-methyl rhamnitol	0.47	0.47	0.35	0.35	0.8	0.8	1.7
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol	1.00	1.00	1.00	1.00	18.7	19.1	14.0
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl mannitol	1.00	1.00	1.00	0.99	7.8	8.4	6.7
1,2,5-Tri-*O*-acetyl-3,4,6-tri-*O*-methyl mannitol	1.95	1.95	1.82	1.82	—	21	—
1,2,5-Tri-*O*-acetyl-3,4,6-tri-*O*-methyl glucitol	1.95	1.98	1.87	1.83	9.0	—	5.1
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol	1.95	1.95	1.85	1.82	4.7	—	—
1,3,4-Tri-*O*-acetyl-2,5,6-tri-*O*-methyl galactitol	2.24	2.25	1.95	1.95	2.3	1.7	1.6
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methyl mannitol	2.48	2.48	2.20	2.19	—	—	—
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucitol	2.48	2.49	2.21	2.20	8.3	8.6	—
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucitol	2.51	2.50	2.30	2.32	—	—	1.0
1,4,6-*O*-Acetyl-2,3,5-tri-*O*-methyl galactitol	3.28	3.28	2.76	2.76	4.4	1.0	1.6
1,2,3,5-Tetra-O-acetyl-4,6-di-*O*-methyl mannitol	3.28	3.29	2.90	2.92	1.0	4.3	—
1,2,5,6-Tetra-*O*-acetyl-3,4-di-*O*-methyl mannitol	5.33	5.37	4.32	4.36	4.3	3.7	—
1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-methyl mannitol	5.42	5.44	4.48	4.51	—	—	5.8
1,2,4,6-Tetra-*O*-acetyl-3,5-di-*O*-methyl galactitol	6.34	6.35	5.12	5.10	21.2	19.2	17.2

the solvent system C, three fractions corresponding to tetra-, tri-, and di-*O*-methylated monosaccharides were detected. Approximate molar ratios of these fractions were 1.0: 1.0: 1.0, 1.0: 1.2: 1.0, and 1.8: 1.0: 1.8, respectively. From the results of PE, tri-*O*-methyl-monosaccharide fractions obtained from methylated ASP-1, ASP-2, and FSP-1 proved to be a mixture of 3,4,6-(MG value, 0.34) and another tri-*O*-methyl monosaccharide (MG value, 0.00). Reported values[18] for tri-*O*-methyl mannose: 0.36 (3,4,6-), 0.00 (2,3,6-, 2,4,6-, and 2,3,4-). Di-*O*-methyl monosaccharide fractions from ASP-1 and ASP-2 were considered as 3,4-di-*O*-methyl mannose (MG value, 0.51) and that of FSP-1 was as 2,4-di-*O*-methyl mannose (MG value, 0.00) by PE. Another fast moving di-*O*-methyl-monosaccharide (MG value, 0.72) detected in ASP-1, ASP-2, and FSP-1 could not be identified but, from the data of GLC–MS as described below, it should be 3,5-di-*O*-methyl galactose. Reported values[19] for di-*O*-methyl mannose: 0.09 (2,6-), 0.14 (2,3-), 0.39 (3,6-), 0.43 (4,6-), and 0.49 (3,4-).

The fully methylated ASP-1, ASP-2, and FSP-1 were converted to alditol acetates as described in our previous paper,[4] and the products were analyzed by GLC. GLC analyses of these alditol acetates were carried out under two different conditions using ECNSS-M and OV-225 columns, and these results were compatible each other as shown in Table V.

Result of the GLC–MS spectrometry of the peaks in each gas chromatogram were consistent with those reported in the literature.[20] In the cases of ASP-1 and ASP-2, 1,2,4,6-tetra-*O*-acetyl-3,5-di-*O*-methyl-D-galactitol was confirmed by change of *m/e* in deuterium labeling experiments; as shown in Table VI, the original peaks at *m/e* 87, 129, and 189 shifted to 88, 130, and 190. The value of *m/e* 117 of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-galactitol shifted completely to 118 by deuterium labeling experiment. This fact proves that 1,2,4,6-tetra-*O*-acetyl-3,5-di-*O*-methyl-D-galactitol can be distinguished from 1,3,5,6-tetra-*O*-acetyl-

Table VI. Comparison of the Values of *m/e* in 2,4-Di-O-methyl- and 3,5-Di-O-methyl-galactitol-tetra Acetates prepared with $NaBD_4$ and $NaBH_4$



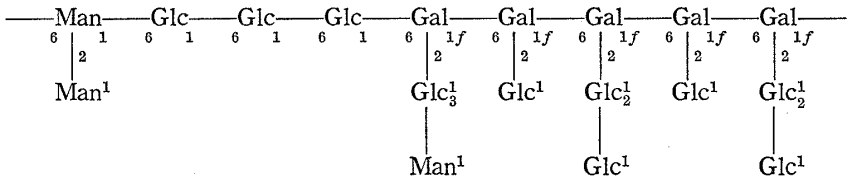
18) H. Weigel, *Adv. Carbohydr. Chem.*, **18**, 61 (1963).
19) T. Miyazaki, *Chem. Pharm. Bull.* (Tokyo), **9**, 831 (1961).
20) H. Bjorndal, B. Lindberg, and S. Svensson, *Carbohydr. Res.*, **4**, 433 (1967).

