ELSEVIER
Carbohydrate Research 274 (1995) 245–249
CARBOHYDRATE RESEARCH

# Structural studies on a cell wall polysaccharide from *Bifidobacterium longum* YIT4028

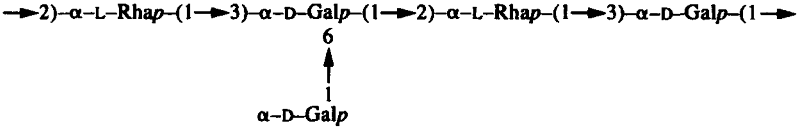
Masato Nagaoka *, Hideyuki Shibata, Itsuko Kimura,
Shusuke Hashimoto, Kazumasa Kimura, Haruji Sawada,
Teruo Yokokura

*Yakult Central Institute for Microbiological Research, 1796 Yaho, Kunitachi, Tokyo 186, Japan*

Received 23 September 1994; accepted in revised form 20 January 1995

## Abstract

The major fraction of rhamnogalactan was isolated from the cell wall of *Bifidobacterium longum* YIT4028 by treatment with *N*-acetylmuramidase. This polysaccharide was composed of rhamnose and galactose in a molar ratio of about 2 : 3. The $^{13}$C NMR spectrum indicated that it contained a pentasaccharides repeating unit. This observation, and the results of Smith degradation, partial acid hydrolysis and methylation analysis led to the conclusion that its structure is



$$\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\underset{\substack{6\\ \uparrow \\ 1 \\ \alpha\text{-D-Gal}p}}{\alpha\text{-D-Gal}p}\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow$$

*Keywords: Bifidobacterium longum*; Cell wall; Polysaccharide

## 1. Introduction

In previous papers, we described the polysaccharide components and their structures of the cell walls of some Bifidobacteria [1,2]. These cell wall polysaccharides could be

---

* Corresponding author.

*SSDI* 0008-6215(95)00076-3

classified into two types, i.e., galactan and 6-deoxyhexose (such as rhamnose or 6-deoxytalose)-containing polysaccharides. These polysaccharides are typical of Bifidobacteria. *Bifidobacterium longum* was isolated from human feces [3]. The cell wall polysaccharide of this organism also contains rhamnose residues, and it has pentasaccharides as the repeating unit [1]. In this paper, we report the structure of the cell wall polysaccharide of *Bifidobacterium longum* YIT4028.

## 2. Materials and methods

*Organism.—Bifidobacterium longum* YIT4028 (ATCC15707) was used in this study. The basal medium and culture conditions for this organism were described previously [1]. Cells were harvested by centrifugation (20,000 × *g* for 45 min at 4°C) and washed three times with distilled water. They were then suspended in 5 mM Tris-malate buffer (pH 6.5) and heated at 100°C for 10 min. The heat-killed cells were centrifuged, and resuspended in the same buffer containing 0.9% NaCl and 2 mM $MgCl_2$.

*Isolation and fractionation of cell wall polysaccharide.*—The method used for the preparation of cell wall polysaccharide was reported previouly [1,2]. In brief, a suspension of this organism was treated with *N*-acetylmuramidase. Insoluble material was removed by centrifugation, and the supernatant was treated with DNase and RNase. This solution was digested with trypsin and then dialyzed against distilled water. The dialyzate was chromatographed on a column of Sephacryl S-200 HR (2 × 90 cm) in 0.05 M $(NH_4)_2CO_3$. Fractions were collected and assayed for hexose. Only one peak was obtained, being designated as the polysaccharide fraction (PSF).

*Component analyses.*—In general, the methods used for the release, identification and determination of monosaccharides were those used previously [1,2,4]. The absolute configuration of the galactose residue of PSF was determined to be the D-configuration, as described in a previous paper [1]. The absolute configuration of the rhamnose residue of PSF was determined by the method of Gerwig et al. [5].

*Partial acid hydrolysis of PSF.*—Partial acid hydrolysis of PSF (10 mg) was carried out by treatment with 30 mM trifluoroacetic acid for 20 h at 100°C. The product was then lyophilized and dissolved in a small volume of water. The resulting solution was subjected to descending paper chromatography (Toyo No.50 filter paper in 1-butanol–acetic acid–water 2:1:1). Oligosaccharides were detected with silver nitrate reagent [6].

*Periodate oxidation and Smith degradation.*—Smith degradation was performed in the same manner as described previously [2,3]. In brief, PSF was oxidized with 0.2 M $NaIO_4$ in 0.5 mL of sodium acetate buffer (pH 5.0) for 20 h at 4°C. After destruction of residual $NaIO_4$ by the addition of ethylene glycol, the product was reduced by treatment with $NaBH_4$ for 20 h at 4°C and then dialyzed. A portion of the resulting non-dialyzable fraction (300 nmol as hexose) was hydrolyzed with 2 M trifluoroacetic acid at 100°C for 2 h for component analysis by GLC. The remaining material was treated with 0.5 M HCl at 20°C for 20 h. The mild acid-treated material was then concentrated and subjected to paper chromatography under the same conditions as described above.

