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Isolation and Structural Analysis of Polysaccharide Containing Galactofuranose from the Cell Walls of *Bifidobacterium infantis*

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We isolated cell wall polysaccharides (PS-1 and PS-2) from *Bifidobacterium infantis* Reuter ATCC 15697 and found that the backbone of PS-2 is →3)-β-D-Gal_f-(1→3)-α-D-Gal_p-(1→ in which β-D-Gal_f and α-D-Gal_p are partially substituted at O-6 with β-D-Glc_p. This is the first report of the presence of this disaccharide backbone in a gram-positive bacterium; it resembles the O antigen of some bacteria.

Bifidobacteria are anaerobic gram-positive bacteria and are believed to be associated with health-promoting effects in the host intestine. Recent studies with several *Bifidobacterium* strains have offered structural information concerning the cell surface construction, such as lipoteichoic acids (4, 5, 10, 18), teichoic acids (26), and cell wall polysaccharides (7, 17). Op den Camp et al. have isolated from *Bifidobacterium bifidum* a new type of lipoteichoic acid, containing a 1,2-linked polyglycerophosphate backbone between a glycolipid moiety and a linear polysaccharide, and found a unique structure, 1,5-linked β-D-galactofuranose homopolymer (galactofuranan), in the polysaccharide chain (18). On the other hand, Fischer et al. have provided another structure for lipoteichoic acid-like amphipathic polymers, which consist of a glycolipid moiety, 1,6-linked β-glucan, and 1,5-linked β-galactofuranan with side chains of monomeric glycerophosphate (4, 5). Such macroamphiphiles do not link to peptidoglycan but are considered to be anchored to membranes through their lipid moiety and should be common in several species of *Bifidobacterium* (10).

Previously, we reported the carbohydrate composition of purified cell walls of *Bifidobacterium infantis* Reuter ATCC 15697 (24). In the present study, we isolated two distinct neutral polysaccharides, PS-1 and PS-2, from the purified cell walls of the same strain and found that the backbone of PS-2 is a 1,3-linked galactan consisting of β-D-galactofuranose and α-D-galactopyranose in which more than 90% of the β-D-galactofuranosyl residues and about 30% of α-D-galactopyranosyl residues are occupied by β-D-glucopyranose at position 6. The backbone structure, →3)-β-D-Gal_f-(1→3)-α-D-Gal_p-(1→, has been identified also in the lipopolysaccharide of *Klebsiella pneumoniae* (12–15, 27, 28), *Pasteurella haemolytica* (22, 23), *Serratia marcescens* (19, 20), and *Serratia plymuthica* (1). It is of interest that the cell wall polysaccharide from *B. infantis* contains the same structure as O-specific antigen of some gram-negative bacteria.

Preparation and carbohydrate composition of cell wall polysaccharides, PS-1 and PS-2. We previously described the growth conditions of *B. infantis* and the isolation procedure of purified cell walls (24). Determinations of amino acids, monosaccharides, fatty acids, and nucleic acids of the cell walls were also carried out by the same methods as previously reported (24). *B. infantis* cell walls consisted of neutral sugars (glucose and galactose), amino sugars (glucosamine and muramic acid), peptidoglycan-derived amino acids (alanine, glutamic acid, threonine, serine, and ornithine), and a small amount of fatty acids, so that they were considered to be free from contamination with nucleic acids and proteins (24).

The purified cell walls (300 mg) were treated with 5% trichloroacetic acid at 4°C overnight and then centrifuged, and the supernatant was thoroughly dialyzed against deionized water. The nondialyzable material was lyophilized and used as the acid extract (220 mg). Trichloroacetic acid released more than 95% of glucose and galactose as water-soluble polymers from the purified cell walls. An aliquot of acid extract (50 mg) was reconstituted with deionized water (5 ml) to be subjected to gel filtration chromatography on Sephacryl S-300 (2.5 by 95 cm; Pharmacia Biotech) and eluted with 50 mM ammonium bicarbonate. According to the carbohydrate content monitored by phenol-sulfuric acid assay (3), the acid extract was separated into two hexose-containing peaks by the gel filtration chromatography (Fig. 1). The first peak, which eluted at the same position as that of standard pullulan P-100 (molecular weight, 100,000), was named PS-1, and the second peak, which eluted after standard pullulan P-5 (molecular weight, 5,800), was named PS-2. The corresponding fractions were pooled and lyophilized to give PS-1 (20 mg) and PS-2 (25 mg). While PS-2 was eluted after the P-5 standard in Fig. 1, the apparent molecular weight of PS-2 was evaluated to be 9,400 by size exclusion high-performance liquid chromatography (HPLC) with a TSK-GEL G-3000 column (0.8 by 30 cm; TOSO) in 0.15 M sodium chloride using pullulan standards, and that of PS-1 was found to be 100,000. Since the position of PS-2 on the Sephacryl S-300 chromatograph was affected by the ionic strength of the elute solution (data not shown), the disagreement between the result of HPLC and that of Sephacryl S-300 chromatography might be due to some interaction of PS-2 with the Sephacryl matrix.

