Chemical and Immunological Properties of Cell Wall Polysaccharide of Leuconostoc mesenteroides subsp. cremoris Isolated from the Commercial Fermented Milk, “Filmjolk"

その他（別言語等）のタイトル	市販発酵乳“フィルムヨーク”から分離したLeuconostoc mesenteroides subsp. cremorisの細胞壁多糖の化学的，免疫学的性質の解析
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原　報

Chemical and Immunological Properties of Cell Wall Polysaccharide of *Leuconostoc mesenteroides* subsp. *cremoris* Isolated from the Commercial Fermented Milk, "Filmjolk"

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Summary

Cell wall polysaccharides were separated from *Leuconostoc mesenteroides* subsp. *cremoris* isolated from "Filmjolk", a fermented milk made commercially in Sweden. Two components (CNP1 and CNP2) were further purified by gel filtration on HPLC and the molecular weights were determined to be 850,000 and 81,000. CNP–2 was shown to contain two polysaccharides in major and minor by an ^{1}H–NMR. The major polysaccharide was shown to have a heptasaccharide repeating unit which contained five α and two β anomers, and also contained non reducing galactopyranose, O–2,3 disubstituted glucopyranose, O–3 substituted galactopyranose and O–3,6 disubstituted galactopyranose as units in a molar ratio of 3 : 1 : 1 : 2. On the other hand, the minor polysaccharide was shown to have a tetrasaccharide repeating unit which contained three α and one β anomers, and also contained non reducing glucopyranose, O–3,6 disubstituted glucofuranose, O–4 substituted glucopyranose and O–6 substituted N–acetylglucosamine as units in a molar ratio of 1 : 1 : 1 : 1. Both CNP1 and CNP2 significantly stimulated the growth of mice spleen cells in vitro.

Introduction

Dairy lactic acid bacteria produce lactate and acetate from lactose during the process of manufacturing fermented milk. Some of the dairy starters such as Bifidobacterium and Acidophilus colonize in the human large intestine to acidfy and then repress the growth of injurious bacteria such as Enterococcus as well as the activities of some enzymes produced by such harmful bacteria[1,2,3]. Thus it has been reported that some dairy starters improve the microflora in the human intestine.

Moreover, the polysaccharides produced by lactic acid bacteria have been reported to have biological functions such as mitogenic activities and antitumor activities. Kitazawa *et al.*[4,5,6] reported that exocellular polysaccharides produced by *Lactococcus lactis* subsp. *cremoris* KVS20 activated macrophage in vitro. Barry *et al.* reported that the growth of the intestinal tumors induced by DMH were inhibited by oral administration of *Lactobacillus* GG[7].

In this study, we try to clarify the chemical property and mitogenic activity of cell wall polysaccharides produced by *Leuconostoc mesenteroides* subsp. *cremoris* isolated from the fermented milk, "Filmjolk", commercially made in Sweden.

Materials and methods

Materials

"Filmjolk" was purchased from a local supermarket in Stockholm in 1996. Specific pathogen-free male C57BL/66 mice were purchased from Japan SLC, Inc (Hamamatsu, Japan). All mice were used at 6 to 7 weeks of age.

Microorganism

The strain of *Leuconostoc mesenteroides* subsp. *cremoris* was isolated by plating diluted "Filmjolk". The identification of the strain was performed according to Bergey's Manual of Systematic Bacteriology[8,9].

Preparation of cell wall polysaccharide

The preculture of the strain was inoculated into the partially deproteinized whey containing 1% peptone and incubated at 30℃ for 72 hr, followed by centrifugation at 7000 r.p.m. for 20 min to collect the precipitate of the cell body. The cell body was suspended in the medium and disrupted by sonification at 9 KHz, 200 W for 10 min, followed by another centrifugation. After heating of the precipitate at 100℃ for 10 min, the pellet was treated with DNase and RNase (2 mg/m*l*, DNase: Funakoshi, Japan, RNase: Sigma, USA) at 37℃ for 24 hr, followed by re-centrifugation. The pellet was extracted with 4% SDS solution for 30 min. The contaminant of the protein was then digested with Pronase E (2 mg/m*l*: Cosmo Bio Co., Japan). The cell wall component was re-extracted with a 1% SDS solution at room temperature for 30 min and then a 3% TCA at 37℃ for 48 hr, followed by centrifugation at 7000 r.p.m. for 20 min. The supernatant was recovered and dialyzed with distilled water. The retentate was lyophilized.