*Other materials and methods.*—Unless otherwise indicated, the Materials and methods were the same as described in previous papers [2,4]. Total hexose was determined

Table 1
Methylation analysis of PSF

| Partially methylated alditol acetate [a] | Peak area ratio [b] |
|---|---|
| 3,4-Di-*O*-methyl-rhamnitol | 2.11 |
| 2,3,4,6-Tetra-*O*-methyl-galactitol | 1.00 |
| 2,4,6-Tri-*O*-methyl-galactitol | 1.00 |
| 2,4-Di-*O*-methyl-galactitol | 0.99 |

[a] Assignment was carried out on the basis of retention times and fragmentation pattern on GLC-MS.
[b] The peak area ratio; 2,3,4,6-tetra-*O*-methyl-galactitol taken to be 1.00.

by the phenol/$H_2SO_4$ method [7]. Permethylation was carried out by the method of Ciucanu and Kerek [8]. The permethylated material was subjected to hydrolysis, and the resulting monosaccharide derivatives were converted to alditol acetates and then analyzed by GLC-MS [4]. $^{13}$C NMR spectra were recorded (with acetone as the internal standard, $\delta$30.5), for a solution in $D_2O$ at 50°C on a Jeol FX400 NMR spectrometer. $^1$H NMR spectra were recorded at 60°C in $D_2O$ with tetradeuterio-3-trimethylsilylpropanoate as the internal standard. The $^{13}$C–$^1$H coupling constants were determined by distortionless enhancement according to the polarization transfer non-decoupling method (DEPT) [9].

## 3. Results and discussion

The acid hydrolysate of the polysaccharide moiety was composed of galactose and rhamnose in a molar ratio of about 3 : 2. A small amount of peptidoglycan component was also detected, but was not investigated in this study. The absolute configuration of the galactose was previously determined to be the D-configuration, [1]. The absolute configuration of the rhamnose residue was determined to be the L-configuration by means of the (−)-2-butanolysis procedure, followed by GLC analysis [5].

The results of methylation analysis of PSF are shown in Table 1. These data indicate that the repeating unit of PSF consists of two galactose and two rhamnose residues as a backbone, with one galactose residue as a side chain.

$^{13}$C NMR spectra of PSF were recorded and the anomeric signals of this polysaccharide are summarized in Table 2. The data indicate that this polysaccharide contains a

Table 2
The anomeric carbon signals of PSF

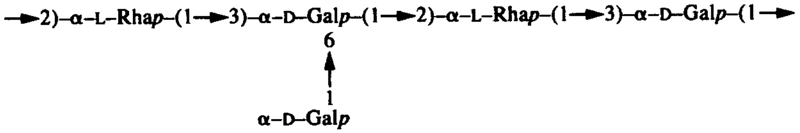
| Chemical shifts ($\delta$) [a] | Assignment |
|---|---|
| 99.84 (173) [b] 99.62 (173) | → 2)Rha α(1 → → 2)Rha α(1 → |
| 98.95 (171) | Gal α(1 → |
| 98.18 (171) 97.93 (173) | → 3)Gal α(1 → [c] → 3,6)Gal α(1 → [c] |

[a] Minor differences between these data and those previously reported [1] may be due to differences in the recording temperature.
[b] $^1J_{C1,H1}$ values (Hz) are given in parentheses.
[c] Assignments may be interchanged.

pentasaccharide repeating unit and that all component sugars are α-linked [1]. This interpretation was confirmed by $^1$H NMR and $^1J_{C,H}$ values. The $^1$H spectrum of PSF exhibited signals of three different anomeric protons (δ4.99, 1 H; δ5.07, 2 H; and δ5.18, 2 H). The doublet signals at δ4.99 and δ5.18 were assigned to α- linked galactopyranose residues; this assignment is consistent with the observed $^1$H–$^1$H coupling constants ($^3J_{H1,H2} = 3.29$ and 3.23 Hz, respectively). The semidoublet signal ($^3J_{H1,H2} \fallingdotseq 1.5$ Hz) at δ5.07 ppm was tentatively assigned to α-linked rhamnose residues on the basis of its chemical shift [10]. Furthermore, all $^1J_{C1,H1}$ values for these component sugars exceeded 170 Hz, which also support the presence of α-linkages (Table 2) [11]. $NaIO_4$ oxidation was carried out on PSF, and after reduction of the oxidized material with $NaBH_4$, component analysis was performed. The resulting material gave galactose and glycerol in a molar ratio of about 1.89 : 1.00. Considering the results of methylation analysis of PSF, propanediol should also be produced by this procedure, but could not be detected due to overlapping of the solvent peak under the GLC conditions used. The proportion of resistant galactose residues was two-thirds, and one part of glycerol was obtained with this procedure. The glycerol may derive from oxidized galactose residues, since glycerol was not formed from rhamnose residues by this procedure.

