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A mannofucogalactan, fomitellan A, with mitogenic effect from fruit bodies of *Fomitella fraxinea* (Imaz.)

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Abstract

A heterogalactan, fomitellan A, was isolated from a 0.9% sodium chloride extract of the fruit bodies of *Fomitella fraxinea* by a combination of fractionation procedures, including precipitation with ethanol and chromatography on columns of DEAE–cellulose and Toyopearl HW 65F. On employing gel permeation–high performance liquid chromatograpy, fomitellan A exhibited a single peak with a molecular weight of 15 kDa. The results of methylation, and ^{1}H and ^{13}C NMR spectroscopic analyses indicated that the heterogalactan fomitellan A was a repeating unit (pentasaccharide) composed of a backbone with (1 → 6)-linked D-galactopyranosyl residues. Its C-2 atom position was substituted with disaccharide units of 3-*O*-D-mannopyranosyl-L-fucopyranosyl residues. The fucomannaogalactan (fomitellan A) from *Fomitella fraxinea* showed pronounced immunostimulating activity in an in vitro lymphocytes proliferation assay.

Keywords: Mannofucogalactan; Fomitellan A; Mitogenic effect; *Fomitella fraxinea*

1. Introduction

Polysaccharides are essential constituents of all living organisms and are associated with a variety of vital functions (Srivastava & Kulshreshtha, 1989). They are found most abundantly in seaweeds, plants and fungi (Jong & Donovick, 1989). Fungi produce remarkable amounts of polysaccharides with diverse structures, including starch-like polymers, glycogen, pullulan, mycodextran, cellulose, (1 → 3)-β-D-glucan, α-D-mannans, β-D-mannans, phosphonomannans, galactans, phosphonogaclactans, chitin, polysaccharides containing *N*-acetylgalactosamine, and heteropolysaccharides containing such widely varying components as xylose, arabinose, fucose, glucuronic acid, and rhamnose (Gojin & Barreto-Bergter, 1980).

In recent decades, mushrooms have received much interest with the realization that they are a good source of delicious food with high nutritional attributes and that some have medicinal values also (Farr, 1983; Kawagishi, 1994; Mizuno, 1993; Molitoris, 1994). More recently, polysaccharides derived from mushrooms have emerged as an important class of bioactive substances. Their antitumor, immunological, anticomplementary, anticoagulant, hypoglycemic and antiviral activities have been investigated (Chihara et al., 1970; Chihara et al., 1982; Kawagishi, 1994; Komatsu et al., 1969; Molitoris, 1994).

Most of the polysaccharides possessing antitumor and immunological activities have been reported as β-glucan and heteroglucan derivatives such as schizophyllan (Komatsu et al., 1969), lentinan (Chihara et al., 1970; Chihara et al., 1982), PSK (Tsukagoshi & Ohashi, 1974). There are several studies reporting the isolation and characterization of heterogalactans from mushrooms such as *Lentinus edodes*, *Ganoderma applanatum*, and *Fomitopsis pinicola* (Shida et al., 1975; Usui et al., 1981a; Usui et al., 1981b). Most of these heterogalactans have a backbone of (1 → 6)-α-D-galactopyranosyl residues, which are substituted at the C-2 atom position either with L-fucopyranose or 3-*O*-α-D-mannopyranosyl-α-L-fucopyranosyl residues. To our knowledge, biological activity of the heterogalactans has not been demonstrated, and purified polysaccharides from *Fomitella fraxinea* have not previously been investigated

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for their immunostimulating properties. In order to determine whether the polysaccharides might contribute in part to the claimed pharmacological activity of *F. fraxinea*, a chemical and immunological investigation of polysaccharides from the mushroom was undertaken.

