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Isolation and Characterization of a Water-Soluble Polysaccharide from the Mycelia of Solid Cultured *Phellinus linteus*

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Abstract Fraction (PMEx-AH-β) of water-soluble polysaccharide, showing stimulating activity against macrophages, was isolated from mycelia of solid cultured *Phellinus linteus* by hot-water extraction, ethanol precipitation, and chromatography. Chemical characteristics of PMEx-AH-β were as follows: carbohydrate content, 71%; monosaccharide composition, Man: Glu:Gal (9:64:27); molecular weight, $1\text{-}7\times10^4$; uronic acid content, 6.8%. Fundamental structure of PMEx-AH-β is deduced as β-(1 → 6)-D-glucan with β-(1 → 3)-D-glucosidic side chains based on methylation analysis.

Keywords: *Phellinus linteus*, chemical characteristics, fundamental structure

Introduction

Increasing evidences have placed focus on mushrooms as sources of alternative medicines. In particular, *Phellinus linteus* is a well known source of oriental medicine in accordance with its high level of inhibition against sarcoma 180 (1). A polysaccharide from the mycelia of *P. linteus* was reported to stimulate the polyclonal antibody production *in vitro*, as well as immune functions of T lymphocytes and nonspecific immune functions mediated by natural killer cells and macrophages (2,3). Its beneficial effects including antitumor (2,3), hypoglycemic (4), anti-inflammatory (5), and anti-mutagenic (6), activities have been well demonstrated in a number of biochemical studies, among which antitumor activity via immunological enhancement has received the most interest, because cancer still remains one of the main human death causes.

β-D-Glucans, in particular, are thought to be responsible for anti-cancer effects not by direct actions to tumor cells, but by potentiating the state of health (7). In a previous study, we reported the antitumor activity of water-soluble polysaccharide extract (CMEx) from the cultured mycelia of *Agaricus blazei* on mice carrying sarcoma 180 (8). Fundamental structure of the β-D-glucan purified from the CMEx was deduced as β-(1→6)-D-glucan with β-(1→3)-D-glucosidic side chains (9). β-Glucans of *P. linteus* also have received special interests due to their immuno-modulating antitumor activity. However, only few reports are available on the structural information of *P. linteus* β-glucan. In the present study, we prepared a water-soluble polysaccharide extract (PMEx) from mycelia of solid cultured *P. linteus* as previously described (10) and examined its stimulating activity on macrophages with nitric oxide (NO) production. β-Glucan fraction was also isolated from PMEx, and its chemical composition and fundamental structure were examined for its use as a potential health food ingredient.

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Materials and Methods

Materials The freeze-dried mycelia (300 g) of solid cultured *P. linteus* were extracted with 2 L hot water three times. The water-soluble extract was concentrated to 600 mL and precipitated with ethanol. The precipitate was dialyzed and freeze-dried. The obtained fraction (19.9 g) was designated as PMEx and used for further experiments. CMEx (18.5 g) was also prepared from mycelia (300 g) of solid cultured *Agaricus blazei* using the same procedure for PMEx preparation.

Measurement of nitrite production NO production in the supernatants obtained from RAW264.7 cells at the indicated time points was spectrophotometrically evaluated by measuring the concentration of nitrite, a stable end-product of NO, by the Griess reaction (11). Briefly, 100 mL culture supernatant was mixed with an equal volume of the Griess reagent [1% (w/v) sulfanilamide in 5% (w/v) phosphoric acid and 0.1% (w/v) *N*-(1-naphthyl) ethyl-enediamine solution (Sigma Chemical Co., St. Louis, USA)] at room temperature for 10 min, and absorbance was measured in a microplate reader (TECAN) at 540 nm. The concentration of nitrite was determined based on the calibration curve with sodium nitrite standards.

Isolation of β-glucan PMEx (0.4g) was dissolved in 1 mL distilled water and passed through a DEAE-cellulose column (Macro-Prep DEAE Support, 2.5×40 cm, Bio-Rad Lab., USA; 0-2 M NaCl gradient elution; total elution volume, 544 mL; volume of 1 fraction, 6.8 mL; flow rate, 1.25 mL/min). The ion exchanged fractions were gel-filtrated with Superose 6 10/300 GL column (1×30 cm, Amersham Biosciences, USA; 0-0.5 M NaCl gradient elution; total elution volume, 60 mL; volume of 1 fraction, 1 mL; flow rate, 0.5 mL/min). Subsequently, the gel-filtrated fractions were separated into α-glucans (absorbed) and β-glucans (non-absorbed) through HI-Trap Con A column (1 mL column, Amersham Biosciences, USA; 0.1 M NaCl, 0.5 M Methyl-α-D-glucopyranoside gradient elution; total elution volume, 27 mL; volume of 1 fraction, 1.5 mL;

flow rate, 0.5-0.15-0.25-0.5 mL/min). The purification procedure of PMEx was repeated several times until collection of 0.1 β-glucan fraction of PMEx.

