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Isolation and Characterization of a Water-Soluble Polysaccharide from the Mycelia of Solid Cultured *Agaricus blazei* Murill

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Abstract A fraction (CMEx-AH-β) of water-soluble polysaccharides, showing selective antitumor activities, was isolated from the mycelia of solid cultured *Agaricus blazei* Murill by hot-water extraction, ethanol precipitation, and series of chromatography. Chemical characteristics of CMEx-AH-β, were as follows: carbohydrate content, 48%; monosaccharide composition, Man:Glu:Gal (2:93:5); molecular weight, 2×10^5; uronic acid content, 6.2%. Fundamental structure of CMEx-AH-β, was deduced as β-(1 → 6)-D-glucan with β-(1 → 3)-D-glucosidic side chains based on results of methylation and ^{13}C-NMR spectroscopic analyses.

Keywords: *Agaricus blazei* Murill, chemical characteristics, fundamental structure

Introduction

Agaricus blazei Murill, once a part of the regular diet in the Piedade region of Brazil, has been well evaluated as a source of neutraceuticals in accordance with the accumulating evidences on their antitumor effects in tumor-bearing animals (1), as well as hypoglycemic (2), anti-inflammatory (3), and antibacterial (4) effects. Among the numerous beneficial properties of *A. blazei,* antitumor activity via immunological enhancement has received the most interest, because cancer still remains one of the main causes of death in humans. Although polysaccharides, nucleic acids, and steroids are reported to be the major antitumor components of *A. blazei,* 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 (5). Because current anti-cancer therapies such as chemotherapy, surgery, and radiation are often accompanied by severe side effects, development of health functional products with minimal side effects is in much demand (6). Thus, special attention has been given to the protein-bound polysaccharides of *A. blazei* due to their immunomodulating antitumor activity.

In a previous study, the mycelia of *A. blazei* was cultured according to our solid-cultural system, to develop a functional food ingredient with antitumor potency (7). CMEx, a water-soluble extract prepared from the mycelia of solid cultivated *A. blazei,* showed significant antitumor activity in mice carrying Sarcoma 180 when administered orally at 100 mg/kg for 10 consecutive days, as well as with syngeneic tumor models, which were developed by injecting P388 leukemia cell lines (H-2d) into BDF1 mice (H-2b/d) or B16F0 melanoma cell lines (H-2b) into C57BL/6 (H-2b) mice (8). In the present study, we isolated an active fraction from CMEx and examined its chemical composition and structure.

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Materials and Methods

Material Water-soluble extracts from the cultured mycelia of *A. blazei* Murill, which were cultivated according to our culture system, were prepared as previously described (7) and designated as CMEx.

Isolation CMEx (0.4g) was dissolved in 1 mL distilled water and passed through a DEAE-cellulose column (2.5 × 40 cm; 0-2 M NaCl gradient elution; flow rate, 1.25 mL/min). The ion exchanged fractions were gel-filtrated with Superose 6 HR 10/30 column (2.5 × 60 cm; 0-0.5 *M* NaCl gradient elution; flow rate, 0.5 mL/min). Subsequently, the gel-filtrated fractions were separated into β-glucans (absorbed) and β-glucans (non-absorbed) through HI-Trap Con A column (0.1 M NaCl, 0.5 M Methyl-α-D-glucopyranoside gradient elution; flow rate, 0.5-0.15-0.25-0.5 mL/min).

General analyses Total sugar, protein, reducing sugar, and uronic acid contents were measured by the Phenol-sulfuric acid method (9), Lowry method (10), Somogyi-Nelson method (11), and carbazole-sulfuric acid method (12), 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 analyses The sugar constituents of CMEx-AH-β, fraction were transformed into the corresponding alditol acetates and subsequently detected by GC/MS. Agilent 6890 GC was equipped with a 5973 MSD (Hewlett - Packard, Avondale, PA, 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., PA, 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.

Methylation analysis Each polysaccaride fraction was methylated according to the Hakomori method (13), hydrolyzed, reduced with $NaBH_4$ and acetylated with an acetic anhydride. The partially methylated alditol acetates were detected by GC/MS under the same conditions as those applied to the sugar analyses.

Smith degradation (14) A water-soluble polysaccharide (50 mg) was dissolved in 50 mL of 0.02 M sodium periodate, adjusted to pH 4.5, and kept at 4°C for 96 hr. At the end of storage, 0.2 mL ethylene glycol was added to the reaction mixture, which was then dialyzed against distilled water. The nondialyzate was collected and subjected to methylation analysis.