We isolated two kinds of polysaccharides by 0.9% sodium chloride extraction from fruit bodies of *F. fraxinea*, which belongs to the aphylloporales of the family *Polyporaceae*. They were identified as heteroglucan and heterogalactan derivatives by gas chromatography (GC), GC–mass spectroscopy (MS), and nuclear magnetic resonance (NMR) spectroscopy. These polysaccharides have demonstrated that they possess immunostimulating activity by using splenetic lymphocytes from mice. In this study, we present the structure and mitogenic effects of heterogalactan obtained from a 0.9% sodium chloride extract of *F. fraxinea*.

2. Materials and methods

2.1. Isolation and purification of the polysaccharide

Fruit bodies of *F. fraxinea*, which is a bracket fungus (*Aphylloporales Polyporaceae*) growing on recently killed acacia trees were collected in forests near Wonju, Korea. The ground fruit bodies (1.3 kg), after successive extraction with 10 l of 90% methanol, were extracted twice with 6 l amounts of 0.9% sodium chloride at room temperature for 24 h. The extract was desalted by dialysis (MWCO: 3500; Spectrum, USA). The non-dialyzable solution was treated with four volumes of ethanol at 10°C overnight. The precipitates were collected by centrifugation and were suspended in distilled water. The ethanol treatment was repeated once more. The suspended polysaccharide was dialyzed and filtered through a cotton plug to remove water-insoluble materials. The filtrate was then lyophilized, yielding the pale-brownish polysaccharide powder. The lyophilized sample was loaded on DEAE–cellulose in sodium phosphate (pH 7.7) and the column was washed with same buffer. The unbound fraction was collected, dialyzed and freeze-dried. The sample was dissolved in the same buffer (2 g per 0.2 l).

2.2. Gel chromatography

Gel permeation chromatography was conducted on a Toyopearl HW 65F (Tosoh, Japan), 2.1 × 100 cm column and elution was effected with H_2O. The sample, dissolved in H_2O (50 mg ml^{-1}) was applied to the column, and 5 ml fractions were collected. Carbohydrates in the fractions were determined by the phenol–sulfuric acid method (Dubois et al., 1956).

2.3. Measurement of molecular size

The molecular size of the polysaccharide was measured by gel permeation–high performance liquid chromatography (GP–HPLC). The HPLC conditions were as follows: column, Tosoh GMPW (7.8 × 30 cm^2; Tosho, Japan); column temperature, 25°C; detection, refractive index (RI-8010; Tosoh, Japan); mobile phase, 0.1 M NaCl; flow rate, 1 ml min^{-1}. Standard calibration curves were constructed using dextrans (Sigma, USA).

2.4. Analysis of component sugars

The polysaccharides were hydrolyzed in 2.0 M CF_3CO_2H for 1 h at 121°C. CF_3CO_2H was removed by evaporation, and the carbohydrates in the hydrolyzate were analyzed by thin layer chromatograpy and GC as described previously (Song et al., 1995).

2.5. Methylation analysis

Methylation analysis was carried out by the Hakomori method (Hakomori, 1964). The permethylated polysaccharide showed no hydroxyl group absorption band in the infrared spectrum. Upon hydrolysis of the fully *O*-methylated polysaccharides, the hydrolyzate was converted into alditol acetates, which were subsequently analyzed by GC–MS (VG Trio-1000; Fisons instruments, UK). The GC–MS conditions were as follows: fused-silica column of SP-2330 (Supelco; Bellefonte, PA) of size 52 m × 0.25 mm; film thickness, 0.20 μm; column temperature program, 160–210°C at 2°C min^{-1}, and 210–240°C at 5°C min^{-1}; carrier gas, helium. Electron impact mass spectra were recorded with use of a Fision Trio-1000 Lab Base data system. The molar ratio of each *O*-methylated sugar was calculated in comparison with 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol.

2.6. NMR spectroscopy

NMR spectra were recorded with use of a JEOL JNM A-600 spectrometer. The polysaccharide (25 mg) was dissolved in 0.6 ml of D_2O. All spectra were recorded at 313 K. HOD at 4.65 for the ^{1}H NMR and 1,4-dioxane at 67.40 for the ^{13}C NMR spectra were used as the internal and external references, respectively.

