Further Studies on the Structure of Polysaccharides from the Lichen *Flavoparmelia caperata* (L.) HALE

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A water-soluble polysaccharide called PC-2 was previously isolated from *Flavoparmelia caperata* (L.) HALE and assigned to α(1-3)(1-4)glucan. However, PC-2 separated into three components, PC-2A, PC-2B, and PC-2C (a single peak in HPLC, respectively) on further purification. Methylation analysis and ^{1}H- and ^{13}C-NMR spectroscopic studies suggested that PC-2A is composed of repeating units of [α-D-Glc1-3]$_3$, : [α-D-Glc1-4]$_2$, while PC-2B and PC-2C are partly branched galactoglucomannans consisting of (1-3)- and (1-4)-linked α-D-glucopyranosyl units as the main chain. In addition we confirmed that the polysaccharide fraction PB-2 from the lichen of the same genus, *Flavoparmelia baltimorensis* (GYELNIK & FORISS) HALE, is identical to PC-2 based on the chemical and spectroscopic data.

Key words *Flavoparmelia caperata*; *Flavoparmelia baltimorensis*; α(1-3)(1-4)glucan (3 : 2)

Apart from low molecular-weight secondary metabolites such as fatty acids, terpenoids, and aromatic polyketides, several high molecular-weight polysaccharides have been isolated from lichens. They are classified into homoglucans (such as lichenan, isolichenan, pustulan, evernan, acroscyphan, and nigeran-type glucans) and heteroglycans (such as galactomannan and glucomannan) with some additional more complex members. Some significant biological activities such as antitumor, immunomodulatory, antiviral, and memory-enhancing effects in these lichen polysaccharides have already been reviewed.[1]

In 1970, Takeda and coworkers[2] isolated an α-glucan fraction, PC-1, from the aqueous extract of *Parmelia caperata* (L.) ARG. (=*Flavoparmelia caperata* (L.) HALE)[3] through precipitation with ethanol. Repeated freezing and thawing yielded PC-2, a major cold water-soluble fraction, and PC-3, a minor cold water-insoluble fraction, from the above PC-1 fraction in a yield of 5.0% and 3.9%, respectively. Recently, we have investigated the hippocampus-activating effect of PC-2,[4] while PB-2,[5] a corresponding fraction isolated from the lichen of the same genus, *Flavoparmelia baltimorensis*,[3] showed the same activity as that of PC-2. In this paper, we report the structural revision of PC-2, which is the major cold water-soluble polysaccharide fraction obtained from *F. caperata* with the aid of chromatography, and its identity with PB-2 from *F. baltimorensis*.

Results and Discussion

We subjected PC-2 (1.27 g) to chromatography on DEAE Sephadex A-25 (acetate form) eluted with water and ammonium acetate. An aqueous eluate (0.62 g) was further purified by Sephacryl S-300 column chromatography to afford three fractions, PC-2A (0.34 g), -2B (0.16 g), and -2C (0.12 g), each of which showed a single peak on HPLC. The gel chromatography gave molecular masses of 53700, 20400, and 13200 for PC-2A, -2B, and -2C, respectively. The high positive specific optical rotation values for each fraction, $[\alpha]_D$ $+240.2°$, $+175.8°$, and $+153.4°$ respectively, revealed their α-anomeric linkages.[6] PC-2A is a homoglucan, whereas PC-2B and PC-2C contain some galactose and mannose other than the main glucose portion in the ratio of Glc : Gal : Man of 10 : 2 : 2 and 10 : 1 : 2 by acid hydrolysis, respectively. No nitrogen exists in the molecules of these polysaccharides.

Structure of PC-2A The ^{1}H-NMR spectrum of PC-2A gave anomeric proton signals at δ 5.37 (br s) and δ 5.41 (br s) integrating three and two protons, respectively, in agreement with the ratio of 3 : 2 of the (1-3)- and (1-4)-linkages shown by methylation analysis. We confirmed the α-glycosidic linkages in PC-2A based on the anomeric proton signals at δ 5.3—5.5 ppm in the ^{1}H-NMR spectrum and the anomeric carbon signals at δ 101.6, 101.7, 101.9, and 102.6 ppm in the region of δ 98—103 ppm in the ^{13}C-NMR spectrum.[7]