^{13}C-NMR, FT-IR spectroscopy To identify the ^{13}C-NMR spectrum of the purified polysaccaride, each fraction (50 mg) was dissolved in 0.5 mL Me_2SO-d_6 and measured using a Jeol ECP-400 MHz instrument (Jeol Ltd., Tokyo, Japan). 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

Chemical composition CMEx, a water-soluble extract prepared from the mycelia of solid cultivated *A. blazei,* showed selective antitumor activities (8). It was found to contain carbohydrate (48%), protein (30%) and uronic acid (6.2%) as major components (Table 1). Recently, Dong *et al.* (15) reported that the crude polysaccharide extracted from the fruiting bodies of *A. blazei* contained carbohydrate (57.5%) and protein (21.6%). Kawagishi *et al.* (16) 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. However, polysaccharide-protein complexes have not yet been fully elucidated. The carbohydrate in CMEx, mainly composed of glucose with small amounts of galactose and mannose (Fig. 1), was eluted with a protein moiety on a DEAE-cellulose chromatography (Fig. 2-A). The components (CMEx-A) obtained mostly in the acidic fractions with 0.5-2 M gradient of aqueous NaCl were purified on Superose 6HR (2 × 60 cm) with 0.5 M NaCl as the eluant. The eluate (CMEx-AH) was shown as a symmetrical peak with respect to the contents of carbohydrate and protein

Table 1. Total sugar, reducing sugar, glucuronic acid and protein contents of the extracts of *Agaricus blazei*

	CMP[a)]	CMEx[b)]
Total sugar (%)	15.9±0.2	48.0±0.4
Reducing sugar (%)	4.8±0.1	2.8±0.2
Uronic acid (%)	2.0±0.1	6.2±0.0
Protein (%)	14.2±0.4	30.1±1.0

[a)]CMP : Dried powder of the cultured mycelia of *A. blazei*
[b)]CMEx : Water-soluble extract of the cultured mycelia of *A. blazei*

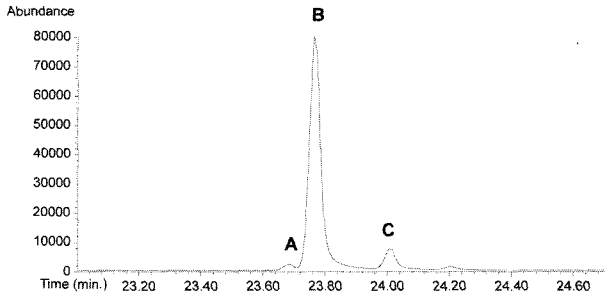

Fig. 1. Total ion current chromatogram of hexa-acetyl hexitols obtained from water-soluble extract of the cultured mycelia of *Agaricus blazei.* A: Mannitol acetate B: Glucitol acetate C: Galactitol acetate.

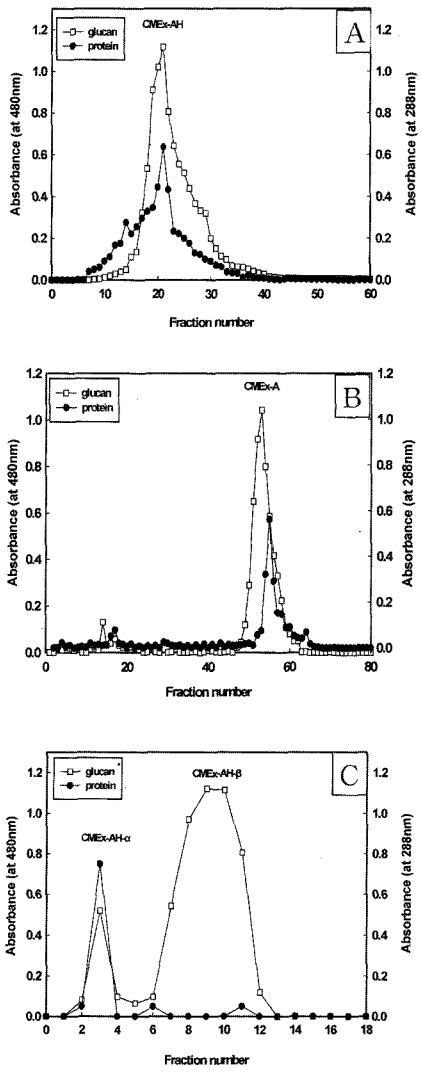

Fig. 2. Ion exchange (A), gel permeation (B) affinity (C) chromato-gram of water-soluble extract of the cultured mycelia of *Agaricus blazei.*

(Fig. 2-B). CMEx-AH was further separated into non-absorbed β, type (CMEx-AH-β) and absorbed α type (CMEx-AH-α) through Hi-Trap Con A (Fig. 2-C). The molecular weight of CMEx-AH was estimated to be 2.0 × 10^5 based on the dextrans of known molecular weights.