2.7. Mitogen activity

Polysaccharide dissolved in 0.85% NaCl was used in all the experiments. Proliferation of the lymphocytes was investigated by a direct proliferation assay (Andersson et al., 1972; Kaldjian et al., 1992). The lymphocytes (1 × 10^6 cells ml^{-1}) were placed in a 96-well microplate (200 μl per well) with various B- and T-cell mitogens. The polysaccharide was added to the lymphocyte culture with or without mitogens at final concentrations of 1 and 10 μg ml^{-1}. Lipopolysaccharide (LPS) and concanavalin A (Con A) were used as B-cell and T-cell mitogens, respectively.

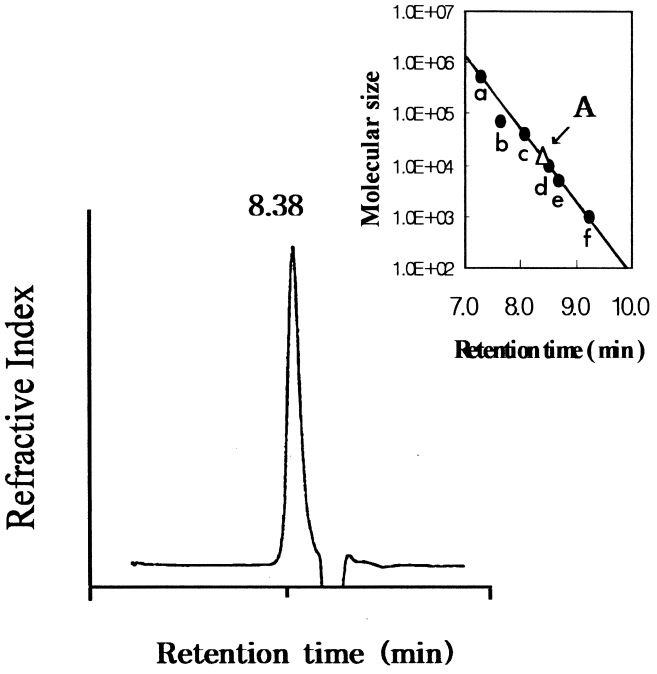

Fig. 1. HPLC profile of heterogalactan obtained from the fruit bodies of *F. fraxinea*, and a calibration curve used in the determination of its molecular weight. Inset: A, heterogalactan from *F. fraxinea*; a, b, c, d, e, and f, standard dextrans with molecular weights 50×10^4, 7×10^4, 4×10^4, 5×10^3 and 1×10^3, respectively.

Pokeweed mitogen (PWM) was used as a B- and T-cell common mitogen. The mitogens were added to the culture at a final concentration of 1 μg ml^{-1}. The lymphocytes were incubated for 72 h. ^{3}H-thymidine (1 μCi per well) was added to the culture 18 h before the incubation ended. After the incubation, the lymphocytes were harvested onto a glass fiber filter using a semi-automatic cell harvester (Inotech, Switzerland) and the radioactivity was counted with a liquid scintillation counter (Beckman LS6000 series, Germany). The mitogenic activity was evaluated using the splenetic lymphocyte reaction by the measurememt of tritiated thymidine incorporation into cultured mice spleen cells by pulse labeling.

3. Results and discussion

The polysaccharide obtained from the 0.9% sodium chloride extract of fruiting bodies of *Fomitella fraxinea* was purified by dialysis, ethanol precipitation, and DEAE–cellulose column chromatography. This polysaccharide showed a single peak when chromatographed on Toyopearl HW-65F gel, and was named fomitellan A. Fomitellan A had $[\alpha]_D = +90°$ (*c*:0.2) and, on acid hydrolysis, yielded D-galactose, D-mannose, and L-fucose in the molar ratio 3:1:1. The yield of fomitellan A was 0.24%. It consisted mainly of neutral heteropolysaccharides. To measure the average molecular size, fomitellan A was subjected to gel permeation–HPLC (Fig. 1) and exhibited a symmetrical and single peak, indicating homogeneity. By comparison of the retention time with that of standard dextran molecules, the average molecular size of fomitellan A was determined to be 15 000 (Fig. 1).