For determination of carbohydrates, samples in Teflon-sealed screw-cap tubes were hydrolyzed at 105°C for 4 to 6 h with 2 M trifluoroacetic acid. The carbohydrate composition of the hydrolysates was analyzed by HPLC with an Ionpak KS-801 column (0.8 by 30 cm; Shodex) in distilled water. Table 1 shows that both polysaccharides contained only glucose and galactose in molar ratios of 1:3 for PS-1 and 1:1.7 for PS-2. To exclude the possibility that PS-2 had been generated from PS-1 by degradation during acid extraction, PS-1 was extracted again with trichloroacetic acid and rechromatographed on the same

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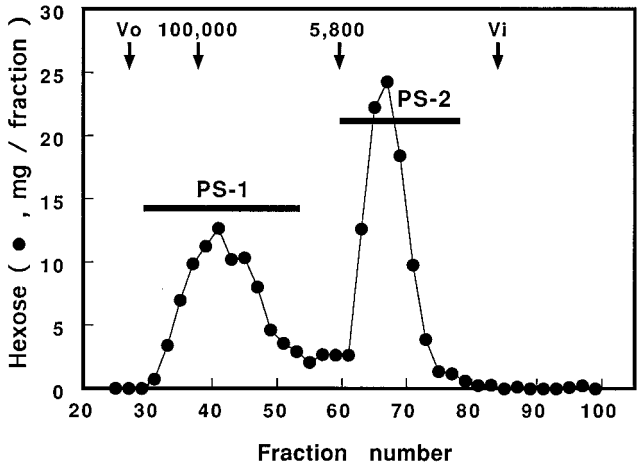

FIG. 1. Preparation of PS-1 and PS-2 by Sephacryl S-300 gel filtration chromatography. Arrows at V_o, 100,000, 5,800, and V_i indicate the elution positions of Blue Dextran 2000, standard pullulan P-100, P-5, and glucose, respectively. Fractions indicated by bars were pooled and designated PS-1 and PS-2.

column, but the position of PS-1 was unchanged and PS-2 was not detected (data not shown).

Examination by nuclear magnetic resonance (NMR) was done to obtain information concerning anomeric conformation and ring size of hexose residues. All NMR spectra were recorded on a JEOL ALPHA-500 spectrometer. Dry PS-1 or PS-2 (4 to 20 mg) was dissolved in 0.5 ml of D_2O, placed in tubes 5 mm in diameter, and deoxygenized by bubbling with nitrogen gas. ^1H NMR spectra were obtained at 30 or 70°C and ^{13}C NMR spectra were obtained at 30°C, using sodium 2,2,3,3-tetradeuterio-3-trimethylsilylpropionate (δ 0.00 ppm) as an internal reference for ^1H spectra and acetonitrile (δ 118.2 ppm) for ^{13}C spectra. PS-1 exhibited eight signals in the combined area of the ^1H NMR spectrum. As each signal integrated for one proton, PS-1 was supposed to have repeating units composed of eight sugar residues. One singlet signal at 5.467 ppm was indicative of an anomeric proton for a β-hexofuranosyl residue, and the other seven signals indicated hexopyranosyl residues. Two of these signals, at 5.263 and 5.152 ppm, were tentatively assigned to α-hexopyranosyl residues, since their coupling constants were 2.6 and 3.8 Hz. The remaining five signals, at 4.730, 4.710, 4.618, 4.548, and 4.466 ppm, were considered to arise from β-hexopyranosyl residues because of their large coupling constants ($J_{1,2} \sim 8$ Hz).