The ^{13}C-NMR signals at δ 82.5, 82.1, and 82.0 ppm assigned to C3 were involved the (1-3)-linkage, and a signal at δ 79.6 ppm was assigned to C4 in the (1-4)-linkage. Starting from H-1 (δ 5.41 ppm) of (1-4)-glucose, H-2 was identified from the cross peak at δ 3.68 ppm. We assigned H-3, H-4, H-5, H-6a, and H-6b in a similar manner. Starting from H-1 of the (1-3)-glucose linkage at δ 5.37 ppm, each of the other proton signals was assigned from the cross peak in a similar manner as above. Based on the ^{1}H-detected heteronuclear multiple-quantum coherence (HMQC) experiments on PC-2A, we assigned all the proton and carbon signals of PC-2A, as shown in Table 1. Then we analyzed PC-2A by methylation, and it converted into alditol acetates. Each of the partially methylated alditol acetates was identified based on the retention time on gas-liquid chromatography and the fragmentation pattern seen in mass spectrometry. The proportion of 2,4,6- and 2,3,6-tri-*O*-methylglucitol acetate determined by GC was 3 : 2. This is in agreement with the result observed in the ^{1}H-NMR analysis of PC-2A indicating the linear (1-3)(1-4)-α-D-glucan (3 : 2) structure. These results revealed that the minimal repeating unit of the polysaccharide moiety of PC-2A consists of three types of component sugar units, as shown in Chart 1.

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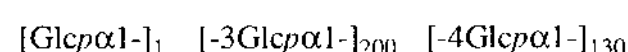
Table 1. ^{1}H- and ^{13}C-Chemical Shifts (δ) Obtained from PC-2A[a)]

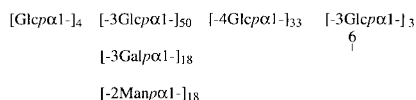
H and C units	Chemical shift (δ)			
	α(1-3)		α(1-4)	
	^{1}H(δ)	^{13}C(δ)	^{1}H(δ)	^{13}C(δ)
H-1/C-1	5.37	101.9, 101.7, 101.6	5.41	102.6
H-2/C-2	3.60	74.2	3.68	73.1
H-3/C-3	3.83	82.5, 82.1, 82.0	3.74	75.2
H-4/C-4	3.68	72.6, 72.5, 72.4	3.68	79.6
H-5/C-5	4.03	76.0	4.14	73.0
H-6/C-6	3.82, 3.86	62.9	3.76, 3.85	63.1

a) Data obtained from ^{1}H (in D_2O), ^{13}C, COSY, and HMQC experiments.

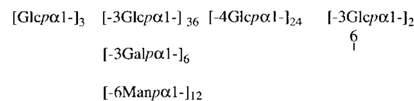
PC-2A



PC-2B



PC-2C



Component sugar Residues in the Minimal Unit in the structure of PC-2A, -2B and -2C

Chart 1

Structures of PC-2B and PC-2C Based on the gas–liquid chromatography of the acid hydrolysates, we confirmed that PC-2B consists of glucose, galactose, and mannose and estimated their molar ratio to be 10 : 2 : 2. We also showed that PC-2C consists of glucose, galactose, and mannose in the ratio of 10 : 1 : 2. In the ^{13}C-NMR spectrum of PC-2B and PC-2C, most carbon signals are very similar to those of PC-2A, except for the signals based on galactose and mannose moieties. Gas–liquid chromatography of the methylation analysis products of PC-2B methyl ether revealed the liberation of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol, and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol in a 5 : 3 : 2 : 2 ratio and a small amount of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylglucitol. Based on the above evidence, we recognized that PC-2B is a heteroglycan consisting of six types of sugar components, as shown in Chart 1. Using similar methods, we propose that PC-2C has a heteroglycan structure with a main chain of (1-3)(1-4)α-D-glucopyranosyl units substituted occasionally at the 6-position with glucopyranose, which has a relatively low content of α(1-6)-linked mannnopyranosyl and α(1-3)-linked galactopyranosyl residues. The results of methylation analysis of PC-2A, PC-2B, and PC-2C are summarized in Table 2.

In the present study, we determined that polysaccharides isolated from *F. baltimorensis* are identical to those isolated from *F. caperata*. The fractions tentatively called PB-2 ($[\alpha]_D$ +230.2° (H_2O)) and PB-3 ($[\alpha]_D$ +201.5° (2 N-NaOH)), which were fractionated from PB-1 of *F. baltimorensis* by the freeze–thaw method, gave the same results as for PC-2 and PC-3 in the NMR experiment, $[\alpha]_D$ values and complete acid hydrolysis.[2)] PB-2 also showed hippocampal-activating activity enhancing LTP[5)] in anesthetized rats.