Structural characterization For identification of structural features, CMEx-AH-β was treated for methylation analysis, giving 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylglucitol as the major peak (m/z 233, 189, 161, 117, 101, 43) (Fig. 3-C), along with 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol (m/z 205, 161, 145, 129, 117, 101, 43) (Fig. 3-A). A small portion of (1 → 3)-linked β-D-glucopyranosyl residues were also observed with the peak of 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylglucitol (m/z 233, 161, 129, 117, 101, 43) (Fig. 3-B). These results indicated that CMEx-AH-β, consist of a backbone chain of (1→6)-linked β-D-glucopyranosides. A backbone chain of β-(1→6)-D-glucopyranosides appear to be branched with β-(1→3)-linked oligisaccharide units, as indicated by the presence of 1,3,5,6-tetra-O-acetyl-2,4-di-O-methylglucitol (m/z 233, 231, 189, 159, 139, 129, 117) (Fig. 3-D). Kawagishi *et al.* (17) reported that methylation analysis of the water-insoluble residue of *A. blazei* fruiting bodies gave 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylglucitol (m/z 233, 161, 129, 117, 101, 43) as the major peak, together with 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-glucitol (m/z 205, 161, 145, 129, 117, 101, 43), indicating that the fraction contains simple (1→6)-β-glucopyranosyl chains as the glucan part. Moreover, Q. Dong *et al.* (15) confirmed the presence of branches attached at O-3 on a (1→6)-D-glucan backbone. Similar to our study, the components of 1,3,5,6-tetra-O-acetyl-2,4-di-O-methylglucitol (m/z 233, 231, 189, 159, 139, 129, 117) and 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylglucitol (m/z 233, 161, 129, 117, 101, 43) were reported through the methylation analysis of a water-soluble fraction from the fruiting bodies of *A. blazei*. Most of the antitumor-active polysaccharides such as lentinan, schizophyllan, and sclereglucan have been reported as β-D-glucans, particularly (1→3)-linked with (1→6)-β-D-glucosidic side chains (18). However, according to the results of methylation analyses, the fundamental structure of *A. blazei* is expected to be (1→6)-β-D-glucan with (1→3)-β-D-glucosidic side chains.

CMEx, upon examination of its structure by Smith degradation, yielded glycerol and some oligosaccharides. Methylation analysis of the nondialyzate showed the peaks of 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylglucitol and 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-glucitol, indications of the presence of a branched chain of (1→3)-linked D-glucopyranosyl residues in CMEx and the degradation of a backbone chain of (1→6)-linked D-glucose residues.