On the other hand, PS-2 gave four anomeric signals in the ^1H and ^{13}C NMR spectra. Signals at 5.258 ppm in the proton spectra and 110.26 ppm in the carbon spectra indicated that PS-2 also contained β-hexofuranose. Because of a vicinal proton coupling constant (2.5 Hz) and chemical shifts, a proton signal at 5.103 ppm and a carbon signal at 100.19 ppm were considered to arise from an α-hexopyranosyl residue. Two partially overlapping proton signals at around 4.51 ppm, along with carbon signals at 103.52 and 103.58 ppm, were assigned to β-hexopyranosyl residues because of the large coupling constants of vicinal protons ($J_{1,2} \sim 8$ Hz) and chemical shifts. Although two anomeric signals from β-hexofuranosyl and α-hexopyranosyl residues integrated for 1 proton, the area of the other two anomeric signals for β-hexopyranosyl residues amounted to only 1.3 protons.

The D or L configuration of glucose and galactose was determined with D-glucose oxidase or D-galactose oxidase and peroxidase (21, 25). The enzymatic analysis indicated that both glucose and galactose in PS-2 had the D configuration (data not shown). These results together with the NMR data described above were consistent with PS-2 containing a tetrasaccharide composed of one β-D-hexofuranosyl, one α-D-hexopyranosyl, and two β-D-hexopyranosyl residues and some units lacking β-D-hexopyranosyl residues in the backbone or side chains.

Smith degradation and methylation analysis of PS-2. In order to obtain more structural information about PS-2, this polysaccharide was oxidized as follows. PS-2 (30 mg) dissolved in 3 ml of 50 mM sodium acetate buffer (pH 5.0) containing 0.1 M sodium periodate was oxidized at 4°C for 4 days in the dark. Ethylene glycol (100 μl) was dropped into the vial of oxidized product to exhaust excess periodate, and the reaction mixture was stirred for 2 h. Reduction proceeded in the same vial for 16 h by adding 4 ml of 0.5 M sodium borohydrate in 0.1 M sodium borate buffer (pH 8.0). Excess borohydrate was disrupted by neutralization with diluted acetic acid, and then the product was dialyzed against distilled water and lyophilized. The resulting polyol was hydrolyzed with 2 ml of 0.1 N sulfuric acid at 25°C for 24 h, and then the Smith degradation products of PS-2 were subjected to gel filtration on Cellulofine GCL 25 (1.5 by 100 cm; Seikagaku Kogyo) in distilled water. Fractions were monitored for both hexose content and refractive index. Only one peak was identified by hexose monitoring, although another peak containing salts and glycerol was recorded later than standard glucose by the refractive index detector (data not shown). The fact that glycerol was the only sugar alcohol produced by Smith degradation indicated that the side chain of PS-2 was a nonreducing terminal residue alone or composed of one or more 6-substituted hexopyranosyl residues and a terminal residue. The hexose-containing fractions that eluted at the column void volume were pooled and lyophilized to give PS-2-S1 (14.3 mg). The apparent molecular weight of PS-2-S1 was measured and estimated to be 5,800 by size exclusion HPLC under the same conditions used for PS-1 and PS-2.

PS-2 and PS-2-S1 (2-4 mg) were methylated according to the method of Ciucanu and Kerek (2), and the products extracted with chloroform were purified by passage through a Sep-pak C_{18} cartridge (Millipore). Methylated polysaccharides were hydrolyzed to be converted into partially methylated alditolacetates according to the method of Harris et al. (9) and analyzed by gas chromatography and gas chromatography-mass spectrometry with DB-17, a fused-silica capillary column (0.3 mm by 30 m; J&W Scientific), and the temperature program 150°C (for 5 min) to 250°C at 5°C/min.

The results of the methylation analysis of PS-2 and PS-2-S1 are summarized in Table 2. Two trimethylated compounds, 2,5,6-trimethylgalactose and 2,4,6-trimethylgalactose, arose from PS-2-S1 in equimolar amounts, indicating 3-substituted galactofuranose and 3-substituted galactopyranose. PS-2-S1 was therefore considered to be a 1,3-linked galactan composed

TABLE 1. Carbohydrate composition of purified cell walls, acid extract, PS-1, and PS-2

Sugar residue	Amt (μg/mg of dry material) in:			
	Purified cell walls	Acid extract	PS-1	PS-2
Glucose	181	302	243	343
Galactose	402	685	728	605
Glucosamine	19	0	0	0
Muramic acid	27	0	0	0
Total	629	987	971	948