Table 2. Methylation Analysis of PC-2A,-2B, and -2C

Methylated sugar (as alditol acetate)	Retention time[a)]	Molar ratio		
		2A	2B	2C
2,3,4,6-Me_4-D-glucose	1.00	1	4	3
2,4,6-Me_3-D-galactose	1.32		18	6
2,4,6-Me_3-D-glucose	1.34	200	50	36
3,4,6-Me_3-D-mannose	1.35		18	
2,3,4-Me_3-D-mannose	1.46			12
2,3,6-Me_3-D-glucose	1.52	130	33	24
2,4-Me_2-D-glucose	1.84		3	2

a) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Me, methyl.

Experimental

Optical rotations were measured with a Jasco digital polarimeter DIP-1000. Gas chromatography was carried out on a Shimadzu GC-14A gas chromatograph equipped with a hydrogen flame ionization detector. ^{1}H- and ^{13}C-NMR spectra were recorded with a JMN A500 FT NMR spectrometer in D_2O at 25 °C at 250 MHz for the ^{1}H-NMR and 62.9 MHz for the ^{13}C-NMR. HPLC was carried out using a Shimadzu LC-10 system with a Shodex Asahipak GS-520HQ column (7.6×300 mm), for semipreparatory chromatography with a Shodex Asahipak GS-520 21F (21.5×300 mm).

Plant Materials The lichens, *F. caperata* (L.) HALE and *F. baltimorensis* (GYELNIK & FORISS) HALE were provided and identified by Dr. Chicita F. Culberson. Voucher specimens were deposited at the herbarium in the Department of Botany, Duke University, Durham, NC, U.S.A.

Isolation and Purification The lichen *F. caperata* (dry weight 25.2 g), collected in the U.S.A., was extracted three times with ethanol for 2 h. The residue was extracted with water for 2 h, and the residual lichen was extracted again with hot water for 6 h. To the filtered extracts two volumes of ethanol were added to form precipitates, which were collected by centrifugation, washed thoroughly with ethanol and ether, and dried, yielding PC-1 (2.36 g). PC-1, a grayish white powder, was readily fractionated into a cold water-soluble fraction, PC-2 (1.27 g), and a cold water-insoluble polysaccharide fraction, PC-3 (0.99 g). We dissolved PC-2 in water and applied it to a column (3.5×16 cm) of diethylaminoethyl (DEAE)-Sephadex A-25 (acetate form). After elution with water, the column was eluted with 0.1 M ammonium acetate and subsequently with 0.5 M ammonium acetate. The aqueous eluate (0.62 g) was applied to a column (2.6×96.5 cm) of Sephacryl S-300. The column was eluted with 0.1 M Tris–HCl buffer (pH 7.0), and three fractions were obtained. We collected fractions of 20 ml and analyzed them using the phenol-sulfuric acid method. Eluate PC-2A (0.18 g) was obtained from tubes 33 to 41, eluate PC-2B (0.16 g) from tubes 86 to 93, and eluate PC-2C (0.12 g) from tubes 103 to 110.

Isolation of PB-2 and PB-3 We obtained cold water-soluble polysaccharide (PB-2 0.18 g, 3.4%) and cold water-insoluble polysaccharide (PB-3 0.17 g, 3.2%) from extracts of *F. baltimorensis* (6.22 g) using the same procedure as described above.

Determination of Components Component sugars were analyzed by gas chromatography after conversion of the hydrolysates into the corresponding alditol acetates.

Methylation Methylation was performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide.[8,9)]

Analysis of Methylated Products The methylated products were hydrolyzed with dilute sulfuric acid in acetic acid and then were reduced and acetylated as described previously.[10)] The partially methylated alditol acetates obtained were analyzed by gas chromatography (GC) using a fused silica capillary column of SP-2330 (Supelco Co.) with a programmed temperature increase of 4 °C/min from 160 to 220 °C at a helium flow of 1 ml/min. GC-MS was performed with a JEOL Automass SUN-HP6890 GC system mass spectrometer. The relative retention times of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (internal standard) in gas chromatography are listed in Table 2.

Determination of Homogeneity of Polysaccharides and Their Molecu-

lar Weight The glycans (5 mg) were dissolved in water, and the solution was applied to a column of Toyopearl HW-65F (2.6×90 cm). It was eluted with 0.1 M Tris–HCl buffer and the resulting fractions of 2 ml were tested for carbohydrates. The column was calibrated for molecular weight using pullulans of Mr 18.6×10^4, 4.80×10^4, 2.08×10^4, 1.22×10^4, and 0.58×10^4 as the reference compound.

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