The cell wall polysaccharide was purified from the retentate on the ion exchange column (1.5×25 cm) chromatography with a DEAE–TOYO PEARL 650 M (Tosoh Co., Japan) equilibrated with a 50 mM Tris HCl buffer. The unadsorbed component, named CNP, in column was eluted with 250 m*l* of buffer, and the adsorbed component was eluted with a linear gradient of NaCl from 0 to 1.0 M of a total volume 500 m*l* of the buffer. The components in each fraction were monitored by absorbance at 280 nm, and at 490 nm with the phenol $-H_2SO_4$ method. Two components, named CNP1 and CNP2, were further separated from CNP by gel filtration on HPLC. The conditions on the HPLC is described as below.

Chemical analyses of cell wall polysaccharide

The molecular weights were determined by HPLC equipped with a TSK–Gel G 6000 PWXL (7.8 mm ID×30 cm L, Tosoh Co., Japan) column. The components (CNP1 and CNP2) were eluted with distilled water and monitored with an RI detector. A Shodex standard Pullulan kit P 800 (Showa Denko Co., Tokyo, Japan) was used as the standard.

The methanolyses of CNP1 and CNP2 were performed in 2% HCl–methanol at 80℃ for 20 hr. The 2%HCl–methanol was prepared from 5%HCl–methanol (Wako Co., Tokyo, Japan). The methanolysates were trimethylsilylated with a TMS HT kit (Tokyo Kasei Co., Tokyo, Japan) and subjected to gas chromatography with a Shimadzu 13B gas chromatograph. The chromatograph was equipped with a capillary column DB–17

(0.32 mm × 30 m) and operated at a temperature gradient of 3°C/min from 150 to 250°C.

The CNP2 was methylated by the method of Hakomori[10] and the methylate was purified by passage through a silica gel column of Wakogel S-1 (Wako Co., Japan). The alditol acetates were derived from the permethylate by the method of Stellner *et al.*[11].

The partially methylated alditol acetates were analysed on a Shimadzu GC/MS-QP 2000 mass spectrometer operated at a GC condition with a temperature gradient at 3°C/min from 150 to 250°C. The partially methylated alditol acetates prepared from Galβ1-4GlcNAcβ1-3Galβ1-4Glc, Galβ1-4GlcNAcβ1-6Galβ1-4Glc and Galβ1-4GlcNAcβ1-3[Galβ1-4GlcNAcβ1-6]Galβ1-4Glc separated from horse colostrum[12] were used as the standard.

^{1}H-NMR

The CNP2 was dissolved in 1 ml D_2O (100.00%, atom %D, Aldrich, USA) and put into a NMR tube. Chemical shifts were expressed relative to internal 3-(trimethylsilyl)-1-propane sulfonic acid sodium salt (TPS), but were actually measured by reference to internal acetone (δ 2.225). The ^{1}H-NMR was recorded in D_2O at 600 MHz with a Varian INOVA 600 spectrometer, with the probe temperature at 293.1 K.

Mitogenic response

Spleen cells were prepared from murine spleens by gentle mincing and tapping on a 200-mesh stainless steel screen in a RPMI-1640 medium[5]. The cells were collected by centrifugation and washed twice with a RPMI medium. After hemolysis of erythrocytes with 0.2%NaCl, the cells were washed with saline. The cells were suspended at a concentration of 2×10^6 cells/m*l* in a RPMI-1640 medium, supplemented with a mixture of streptomycin (100 μg/m*l*), penicillin (100 IU/m*l*), fungizon (2.5 μg/m*l*), 0.3% L-glutamin and 2% fetal serum.