The heterogalactan fomitellan A was methylated by the Hakomori method and the mixtures of methylated alditol

Table 1
Partially methylated sugar alditol acetates from the hydrolyzate of permethylated fomitellan A from *Fomitella fraxinea*

Methylated sugar[a]	Linkage indicated	Molar ratio (%)	T[b]	Major mass fragment (*m*/*z*)
2,3,4,6-Me$_4$-Man	Man*p*-(1 →	16.2	0.98	87,101,117,129,145,161,205
2,4-Me$_2$-Fuc	→ 3)-Fuc*p*-(1 →	18.9	0.99	101,117,131,173,233
2,3,4,6-Me$_3$-Gal	Gal*p*-(1 →	1.5	1.00	101,117,129,145,161,189,205
2,3,4-Me$_2$-Gal	→ 6)-Gal*p*-(1 →	44.1	1.41	99,101,117,129,161,189,233
3,4-Me$_2$-Gal	→ 6)-Gal*p*-(1 → 2 ↑	19.3	1.67	87,99,129,189,233

[a] Determined as alditol acetates.
[b] Retention time of the corresponding alditol acetates on the SP-2330 column relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol (1.0).

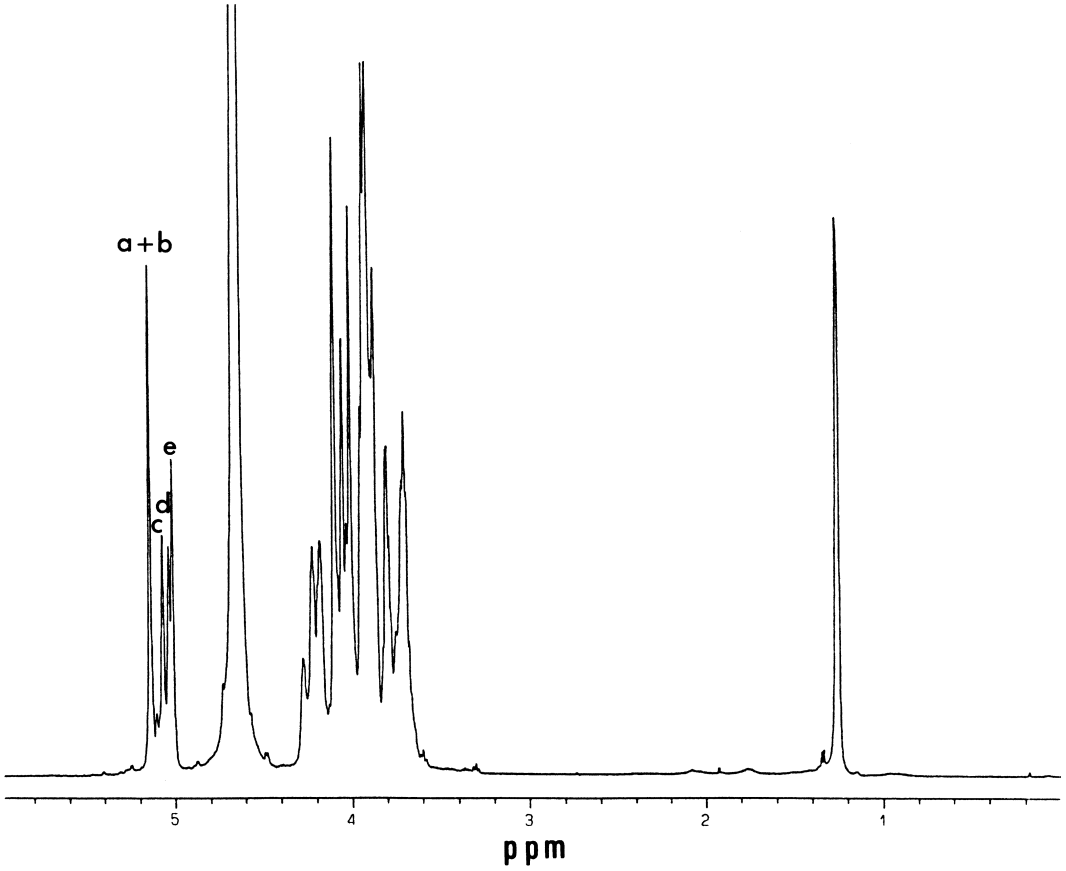

Fig. 2. ^{1}H NMR spectrum (600 MHz) of fomitellan A from *F. fraxinea* recorded at 40°C in D_2O. Anomeric protons are labelled a–e.

acetates obtained were then analyzed by GC–MS spectroscopy. The results are summarized in Table 1. Identification of all the peaks was made on the basis of their mass spectra and characteristic retention times (*T*-values) relative to the standard, 2,3,4,6-D-tetra-*O*-methyl-D-galactopyranose (1.0) (Elaine & Nicholas, 1988). Together with NMR data, the methylation analysis showed that all the sugar components were pyranoses. Methylation analysis