TABLE 2. Methylation analysis of PS-2 and PS-2-S1

Compound[a]	Molar ratio in:	
	PS-2	PS-2-S1
2,3,4,6-Tetra-*O*-methylglucose	1.3	0.0
2,5,6-Tri-*O*-methylgalactose	<0.1	1.0
2,4,6-Tri-*O*-methylgalactose	0.8	1.1
2,5-Di-*O*-methylgalactose	1.0	0.0
2,4-Di-*O*-methylgalactose	0.4	0.0
2,4-Di-*O*-methylpentose	0.0	Trace

[a] Methylated compounds were identified as corresponding alditolacetate derivatives.

of galactofuranose and galactopyranose. A trace of unidentified 2,4-dimethylpentose was speculated to be 2,4-dimethylarabinose derived from 3-substituted arabinose that resulted from the Smith degradation of unbranched 3-substituted galactofuranose in the backbone of PS-2 (see below). On the other hand, five compounds, 2,3,4,6-tetra-*O*-methylglucose, 2,5,6-tri-*O*-methylgalactose, 2,4,6-tri-*O*-methylgalactose, 2,5-di-*O*-methylgalactose, and 2,4-di-*O*-methylgalactose, were produced from PS-2 in a molar ratio of 1.3:<0.1:0.8:1.0:0.4 (Table 2). The production of 2,3,4,6-tetra-*O*-methylglucose indicated that glucopyranosyl residues were located in the side chains as terminal residues. The occurrence of 2,4,6- and 2,5,6-tri-*O*-methylgalactose demonstrated that 3-substituted galactopyranose and a small amount of 3-substituted galactofuranose were present in PS-2. Additionally, two dimethylated compounds, 2,5-dimethylgalactose produced from 3,6-disubstituted galactofuranose and 2,4-dimethylgalactose from 3,6-disubstituted galactopyranose, were found and considered to make up the backbone. If these 3- and/or 3,6-substituted residues had been present not only in the backbone but also in the side chains of PS-2, the branched structure should have remained in PS-2-S1 after Smith degradation because of the resistance of these residues to periodate oxidation. PS-2-S1 was, however, considered to be a 1,3-linked linear galactan as described above; hence, 3- and 3,6-disubstituted galactosyl residues were presumed to be present only in the backbone. The molar ratio of 2,5,6-tri-*O*-methylgalactose to 2,5-di-*O*-methylgalactose, <0.1:1.0 (Table 2), indicated that more than 90% of the galactofuranosyl residues branched at position 6. Similarly, from the molar ratio of 2,4,6-tri-*O*-methylgalactose to 2,4-di-*O*-methylgalactose (0.8:0.4 in Table 2), about 30% of galactopyranosyl residues were substituted at position 6.

The results of Smith degradation and the methylation analysis demonstrated that the backbone of PS-2 was a 1,3-linked galactan in which most of the galactofuranosyl residues and two-thirds of the galactopyranosyl residues were substituted by glucopyranose at position 6. Comparing the NMR data for PS-2 with the results of methylation analysis, β-hexofuranose in the NMR data could be regarded as β-galactofuranose, α-hexopyranose could be regarded as α-galactopyranose, and β-hexopyranose could be regarded as β-glucopyranose.

NMR analysis of PS-2-S1. To confirm that the backbone of PS-2 has the repeating units, it is necessary to obtain several oligosaccharide fragments containing more than two galactosyl residues, for example, $Gal_f\beta1\rightarrow3Gal_p\alpha1\rightarrow3Gal$, $Glc_p\beta1\rightarrow6Gal_f\beta1\rightarrow3Gal$, and $Glc_p\beta1\rightarrow6Gal_p\alpha1\rightarrow3Gal$. Thus, we carried out partial acid hydrolysis on PS-2 under several conditions, but all attempts had yielded only one disaccharide, Glcβ1→6Gal, and free galactose. Hence, we further investigated the structure of PS-2-S1, which was considered to be the 1,3-linked backbone galactan of PS-2.

TABLE 3. ^{1}H NMR chemical shift data of PS-2-S1

Sugar residue	Chemical shift (ppm)[a]						
	H-1	H-2	H-3	H-4	H-5	H-6	H-6
→3)-β-D-Gal_f-(1→	5.242	4.411	4.094	4.276	3.880	3.719	3.692
→3)-α-D-Gal_p-(1→	5.090	3.959	3.937	4.123	4.155	3.916	3.772

[a] Measured at 70°C in D_2O with sodium 2,2,3,3-tetradeuterio-3-trimethylsilylpropionate as the internal chemical shift reference (δ 0.00 ppm).