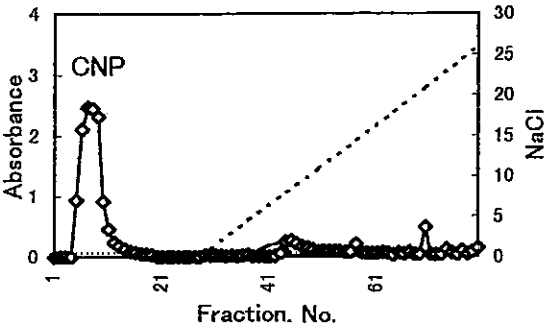


Fig. 1 The anion exchange chromatography of cell wall polysaccharide separated from *Leuconostoc mesenteroides* subsp. *cremoris* with DEAE-TOYO Pearl 650M column (1.5 × 25 cm)
The absorbance of each fraction was determined at 490 nm (◇) with phenol-H_2SO_4 method and 280 nm (—).

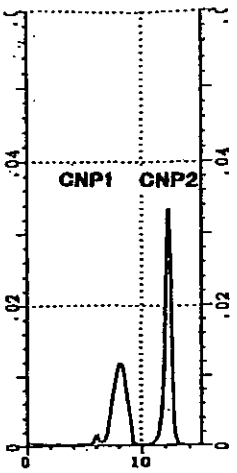
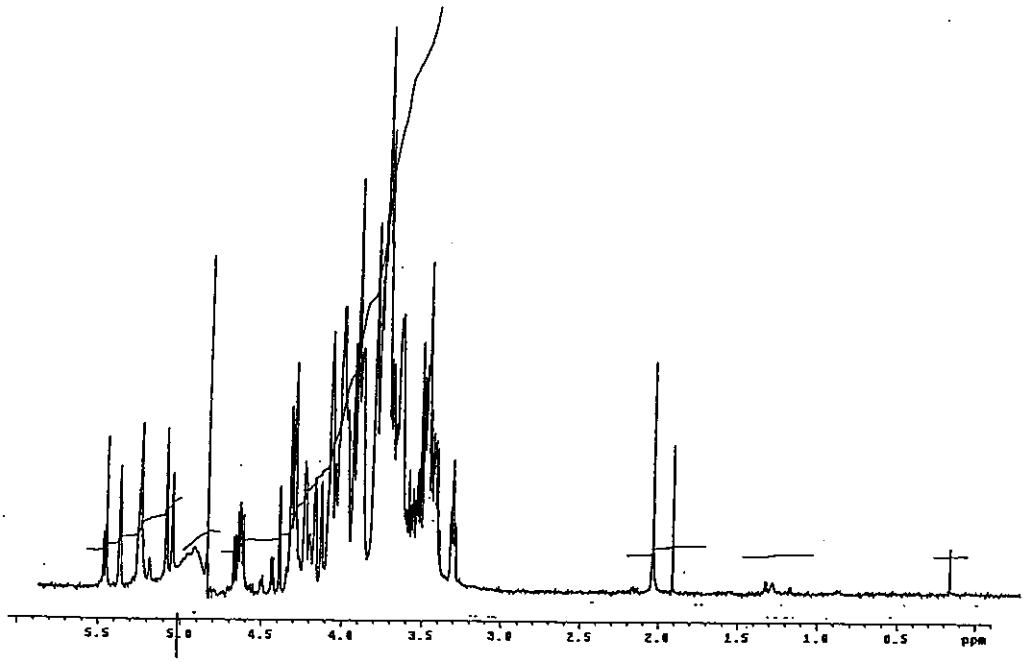


Fig. 2 HPLC of cell wall polysaccharide separated from *Leuconostoc mesenteroides* subsp. *cremoris* with a column of TSKgel G6000 (7.8 mm I.D. × 30 cm).