2,4-di-*O*-methyl-D-galactitol on GLC–MS spectrum by deuterium labeling. In FSP-1, di-*O*-methyl derivative was similarly confirmed as 1,2,4,6-tetra-*O*-acetyl-3,5-di-*O*-methyl-D-galactitol.

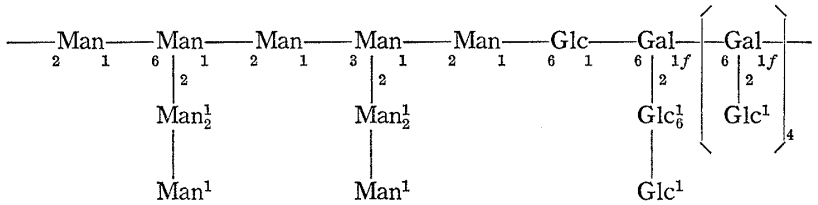
From these data, it is concluded that (1) ASP-1, ASP-2, and FSP-1, are complicated heteroglycans composed of galactose, glucose mannose, and a trace of rhamnose (ASP-1 and ASP-2), and glucuronic acid is also present in the case of FSP-1 as an additional component. (2) These glycans contain furanosyl sugar residues at non-terminal position, because these were easily split off into oligosaccharides by a mild acid hydrolysis such as using 0.01 N H_2SO_4, and 3,5-di-*O*-methyl derivative was detected in the methylation studies. (3) These glycans contain 1,3-linked or branching units, and a small amount of 1,2- or 1,3-linked galactofuranose, because considerable oxidation-resistant sugar residues and small amount of L-arabinose were detected accompanying with polyhydric alcohols in Smith degradation treatment. (4) Non-reducing terminal residues of these glycans are mostly glucopyranose, with a smaller amount of mannopyranose. In the case of FSP-1, a very small amount of rhamnopyranose is also present. These are clear from the results of methylation studies. (5) Main sugar units in ASP-1 were 1,2-, 1,3-, and 1,6-linked glucopyranosyl, 1,2,6-linked galactofuranosyl and mannopyranosyl residues, while in the case of ASP-2, 1,2-linked mannopyranosyl, 1,6-linked glucopyranosyl, 1,2,6-linked galacto-furanosyl, and mannopyranosyl residues. In the case of FSP-1, 1,2-linked glucopyranosyl, 1,2,6-linked galactofuranosyl, and 1,3,6-linked mannopyranosyl residues were present as main sugar units, as evidenced from the results of methylation studies. (6) Glucuronic acid and less amount of rhamnose were detected as the additional component sugar of FSP-1, and the former will be linked through 1,3-position with other residues, because glucuronic acid resisted to periodate oxidation. (7) Identification of 2,4-di-*O*-methyl and 3,5-di-*O*-methyl alditol acetates was difficult even by GLC analyses carried out under two different conditions. However, as described above, both derivatives were clearly differentiated by shifting of the value of *m/e* in deuterium labeling experiments. (8) As shown in Table IV, mild acid hydrolysis of ASP-1, ASP-2, and FSP-1 gave different oligosaccharides. These results will be used for the elucidation of fine structures of these glycans.

Possible structures for the main portions of ASP-1, ASP-2, and FSP-1 will be as follows:

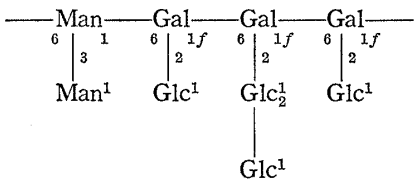
ASP-1;



ASP-2;



FSP-1;



As described above, 1,2,6-linked galactofuranosyl residue was present as a common unit in the core of these glycans, but their fine structures are evidently different from each other, and in particular, 1,3,6-linked mannopyranosyl residues are present only in FSP-1. Therefore, it may be concluded that these polysaccharides have genus or species specificity, and it is of special interest that 1,2,6-linked galactofuranosyl residues were detected for the first time in natural glycans. Examination of selorogical relationship of these glycans is in progress.