Two-dimensional NMR spectra, correlated spectroscopy and heteronuclear multiple quantum correlated spectroscopy spectra, which were obtained with standard pulse sequences, permitted the complete assignment of all proton and carbon signals of PS-2-S1. ^{1}H and ^{13}C NMR chemical shifts for PS-2-S1, summarized in Tables 3 and 4, are consistent with those of →3)-β-D-Gal_f-(1→3)-α-D-Gal_p-(1→ from *P. haemolytica* lipopolysaccharide O antigen (23).

Since the resonances for two anomeric protons were separated fully, nuclear Overhauser effect (NOE) measurements were carried out. The NOE experiment has been widely used to obtain information about the positions of glycosidic linkages. NOE differential spectra were acquired by using a selective presaturation pulse applied to the anomeric signals for 170 ms, followed by a 90° observation pulse. Preirradiation of the anomeric proton of β-galactofuranose (5.242 ppm) caused a negative NOE to the H-3 signal of α-galactopyranose (3.937 ppm) and to the signal of its own H-2 proton (4.411 ppm). The former interresidual NOE was indicative of $Gal_f1\rightarrow3Gal_p$ linkage, and the latter occurrence of the intraresidual NOE might be due to the fact that the conformation of the galactofuranose ring system positions H-2 close to the anomeric proton. Another interresidual NOE, by which the $Gal_p1\rightarrow3Gal_f$ linkage was verified, was also observed at the H-3 signal of β-galactofuranose (4.094 ppm) after irradiation of the anomeric proton of α-galactopyranose (5.090 ppm). These results indicated that PS-2-S1 was a 1,3-linked galactan in which β-galactofuranosyl and α-galactopyranosyl residues alternate.

The proposed structure of PS-2. From the above results, we concluded that PS-2 consists of →3)-β-D-Gal_f-(1→3)-α-D-Gal_p-(1→ backbone disaccharide repeating units in which more than 90% of the β-galactofuranosyl residues and about 30% of the α-galactopyranosyl residues were substituted at O-6 with β-D-glucopyranose. By dividing the apparent molecular weight of PS-2-S1 by that of the repeating unit, the average number of repeating units was estimated to be 18.

Several questions remain unanswerable: (i) which of β-galactofuranose and α-galactopyranose is present at the reducing end of PS-2, (ii) which residue of peptidoglycan is responsible for the linkage between PS-2 and peptidoglycan, and (iii) whether a di- or trisaccharide linkage unit is included as found in some other bacteria (11, 16, 29). We attempted to isolate both PS-1–peptidoglycan and PS-2–peptidoglycan complexes by lytic enzyme digestion using lysozyme and *N*-acetylmurami-

TABLE 4. ^{13}C NMR chemical shift data of PS-2-S1

Sugar residue	Chemical shift (ppm)[a]					
	C-1	C-2	C-3	C-4	C-5	C-6
→3)-β-D-Gal_f-(1→	110.51	81.00	85.77	83.41	72.23	64.14
→3)-α-D-Gal_p-(1→	100.75	68.55	78.33	72.55	40.56	62.50

[a] Measured at 30°C in D_2O with acetonitrile as the internal chemical shift reference (δ 118.2 ppm).

dase SG to obtain information about the linkage region; however, the purified cell walls were resistant to such enzymatic digestions. While the linkage region between PS-2 and peptidoglycan has not been identified yet, PS-2 as well as many other bacterial cell wall polymers is presumed to link to the muramic acid 6-phosphate of peptidoglycan through a phosphodiester linkage, because the purified cell walls contained only glucose, galactose, and peptidoglycan components, except for a slight level of fatty acid.

Recently, human natural antigalactosyl antibody has been reported to interact with some bacteria isolated from human flora (6, 8). We have found that *B. infantis* cell walls activate the alternative pathway of human complement system and that this ability of the cell walls is abolished by removing the polysaccharides (unpublished experiments), suggesting that the cell wall polysaccharides react with the antibody. Since PS-1 and PS-2 amount to 30 and 40% of the purified cell walls, respectively, and occupy the major portion of the cell wall mass, they should be significant to this organism and may participate in the immunopotentiating activity of the cell walls (24). The biological roles of these cell wall polysaccharides and the structure of PS-1 are now under investigation in our laboratory.

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