The product of Smith degradation was subjected to paper chromatography. A single spot was detected and extracted with water. This fraction (Fraction X, $R_{Glc} = 0.66$, 1.08 μmol as hexose) was acid hydrolyzed and analyzed by GLC. Only galactose was identified under the conditions used. However, galactose and glycerol were detected, in a molar ratio of 1.00 : 1.13, after $NaBH_4$ reduction. So Fraction X was identified as galactosylglyceraldehyde. Considering the results of methylation analysis, this glyceraldehyde residue may derive from the 2-*O*-substituted rhamnose residue detected by Smith degradation.

Partial acid hydrolysis of PSF was carried out and the product was subjected to paper chromatography, three main oligosaccharide fractions (Fractions A, B and C) were obtained. The yields of Fraction A ($R_{gal} = 0.736$), Fraction B ($R_{gal} = 0.552$), and Fraction C ($R_{gal} = 0.467$) were 4.8 μmol, 3.2 μmol, and 2.1 μmol as hexose, respectively. The yield of Fraction C was much lower than those of Fraction A and Fraction B, and since the spots of Fraction B and Fraction C were close to each other, we only extracted the central region of the spot of Fraction C. Thus, the molar ratio of these fractions could not be determined in this experiment. Fraction A was composed of galactose and rhamnose in a molar ratio of about 1.3 : 1.0. Fraction B was composed of galactose alone. Fraction C was composed of galactose and rhamnose in a molar ratio of about 1.7 : 1.0. On methylation analysis, 2,3,4,6-tetra-*O*-methyl-galactitol and 3,4-di-*O*-methyl-rhamnitol were detected for Fraction A, 2,3,4,6-tetra-*O*-methyl-galactitol and 2,3,4-tri-*O*-methyl-galactitol for Fraction B, and 2,3,4,6-tetra-*O*-methyl-galactitol, 2,3,4-tri-*O*-methyl-galactitol and 3,4-di-*O*-methyl-rhamnitol for Fraction C. The data indicate the structures of Fraction A and B are galactosyl(1 → 2)rhamnose and galactosyl(1 → 6)galactose, respectively. The reducing terminal of Fraction C was rhamnose, as determined by $NaBH_4$ reduction of Fraction C. From the results of methylation analysis of Fraction C, the structure of Fraction C is deduced to be galactosyl(1 → 6)galactosyl(1 → 2)rhamnose. Taking into account the structures of Fraction X and Fraction A, and the results of methylation analysis, the galactose residue of the backbone chain is linked at C-2 of a rhamnose residue. So the structure of the backbone chain of PSF is → 2Rha1 → 3Gal1 →. In addition, half of the galactose residues of the backbone chain are substituted at the C-6 position by a branched galactose residue, which was supported by the structures of Fraction B and C. These results indicate that the most likely repeating structure of PSF is



$$\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\underset{\substack{6\\ \uparrow\\ 1\\ \alpha\text{-D-Gal}p}}{\alpha\text{-D-Gal}p}\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow$$

The apparent molecular mass of this polysaccharide, as estimated by chromatography on Sephacryl S-200, is about 12.5 kDa. The backbone chain consists of alternating galactose and rhamnose residues. In the case of 6-deoxyhexose-containing polysaccharides in Bifidobacteria, most of the backbone chain has only 6-deoxysugars as the repeating units, and their basic structures resemble each other [1,2,12]. The backbone chain of the polysaccharide of *B. longum* differs from those of these bacteria.

In this study, we did not investigate the linkage region between the polysaccharide and peptidoglycan moieties. The structure of this region is currently under investigation.

## Acknowledgement

We are grateful to Dr Toshiaki Osawa for helpful advice.

## References

[1] Y. Habu, M. Nagaoka, T. Yokokura, and I. Azuma, *J. Biochem. (Tokyo)*, 102 (1987) 1423–1432.
[2] M. Nagaoka, M. Muto, T. Yokokura, and M. Mutai, *J. Biochem. (Tokyo)*, 103 (1988) 618–621.
[3] E. Lauer and O. Kandler, *System. Appl. Microbiol.*, 4 (1983) 42–64.
[4] M. Nagaoka, M. Muto, K. Nomoto, T. Matsuzaki, T. Watanabe, and T. Yokokura, *J. Biochem. (Tokyo)*, 108 (1990) 568–571.
[5] G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.
[6] W.E. Trevelyan, D.P. Procter, and J.C. Harrison, *Nature*, 166 (1950) 444–445.
[7] M. Dubois, J.K. Gilles, J.K. Hamilton, P.A. Roberts, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
[8] I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.
[9] D.M. Doddrell, D.T. Pegg, and M.R. Bendall, *J. Magn. Reson.*, 48 (1982) 323–327.
[10] A.S. Shaskov, E.V. Vinogradv, E.D. Daeva, Y.A. Knirel, G.M. Zdorovenko, N.Y. Gubanoba, L.M. Yakovleva, and I.Y. Zakharova, *Carbohydr. Res.*, 212 (1991) 301–305.
[11] L.D. Hall and G.A. Morris, *Carbohydr. Res.*, 82 (1980) 175–184.
[12] J.H. Veerkamp, G.E.J.M. Hoelen, and H.J.M. Op Den Camp, *Biochim. Biophys. Acta,* 755 (1983) 439–451.