of fomitellan A revealed the presence of at least five major components. These were identified as 2,3,4,6-tetra-*O*-methyl-D-mannopyranosyl (**A**), 2,4-di-*O*-methyl-L-fucopyranosyl (**B**), 2,3,4,6-tetra-*O*-methyl-D-galactopyranosyl (**C**), 2,3,4-tri-*O*-methyl-D-galactopyranosyl (**D**), and 3,4-di-*O*-methyl-D-galactopyranosyl residues (**E**) in the molar ratio 1.6: 1.9: 0.2: 4.4: 1.9. The presence of the 2,4-di-*O*-methyl-L-fucopyranosyl residue indicates that L-fucopyranose exists as residue linked through the C-1 and C-3 atom sites. Also, the presence of the 2,3,4,6-tetra-*O*-methyl-D-mannopyranosyl residue indicates that D-mannopyranose exists exclusively at a non-reducing terminal unit. Three D-galactopyranosyl residues are present as main-chain residues linked at the O-6 position (**D**), as residues with branch points at the O-2 position (**E**), and as a terminal, non-reducing residue (**B**), respectively. These results should suggest that fomitellan A has a backbone chain of (1 → 6)-linked D-galactopyranosyl residues and approximately every other third of these are substituted at the O-2 postion by a disaccharide unit made up of 3-*O*-D-mannopyranosyl-L-fucopyranosyl residues.

The proton NMR spectrum of fomitellan A in D_2O is shown in Fig. 2. This spectrum revealed five anomeric

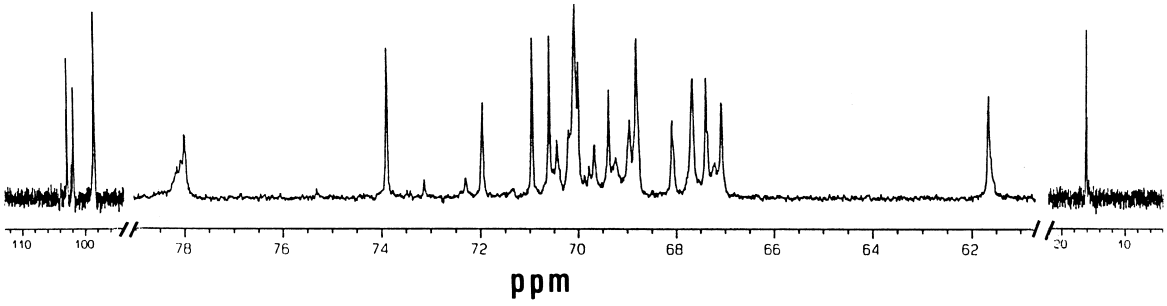

Fig. 3. ^{13}C NMR spectrum at 125 MHz of fomitellan A from *F. fraxinea* recorded at 40°C in D_2O.