General analyses Total sugar, protein, and uronic acid contents were measured by the Phenol-sulfuric acid method (12), Lowry method (13), and carbazole-sulfuric acid method (14), respectively. The molecular-weight of each fraction was determined through gel filtration chromatography by comparing with the standard curve using standard dextrans (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Sugar analysis The sugar constituents of PMEx-AH-β fraction were transformed into the corresponding alditol acetates and detected by GC/MS. Agilent 6890 GC was equipped with a 5973 MSD (Hewlett-Packard, Avondale, USA) and an ethylene glycol-coated fused silica capillary column (Supelcowax-10, 30 m, 0.25 mm i. d., 0.25 μm film thickness, Supelco Inc., Supelco Park Bellefonte, USA). Temperatures of the ion source and injector were 230°C at 70 eV, and 280°C, respectively. The oven temperature was raised from 100 to 280°C at 5°C/min. The flow rate of carrier gas (He) was 1 mL/min, and the split ratio was 1 : 10. The mass chromatogram of hexaacetyl hexitols was reconstructed with the most abundant ion (*m/z* 289) from hexaacetyl hexitols to exclude impurities during the estimation of relative quantities.

Determination of the position of glycosidic linkages Each polysaccaride fraction (5 mg) was methylated with 0.4 mL methyl sulfinyl methyl sodium at 25°C for 6 hr according to the Hakomori method (15). The obtained methylated polysaccharides were depolymerized with 90% formic acid at 105°C for 1.5 hr, followed by hydrolysis with 0.15 M H_2SO_4, and neutralized with barium carbonate. The hydrolyzed fraction was reduced with 10 mg $NaBH_4$ at 22°C for 20 hr and acetylated with an acetic anhydride in pyridine at 105°C for 2 hr. The partially methylated alditol acetates were detected by GC/MS under the same conditions as those applied to the sugar analysis.

FT-IR spectroscopy For FT-IR spectroscopy (JASCO Co., Tokyo, Japan), the samples were mixed with KBr according to the KBr disk method and vacuum-dried at 120°C for 3 hr. The IR spectrum of the samples were compared with those of β-glucan and amylose.

Results and Discussion

Activation of macrophages The authors previously found that CMEx stimulated macrophages to produce NO when RAW264.7 cells were cultured together, resulting in significant antitumor activity in mice carrying Sarcoma 180, as well as in syngeneic tumor models developed with P388 leukemia cell lines (H-2d) or B16F0 melanoma cell lines (H-2b) (8). Given the pivotal roles of macrophages in the immune system, we examined whether PMEx could activate macrophages with such parameters as NO production. When RAW264.7 cells were cultured with PMEx, the production of NO increased in a concentration-dependent manner, and the pattern of NO production was very close to that of CMEx, a water-soluble polysaccharide prepared from the mycelia of solid cultivated *A. blazei,* (Fig. 1). These results suggest that, as with CMEx, PMEx can also activate macrophages to produce NO.

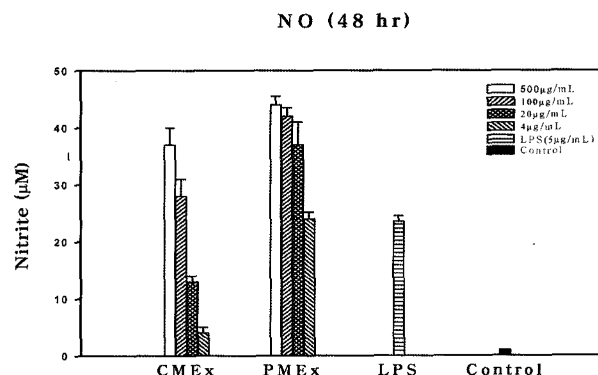

Fig. 1. NO production by RAW 264.7 cell. RAW 264.7 cell were cultured with various concentrations of PMEx or CMEx for 48 hr and NO concentrations in triplicate culture supernatants were determined by nitrite assay.