Chemical shifts

Major polysaccharide of CNP2

α anomer signals	β anomer signals
δ 5.447	δ 4.618
5.363	4.63
5.233 (2)	
5.08	

Minor polysaccharide of CNP2

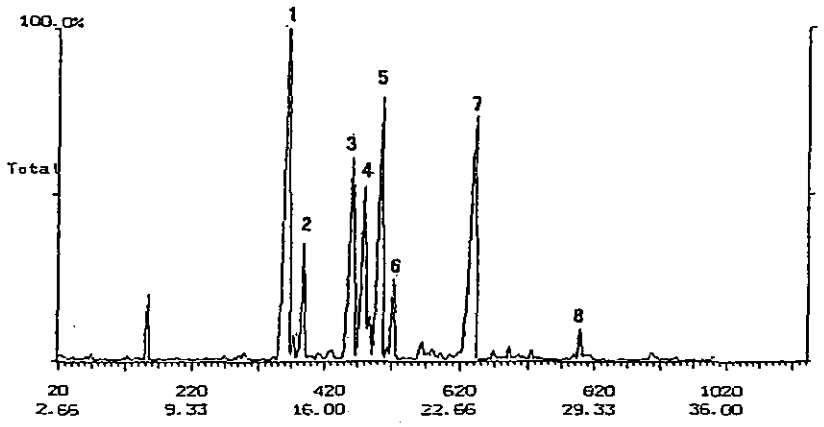
α anomer signals	β anomer signal
δ 5.465	δ 4.667
5.247	
5.041	

Fig. 3 ^{1}H–NMR spectrum of CNP2 separated from *Leuconostoc mesenteroides* subsp. *cremoris*. The spectra was obtained at 293.1 K and recorded in D_2O (100.00%D). Chemical shifts (ppm) are expressed down field from internal TPS, but were actually measured by reference to internal acetone (δ=2.225).

Mitogenic responses were determined by the incorporation of [^{3}H]TdR into spleen cell[5]. An aliquot of the spleen cell suspension was put into the well on a round-bottom microtiter plate. Spleen cells were cultured with each of CNP1 and CNP2 in 5% CO_2 and 95% air at 37°C for 48 hr. [^{3}H] TdR (Amersham Pharmacia Biotech. Tokyo, Japan) was added to all cultures 6 hr prior to harvesting on a glass filter (M & S Co., Tokyo, Japan). The amount of [^{3}H]TdR incorporated into the spleen cells was counted with a scintillation counter. The mitogenic responses were expressed as a Stimulation Index (S.I.) calculated as follows: S.I.=count per minute in treated/count per minute in control.

Results and Discussion

The neutral cell wall polysaccharide, CNP, was purified by an anion exchange chromatography on a DEAE–TOYO Pearl 650M (Fig. 1). Two components, CNP1 and CNP2, were separated from the CNP by gel filtration



Major polysaccharide

1: 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylgalactitol
3: 1,2,3,5-tetra-O-acetyl-4,6-di-O-methylglucitol
5: 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylgalactitol
7: 1,3,5,6-tetra-O-acetyl-2,4-di-O-methylgalactitol

Minor polysacccharide

2: 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol
4: 1,3,4,6-tetra-O-acetyl-2,5-di-O-methylglucitol
6: 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol
8: 1,5,6-tri-O-acetyl-3,4-di-O-methyl-2-N- methylacetamido-2-deoxyglucitol

Fig. 4 Total ion monitored mass spectrum of the partially methylated alditol acetates prepared from CNP2 of *Leuconostoc mesenteroides* subsp. *cremoris*.

on HPLC (Fig. 2). The molecular weights of CNP1 and CNP2 were estimated to be around 850,000 (CNP1) and 81,000 (CNP2). The GC after methanolysis showed that CNP1 and CNP2 are composed of Glc, Gal and GlcNAc in molar ratios of 1.0 : 0.6 : 0.2 and 1.0 : 2.3 : 0.3, respectively. Further analyses were performed on CNP2 only.

From the intensities of the anomeric signals of CNP2 in the ^{1}H–NMR (Fig. 3), it was estimated that CNP2 was composed of two polysaccharides in major and minor. The ^{1}H–NMR of the major polysaccharide had anomeric signals at δ 5.447 (1H), δ 5.363 (1H), δ 5.233 (2H), δ 5.08 (1H), δ 4.618 (1H) and δ 4.63 (1H), whereas the minor polysaccharide had signals at δ 5.465 (1H), δ 5.247 (1H), δ 5.041 (1H) and δ 4.667 (1H). From the intensity of the anomeric signals, the major polysaccharide was assumed to have a heptasaccharide repeating unit of five α and two β anomers, whereas the minor polysaccharide contained a tetrasaccharide repeating unit of three α and one β anomers.