proton signals at 5.14 (2H), 5.07, 5.03, and 5.01 ppm. These five residues were labeled a, b, c, d, and e, according to their anomeric protons, from high to low field. The signals c, d and e were observed from the galactopyranosyl residues. Signals due to the oxygenated methine and methylene group protons from H-2 to H-6 of the component sugars were observed around 3.60–4.30 ppm, but the assignment of each signal was not made because of spectral duplication. Also, a methyl group signal (H-6) was observed at 1.26 ppm due to the L-fucopyranosyl residue (Fig. 2).

The ^{13}C NMR spectrum of fomitellan A shown in Fig. 3 provides useful information on its composition and sequence. All the carbon atom signals were resolved by comparison with reported data (Shida et al., 1975; Usui et al., 1981a; Usui et al., 1981b). Their chemical shifts are summarized in Table 2. The ^{13}C NMR spectrum showed five anomeric carbon atom signals at 102.78, 101.83, 98.64, 98.47 and 98.43 ppm, suggesting that the repeating unit of fomitellan A is a pentasaccharide. The spectrum contains the signals for a methyl group (C-6) of the fucopyranosyl residue at 16.17 ppm, four CH_2OH groups (C-6 atoms of four hexoses) at 61.66, 67.10, 67.22, and 67.67, and 20 other sugar carbon atoms in the region of 67~88 ppm. Chemical shifts at low field, 102.8 ppm and 101.83 ppm, must correspond to the C-1 resonance of the D-mannopyranosyl residue at the non-reducing position and the L-fucopyranosyl residue, respectively (Usui et al., 1981a). Therefore, the remaining anomeric signals at 98.64, 98.47 and 98.43 ppm are considered to be due to the carbon atoms resulting from three galactopyranosyl residues. Considering that the ^{13}C resonance for the C-6 atom of the hexose residue corresponds to the region 61.66 ppm, the signal appears to be that of the mannopyranosyl residue. The presence of the signal for the C-6 atom at 61.66 ppm for the unsubstituted pyranose residue, and of the signals at 67.10, 67.22, and 67.67 ppm for the 6-substituted pyranose residues, the presence or absence of (1 → 6) linkages can be inferred (Lipkind et al., 1988). These data agree with the results obtained by methylation analysis (Table 1).

Although further detailed structural studies by two-dimensional NMR are needed, these results determined by methylation and 1D NMRs suggested that the heterogalactan fomitellan A of *F. fraxinea* has the following structure.

→6)-D-Gal *p*-(1→6)-D-Gal *p*-(1→6)-D-Gal *p*-(1→

|

D-Man *p*-(1→3)-L-Fuc *p*-(1→2)

The *F. fraxinea* heterogalactan has a backbone similar to those of other heterogalactans in its side-chain structure (Shida et al., 1975; Usui et al., 1981a; Usui et al., 1981b). Parts of L-fucose and/or a disaccharide of D-mannose and L-fucose in the side-chains of the other heterogalactans are present as non-terminal residues. However, only a disaccharide of the 3-*O*-D-mannopyranosyl-L-fucopyranosyl residue is present exclusively as a terminal residue in the heterogalactan of *F. fraxinea*.