Chemical composition In general, chemical components of edible mushrooms include steroids, lipids, proteins, and various polysaccharides (16), among which polysaccharide-protein complex has been reported as one of the active antitumor component (16). Therefore, PMEx, an extract of water-soluble polysaccharide, was collected from the mycelia of *P. linteus*. It was found to contain carbohydrate, together with protein and uronic acid (Table 1). Lee *et al.* (17) reported that polysaccharide fractions, showing immunostimulating activity, obtained from hot water extract of mycelium of *P. linteus* were polysaccharide-protein complexes. Kawagishi *et al.* (18) also found the presence of protein in the antitumor-active β-(1 → 6)-glucan fraction and explained that the protein component is essential for antitumor activity of the complex and that the glucan alone cannot exhibit strong activity as does the glucan-protein complex. The carbohydrate in PMEx was mainly composed of glucose, galactose, and mannose (Fig. 2). The mass chromatogram of hexaacetyl hexitols was reconstructed with the most abundant ion (*m/z* 289) from hexaacetyl hexitols. It was eluted with a protein moiety on a DEAE-cellulose chromatography by 0-2 M gradient of aqueous NaCl (Fig. 3-A). The carbohydrate fractions (PMEx-A) with a protein moiety obtained in the acidic fractions were purified on Superose 6 10/300 GL column (1×30 cm) with 0.5 M NaCl as the eluent. The eluate (PMEx-AH) was shown as a symmetrical peak with respect to the contents of carbohydrate and protein (Fig. 3-

Table 1. Total sugar, uronic acid, and protein contents

	PMP[a]	PMEx[b]
Total sugar (%)	14.5±0.5	71.1±2.0
Uronic acid (%)	5.1±0.3	6.8±0.3
Protein (%)	12.6±0.5	18.2±0.6

[a]PMP: Dried powder of the cultured mycelia of *P. linteus*
[b]PMEx: A water-soluble polysaccharide extract of the cultured mycelia of *P. linteus*

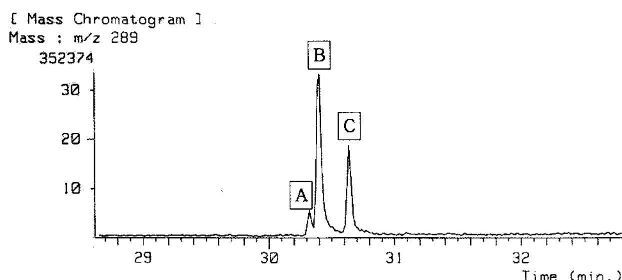

Fig. 2. Mass chromatogram of hexaacetyl hexitols obtained from a water-soluble polysaccharide extract of the cultured mycelia of *P. linteus*. A: Mannitol acetate B: Glucitol acetate C: Galactitol acetate a)reconstructed with the most abundant ion (*m/z* 289) from hexaacetyl hexitols in order to exclude impurities.

B). PMEx-AH was further separated into non-absorbed β type (PMEx-AH-β) and absorbed α type (PMEx-AH-β) through Hi-Trap Con A (Fig. 3-C). The molecular weight of PMEx-AH was estimated to be $1\text{-}7\times10^4$ by comparing with the elution profile of the dextrans of known molecular weights.

Structural characterization PMEx-AH-β was treated for methylation analysis, and its structure was characterized using GC/MS. The mass spectra obtained were compared to the respective standard mass spectra reported by Janson *et al.* (19). Mass spectra of partially methylated alditol acetates may be analyzed with reference to a few simple rules (20) in the fragmentation pattern of 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylhexitol, fission prefers to occur next to the methoxy group, and secondary fragments are derived from the primary fragment by the consecutive elimination of formaldehyde, methanol, ketene or acetic acid (Fig. 4).

Methylation analysis of PMEx-AH-β gave 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol as the major peak (*m/z* 233, 189, 161, 129, 117, 101, 43) (Fig. 5-A) along with 1, 5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol derivatives (*m/z* 205, 161, 145, 129, 117, 101, 43) (Fig. 5-B). In addition, peaks of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol (*m/z* 233, 161, 129, 117, 101, 43) confirmed the presence of (1 → 3)-linked β-D-glucopyranosyl residues (Fig. 5-C). In particular, mass fragment at *m/z* 129 was commonly found in all mass spectra of the hexitol derivatives (Fig. 5). Moreover, a base fragment at *m/z* 43 generated from large primary fragmentation was also found (Fig. 5). These results implied the formation a main backbone chain of PMEx-AH-β with a (1 → 6)-linked β-D-glucopyranosides, which appear to be branched with β-(1 → 3)-D-glucopyranosyl residues. The presence of 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methylhexitol (*m/z* 233, 189, 173, 159, 129, 117, 99, 87) (Fig. 5-D) revealed that β-(1 → 3) residues were branched at C-2 rather than at C-3 as observed in *A. blazei*. In a previous report, the authors deduced the fundamental structure of glucan chain in *A. blazei* as β-(1→6)-D-glucan with β-(1 → 3)-D-glucosidic side chains based on the characteristic mass fragment patterns obtained through the methylation analyses (9), well coinciding with the studies of Kawagishi *et al.* (18) and Q. Dong *et al.* (16). Most of the antitumor-active polysaccharides such as lentinan,

