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Polysaccharides of Lichen Symbionts[1]

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The water-soluble polysaccharides isolated from the laboratory cultures of lichen mycobionts and phycobionts were studied comparatively. The polysaccharides of mycobionts so far tested showed close similarities with those of parent lichens, whereas the polysaccharides of phycobionts gave different features. Therefore, it is highly probable that the water-soluble polysaccharides of lichens are mostly produced by their mycobionts as like as the lichen metabolites of smaller molecular size.

Keywords——polysaccharides; lichen; lichen mycobiont; lichen phycobiont; *Cladonia bellidiflora*; *Cladonia graciliformis*; *Cladonia mitis*; *Cladonia rangiferina*; *Cladonia calycantha*; *Parmelia caperata*; *Ramalina crassa*

Recently Shibata and his coworkers[3–6] have demonstrated that several lichen metabolites of small and medium molecular size are produced by the laboratory cultures of mycobionts isolated from the parent lichens without participation of the phycobionts.

Accordingly, the characteristic water-soluble polysaccharides of lichens have been examined whether they are produced by the mycobiont or the phycobiont or both.

For the present experiments the following lichens and their symbionts were used.

TABLE I. Lichens and Their Symbionts used for Experiments

Cladonia bellidiflora (ACH.) SCHAER.	(CB)[a]	Mycobiont	—
Cladonia graciliformis ZAHLBR.	(CG)	Mycobiont	—
Cladonia mitis SANDST.	(CM)	Mycobiont	Phycobiont
Cladonia rangiferina (L.) G. WEB. ex WIGG.	(CR)	—	Phycobiont
Cladonia calycantha (DEL.) NYL.	(CC)	—	Phycobiont
Parmelia caperata (L.) ACH.	(PC)	Mycobiont	Phycobiont
Ramalina crassa (NYL.) MOT.	(RC)	Mycobiont	Phycobiont

a) Those given in parenthesis are the abbreviateions of the names of lichens; — no testing due to the shortage of material.

The mycobionts and phycobionts of the above lichens were isolated by the method developed by Komiya *et al.*[3,4] and cultured for 6 months on the medium I* for mycobionts, and for 4 weeks on the medium II* for phycobionts *(see experimental part). The mycobionts and phycobionts of lichens harvested were extracted first with ether followed by 80% ethanol to remove lipophilic compounds, then with water to obtain carbohydrates.

The crude polysaccharides (Fract. 1) precipitated from the aqueous extracts of lichen by the addition of ethanol were separated by means of freezing-thawing into a cold water-

1) Part VIII in the series of Polysaccharides of Lichens and Fungi. Part VII: I. Yokota and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), **26**, 2668 (1978).

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3) T. Komiya and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), **17**, 1305 (1969).

4) H. Nakano, T. Komiya and S. Shibata, *Phytochem.*, **11**, 3505 (1972).

5) H. Ejiri and S. Shibata, *Phytochem.*, **13**, 2871 (1974).

6) H. Ejiri and S. Shibata, *Phytochem.*, **14**, 277 (1975).

Table II. The Physical and Chemical Properties of Polysaccharides of Lichens and Their Symbionts

Lichens[a]	Polysaccharide fractions	Yield[b] (%)	$[\alpha]_D$	Solubility in cold H_2O	IR abs. (cm^{-1})	Sugar components
CB	CB-2	0.68	—	sol.	810, 900, 925	Man Gal Glc
	CB-3	0.009	—	insol.	785, 845, 925	Glc
	CB-f-2	1.1	+40	sol.	810, 885	Man Gal Glc (4:4:1)
	CB-f-3	0.76	+177	insol.	820, 845, 865, 895	Man Gal Glc (1:10:20)
CG	CG-2	1.4	—	sol.	—	Man Gal Glc
	CG-3	0.46	—	insol.	—	—
	CG-f-2	1.9	+24	sol.	—	Man Gal Glc (3:3:1)
	CG-f-3	1.6	+342	insol.	845, 865	Man Gal Glc (1:5:30)
CM	CM-f-2	2.3	−36	sol.	879, 895	Man Gal (3:2)
	CM-f-3	0.9	+336	insol.	810, 850, 890	—
	CM-a-2	1.5	−57	sol.	790, 890	Rha Man Gal Glc (5:1:28:3)
	CM-a-3	0.056	−258	insol.	—	Glc
CR	CR-a-2	0.58	−46	sol.	800, 890	Rha Man Gal Glc (7:1:28:3)
	CR-a-3	0.14	−138	insol.	—	—
CC	CC-a-2	2.3	−29	sol.	800, 870	Rha Man Gal Glc (4:1:18:1)
	CC-a-3	0.081	−109	insol.	—	Man Gal Glc (1:2:7)
PC	PC-2	0.6	+165	sol.	805, 845, 935	Glc
	PC-3	3.0	+201	insol.	780, 845, 925	Glc
	PC-f-2	3.3	−8.5	sol.	860, 890	Gal
	PC-f-3	7.7	+200	insol.	780, 840, 925	Glc
RC	RC-2	2.1	+31	sol.	890	Glc
	RC-3	0.3	+140	insol.	780, 840, 925	Glc
	RC-f-2	1.3	+36	sol.	890	Glc
	RC-f-3	1.8	+136	insol.	800, 840, 860	Glc
	RC-a-2	—	−85	sol.	800, 840, 860	Gal

a) Abbreviations of the names of Lichens are given.
b) Calculated from the dried starting materials. — not measured.