The analysis of the partially methylated alditol acetates prepared from CNP2 showed that the repeating unit of the major polysaccharide was composed of non reducing galactopyranose, O–2,3 disubstituted glucopyranose, O–3 substituted galactopyranose and O–3,6 disubstituted galactopyranose in the molar ratio of 3 : 1 : 1 : 2. On the other hand, the minor polysaccharide was shown that it was composed of non reducing glucopyranose, O–3,6 disubstituted glucofuranose, O–4 substituted glucopyranose and O–6 substituted N–acetylglucosamine in the molar ratio of 1 : 1 : 1 : 1. From the above data, the major polysaccharide in CNP2 was shown to have a heptasaccharide repeating unit of six Gal and one Glc, whereas the minor polysaccharide had a tetrasaccharide repeating unit

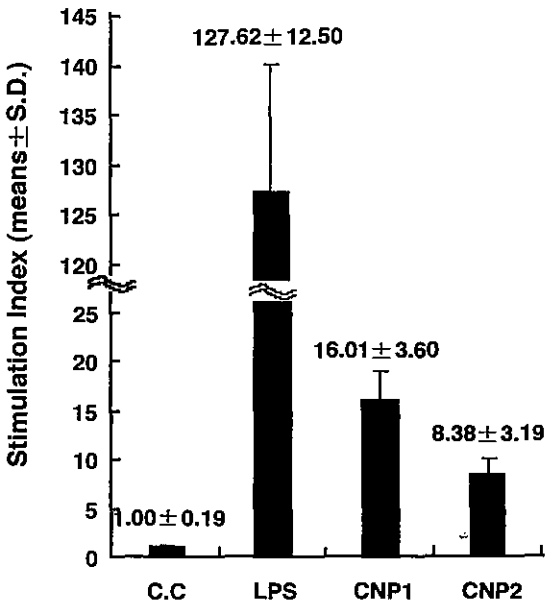


Fig. 5 The mitogenic activities of CNP1 and CNP2 separated from cell wall polysaccharide of *Leuconostoc mesenteroides* subsp. *cremoris*. Values are the means SI and the standard deviation of triplicate cultures.

of three glucose and one N–acetylglucosamine. These types of polysaccharides are assumed to be different from the exocellular and cell wall polysaccharides produced by dairy lactic acid bacteria, which have been previously reported[13~24].

The mitogenic activities on CNP1 and CNP2 are shown in Fig. 5. They significantly stimulated the growth of spleen cells. The acitivity in CNP1 was higher than that in CNP2. This data suggested that the strength of the mitogenic activity may correlate to the molecular weights of the polysaccharides. The positive effects of CNP1 and CNP2 on the mitogenic activities may show that one of the functional activities of "Filmjolk" is caused by the cell wall polysaccharides on the starter strain, *Leuconostoc mesenteroides* subsp. *cremoris*.

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市販発酵乳“フィルムヨーク”から分離した *Leuconostoc mesenteroides* subsp. *cremoris* の細胞壁多糖の化学的，免疫学的性質の解析

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スウェーデンで市販されている発酵乳“Filmjolk”から分離した *Leuconostoc mesenteroides* subsp. *cremoris* より，細胞壁多糖を分離した。同多糖はさらに，HPLC によるゲル濾過で２つの成分（CNP1 と CNP2）に分離され，その分子量は850,000また81,000と推定された。CNP2 は ^{1}H-NMR 分析によって，２種の多糖からなることが示された。そのうちの主要成分は，５個のαアノマーおよび２個のβアノマーと，残基ユニットとして非還元末端ガラクトピラノース，2,3 位置換ガラクトピラノース，３位置換ガラクトピラノースおよび 3,6 位置換ガラクトピラノースを，３：１：１：２のモル比で含むことが示された。一方少量成分は，３個のαアノマーおよび１個のβアノマーと，残基ユニットとして非還元末端グルコピラノース，3,6 位置換グルコフラノース，４位置換グルコピラノースおよび６位置換 N-アセチルグルコサミンを，１：１：１：１のモル比で含むことが示された。CNP1, CNP2 とも，試験管内でマウス脾臓細胞の増殖を有意に促進することが示された。