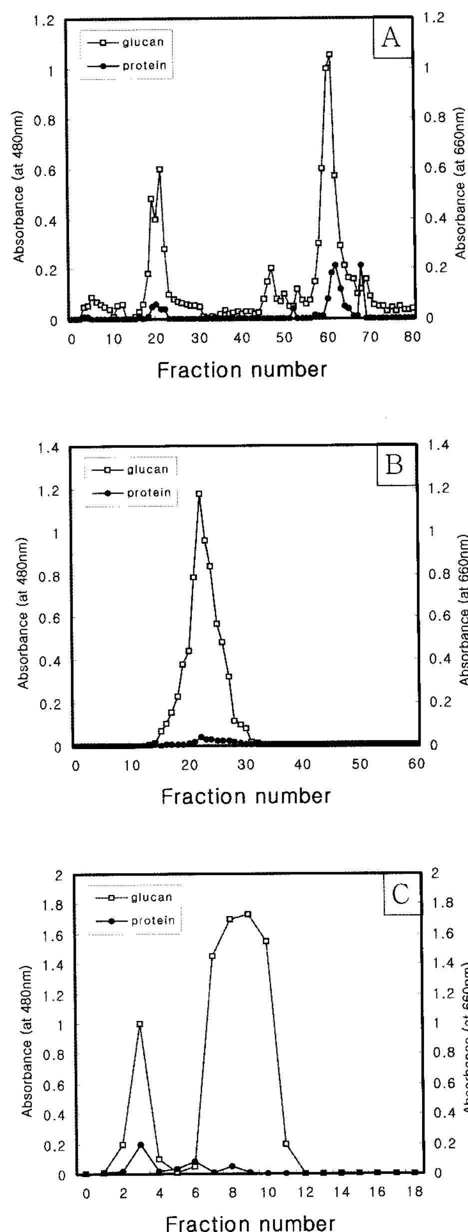

Fig. 3. Ion exchange (A), gel permeation (B) affinity (C) chromatogram of a water-soluble polysaccharide extract of the cultured mycelia of *P. linteus*.

schizophyllan, and scleroglucan have been reported as β-D-glucans, particularly (1 → 3)-linked with (1 → 6)-β-D-glucosidic side chains (21). However, similar to the case of *A. blazei*, a backbone chain of glucan in *P. linteus* is expected to be β-(1→ 6)-D-glucopyranosides branched with β-(1→ 3)-linked oligisaccharide units based on our results of methylation analyses. Unfortunately, no report could be found on the structural characterization of glucan from mycelium of *P. linteus*. Only Lee *et al.* (17) mentioned that the most active immunostimulating fraction (4-III) obtained from mycelium of *P. linteus* could be different from β-(1 → 3)-glucan in structure. Additional structural information on the anomeric configuration of the purified fraction of PMEx was obtained from the infra red absorption band at 890-900 cm^{-1} for β-glucosides (Fig. 6). The pattern of FT-

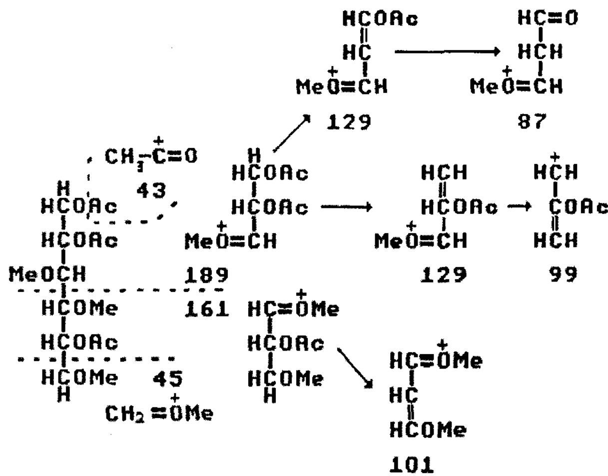

Fig. 4. The fragmentation pattern of 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylhexitol[a]. [a]Ref. 20