soluble(Fract. 2) and a cold water-insoluble fraction (Fract. 3). In the present paper the abbreviation of the name of the original lichen is given as a prefix of the fraction, and *f* indicates the fungal (mycobiont) and *a* the algal (phycobiont) origin of the polysaccharide. The physical and chemical data of the polysaccharide fractions of the lichen symbionts isolated are shown in Table II.

Specific rotation values[7] and the infrared (IR) absorptions[8] show that the cold water-soluble polysaccharides of mycobionts of *Cladonia* spp. so far examined possess β-D-linkage predominantly in the molecules and yield on hydrolysis mannose and galactose as the major components and glucose as the minor one.

CG-f-2 was chromatographed on a column of Sephadex G-200 to give an elution diagram as shown in Fig. 1. CG-f-2-I was shown to be a glycopeptide, since the carbohydrate and the peptide peaks in the elution diagram coincided well each other at this fraction. The sugar components of CG-f-2-I are mannose, galactose and glucose in a ratio of 4: 5: 1 as indicated

7) K. Freudenberg, K. Friedrich and I. Bumann, *Ann. Chem.*, **494**, 41 (1932).
8) S.A. Barker, E.J. Bourn, R. Stephens and D.H. Whiffen, *J. Chem. Soc.*, **1954**, 3468.

with a sugar analyzer. The corresponding polysaccharide fraction (CG-2-I) isolated from the parent lichen, *Cladonia graciliformis*, showed a ratio of mannose, galactose and glucose as 3:5:2. The cold water-soluble polysaccharides, CG-f-3 and CB-f-3, were shown to have α-D-glycosyl linkages predominantly in the molecules.

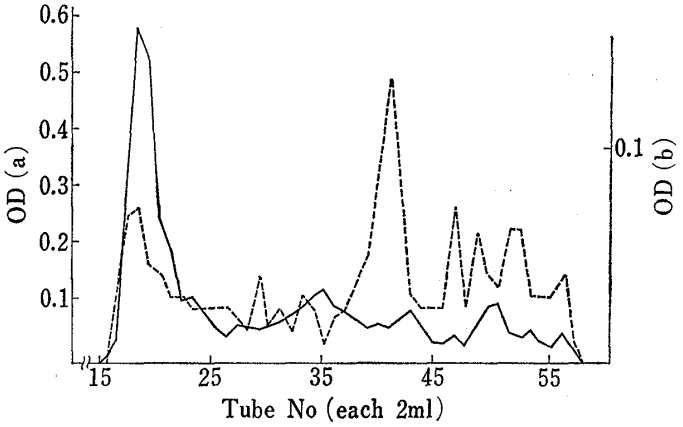

Fig. 1. Elution Diagram on Sephadex G-200

——: Carbohydrate (a) by Phenol-H_2SO_4 method at 490 nm.
----: Peptide (b) by Cu-Folin method at 750 nm.
Column: 2.5×45 cm; Flow rate: 0.65 ml/min; Flow solvent: H_2O; Sample: CG-f-2 8.3 mg.

A cold water-insoluble homoglucan fraction, PC-f-3, isolated from the laboratory culture of the mycobiont of *Parmelia caperata* was proved to be obviously identical with PC-3, an α(1→3) (1→4) glucan (1:1), which was originally isolated from the parent lichen,[9] from the data of specific rotation value, infrared spectrun, solubility and sugar components. The mycobiont isolated from *Ramalina crassa* produced by the laboratory cultivation a cold water-soluble (RC-f-2) and an insoluble (RC-f-3) homoglucan whose properties are almost identical respectively with those of the corresponding glucans of the parent lichen.

On the other hand, the phycobionts were isolated from *Cladonia mitis*, *Cl. rangiferina*, *Cl. calycantha*, *Parmelia caperata* and *Ramalina crassa*. The crude polysaccharide fractions, CM-a-1, CR-a-1 and CC-a-1, were obtained from the laboratory cultivation of the phycobionts of the above lichens respectively, to show as being peptide heteroglycans containing galactose as the major sugar component. By the freezing and thawing procedure, the polysaccharides of the cultivated phycobionts were separated into the major cold water-soluble CM-a-2, CR-a-2, and CC-a-2 fractions, respectively, and the minor cold water-insoluble, CM-a-3, CR-a-3 and CC-a-3 fractions. Their specific rotation values and the IR spectra showed that β-D-configuration is dominant in their molecules.