The immunological activity of fomitellan A was investigated by a direct proliferation assay with or without various B- and T-cell mitogens. Fig. 4 shows that the purified polysaccharide enhances the proliferation of splenetic lymphocytes via the stimulation of DNA synthesis. Fomitellan A exhibited relatively high activity (Fig. 4). To investigate the synergistic effect of fomitellan A, the proliferation of splenetic lymphocytes was induced through combined treatment with B- and T-cell mitogens. Again, fomitellan A stimulated the proliferation of lymphocytes, as determined at a final concentration 10 μg ml^{-1}. When lipopolysaccharide (LPS) was used as a B-cell mitogen, fomitellan A showed a higher activity, the stimulation index being 5.4. In addition, when concanavalin A (Con A) was used as a T-cell

Table 2
Assignments of signals in the ^{13}C NMR spectrum of fomitellan A from *F. fraxinea*

Signal	Assignment
102.78	C-1 of D-mannopyranose non-reducing end units
101.83	C-1 of L-fucopypyranose units
98.64	C-1 of D-galactopyranose units
98.47	C-1 of D-galactopyranose units
98.43	C-1 of -galactopyranose units
78.08	C-2 of 2-*O*-substituted D-galactopyranose units of L-Fuc*p*-(1 → 2)-D-Gal*p*-
78.02	C-3 of 3-*O*-substituted L-fucopyranose units of D-Man*p*-(1 → 3)-L-Fuc*p*-
73.91	C-5 of D-mannopyranose non-reducing end units
71.96	C-4 of L-fucopypyranose units
70.95	C-3 of D-mannopyranose non-reducing end units
70.60	C-2 of D-mannopyranose non-reducing end units
67.67	C-6 of 6-*O*-substituted D-galactopyranose units
67.40	C-4 of D-mannopyranose non-reducing end units
67.22	C-6 of 6-*O*-substituted D-galactopyranose units
67.10	C-6 of 6-*O*-substituted D-galactopyranose units
61.66	C-6 of D-mannopyranose non-reducing end units
16.17	C of methyl group of L-fucopyranose units

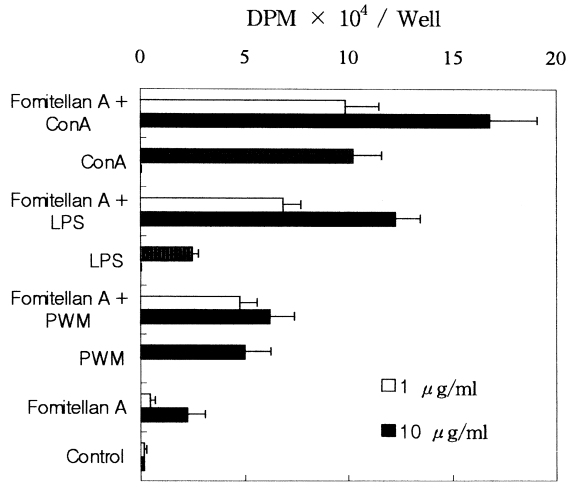

Fig. 4. Induction of the proliferation of spleen lymphocytes by polysaccharides from *F. fraxinea*, mitogens and mixtures of polysaccharides and mitogens. Plates were pulsed with ^{3}H-thymidine, and the cells were harvested and counted by use of a scintillation counter. The scintillation counting was performed as described in Section 2. Open bars and hatched bars represent the results obtained at the final concentrations 1 μg ml^{-1} and 10 μg ml^{-1}, respectively. The data were shown as the geometric mean of three replicates. LPS, Con A, and PWM were treated at a final concentration of 1 μg ml^{-1}.

mitogen and PWM as a B- and T-cell mitogen, fomitellan A showed proliferation effects at a final concentration of 10 g ml^{-1}.

It is interesting to note that the result of the proliferation assay for this mushroom polysaccharide is compararable to that for fungal polysaccharides such as shichophyllan and others (Komatsu et al., 1969; Molitoris, 1994; Song et al., 1995). Although further immunological in-vivo studies are needed, it can be suggested that polysaccharides in *F. fraxinea* contribute to the pharmacological efficacy of mushroom extracts.

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