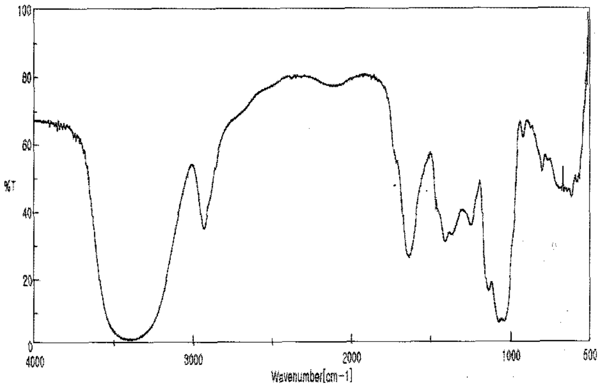

Fig. 6. FT-IR spectrum of PMEx-AH-β from a water-soluble polysaccharide extract of the cultured mycelia of *P. linteus*.

IR spectrum of PMEx-AH-β was similar to that of the 4-III fraction from mycelium of *P. linteus* (17). Conclusively, fundamental structure of PMEx-AH-β is deduced as β-(1 → 6)-D-glucan with β-(1 → 3)-D-glucosidic side chains based on spectral analysis as well as methylation analysis.

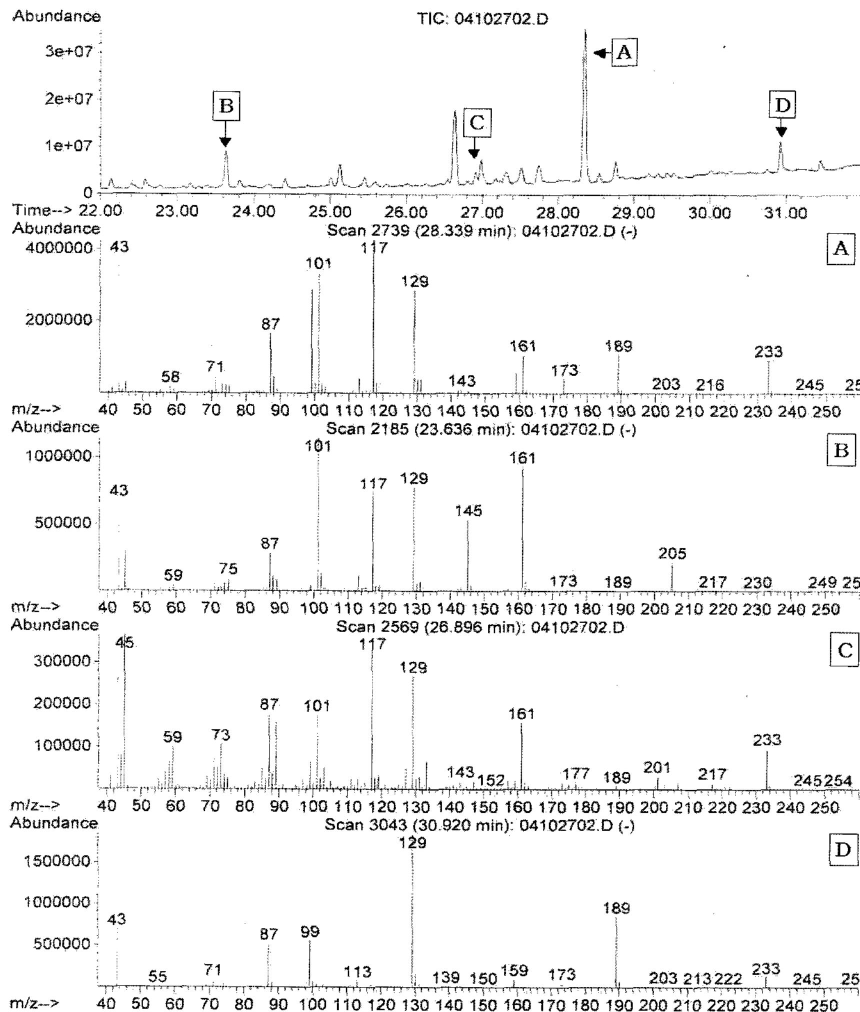

Fig. 5. GC-MS data of PMEx-AH-β from a water-soluble polysaccharide extract of the cultured mycelia of *P. linteus*. A: 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol, B: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol, C: 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol, D: 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methylhexitol

Acknowledgments

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