On hydrolysis of these fractions galactose was the major product, and rhamnose, mannose and glucose were the minor ones.

The polysaccharide fraction of the phycobiont isolated from *Parmelia caperata* was separated by the freezing and thawing procedure into a major cold water-soluble, PC-a-2, and a minor cold water-insoluble PC-a-3 fraction. From the phycobiont of *Ramalina crassa* a major cold water-soluble galactan, RC-a-2, was obtained, whose properties are different from those of the polysaccharides of the parent intact lichen and its mycobiont.

According to the above mentioned results, the polysaccharides of mycobionts are very similar or identical with those of the parent intact lichens, whereas those of the phycobionts are rather different from the parent ones. These results suggest that the water-soluble polysaccharides of lichens are mostly produced by the mycobionts as like as the lichen metabolites of smaller and medium molecular size.

Experimental

Sugar analysis was performed using a liquid chromatographic autoanalyzer, JEOL Model JLC-6AH; the IR spectra were measured with a spectrophotometer JASCO Model DS 420-G; the specific rotations were determined with a Yanagimoto Model OR-50 polarimeter.

9) T. Takeda, Y. Nishikawa and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), 18, 1074 (1970).

Preparation of the Crude Polysaccharide Fractions——The mycobionts and phycobionts of lichens were extracted twice with ether and 80% EtOH for 8 hr subsequently in order to remove soluble components, and the residue was extracted twice with dist. H_2O on a boiling water bath.

To the hot extracts was added EtOH to form precipitates which were collected by centrifugation, washed with EtOH and ether, and dried to obtain a pale brownish water-soluble powder. The yields of the polysaccharide fractions prepared as above are as follows:

From the mycobionts-CB-f-1 (3.7%), CG-f-1 (4.9%), CM-f-1 (5.0%).

From the phycobionts—CC-a-1 (2.7%), CM-a-1 (2.4%), CR-a-1 (2.5%).

Separation of Polysaccharides by Freezing-thawing Method——The crude polysaccharide fraction was dissolved in dist. water on a water bath and filtered. The hot solution of crude polysaccharide was filtered through a glass filter and frozen. The product was allowed to thaw. The cold water insoluble polysaccharide was collected by filtration, and then the filtrate was evaporated to 1/3 vol. Ethanol was added to the concentrated solution to form precipitates, which were collected as a cold water-soluble polysaccharide.

Sugar Analysis——Neutral sugar components of the polysaccharide fractions were determined with a sugar analyzer as follows: Each fraction, 2 mg, was hydrolyzed with 2 N H_2SO_4, 2 ml, for 8 hr at 100°. The hydrolysates were neutralized with Amberlite CG-4B (OH^-) and evaporated to a syrup which was dissolved in 0.13 M of borate buffer, pH 7.5, 1.5 ml, and applied to a column of JEOL resin LC-R-3 of the sugar analyzer.

The determination of sugar components was made by the orcinol-H_2SO_4 method, and the absorbances at 510 and 425 nm were automatically recorded.

Media for Cultivation of Lichen Symbionts——Medium I for mycobionts: Dextrose 10 g, ammonium tartarate 2 g or L-alanine 0.455 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $Fe(NO_3)_3 \cdot 9H_2O$ 0.2 mg, $ZnSO_4 \cdot 7H_2O$ 0.2 mg, $MnSO_4 \cdot 4H_2O$ 0.1 mg, $CaCl_2$ 0.2 mg, thiamine 0.1 mg, biotin 0.005 mg, dist. H_2O ad 1000 ml.

Medium II for Phycobionts (Bald's Mineral Medium): Part A: $NaNO_3$ 10.0 g, $CaCl_2$ 1.0 g, $MgSO_4 \cdot 7H_2O$ 3.0 g, K_2HPO_4 3.0 g, KH_2PO_4 7.0 g, NaCl 1.0 g, dist. H_2O ad 400 ml. Ten ml of the above solution (A) was added into 940 ml of H_2O. Part B: (1) H_3BO_3 11.42 g/1, (2) $FeSO_4 \cdot 7H_2O$ 4.98 g, $ZnSO_4 \cdot 7H_2O$ 8.82 g, $MnCl_2 \cdot 4H_2O$ 1.44 g/1, (3) MoO_3 0.71 g, $CuSO_4 \cdot 5H_2O$ 1.57 g, $Co(NO_3)_2 \cdot 6H_2O$ 0.89 g/1, (4) EDTA 50.0 g/1. One ml of each solution (1)—(4) was added into Part A medium.

Trebouxia Organic Nutrient Medium: Bold's mineral solution 980 ml, Cassamino acids (Vitamin free) 10 g, D-glucose 10 g.

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