FT-IR and ^{13}C-NMR spectroscopy of CMEx-AH-β,

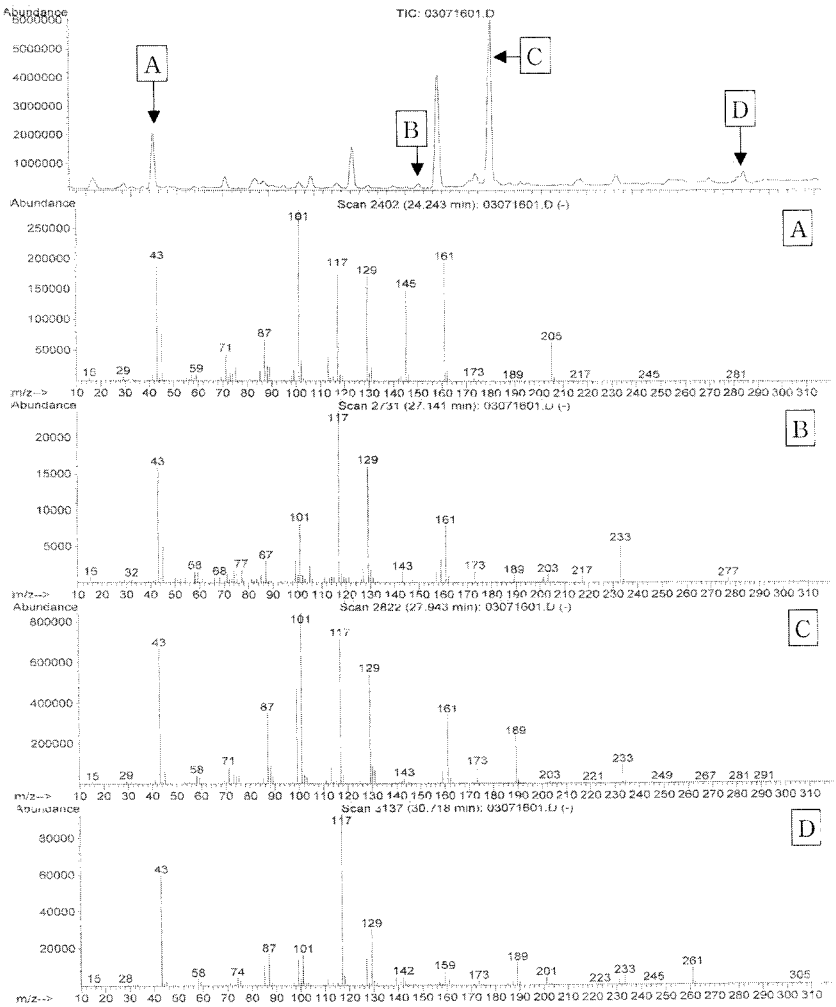

Fig. 3. GC-MS data of CMEx-AH-β from water-soluble extract of the cultured mycelia of *Agaricus blazei*. A: 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylhexitol, B: 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylhexitol, C: 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylhexitol, D: 1,3,5,6-tetra-O-acetyl-2,4-di-O-methylhexitol

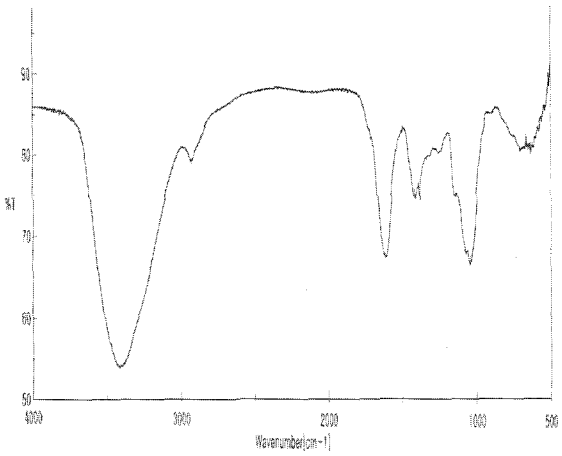

Fig. 4. FT-IR spectrum of CMEx-AH-β from water-soluble extract of the cultured mycelia of *Agaricus blazei*.

Table 2. The ^{13}C-NMR spectral lines of CMEx-AH-β from *Agaricus blazei* and their assignments

peak	δ (ppm)	Assignment	Curdlan[a)]	GE-3[b)]
A	61.1	C-6 (1 → 3)-	62.4	
B	69.2	C-6' (1 → 6)-, (1 → 3)-		70.0
C	70.5	C-4 (1 → 6)-	69.9	71.4
D	72.8	C-2 (1 → 6)-	74.3	75.0
E	77.5	C-5 (1 → 6)-, C-3 (1 → 6)-	77.7	77.1
F	85.5	C-3' (1 → 3)-, (1 → 6)-	87.5	78.1
G	103.2	C-1' (1 → 6)-, (1 → 3)-	104.5	104.9

[a)]Curdlan: β-(1 → 3) glucan, Ref. 18
[b)]GE-3: β-(1 → 6) glucan, Ref. 18

further support the results of methylation analysis. CMEx-AH-β, showed i.r. absorption band at 890 cm^{-1}, characteristic for β-glucosides. In addition, a broad O-H stretching vibration from 3500 to 3200 cm^{-1}, a C-H stretching vibration at 2900 cm^{-1}, and a C-O stretching vibration from 1250 to 1000 cm^{-1} were observed (Fig. 4). The obtained FT-IR spectrum of CMEx-AH-β was similar to that of the bioactive CR-PS fraction from *A. blazei* fruiting bodies (19). In the case of ^{13}C-NMR spectrum, the signals were assigned in reference to the signals of curdlan [linear (1→3)-β-glucan] and GE-3 [linear (1→6)-β-glucan] (Table 2). The signal A at 61.1 ppm was assigned to the non-substituted C-6 of the side chains, and the substituted C-6 signal of the β-glucopyranosyl residues appeared strongly at 69.2 ppm. Regarding the C-1 signal of the β-glucopyranosyl residues, peak G could be considered regardless of where it originated from, either (1→3)- or (1→6)-linked β-glucosyl residues. The signal (peak F) at 85.5 ppm, which was much weaker than that (peak E) of the non-substituted C-3 appeared at 77.5 ppm, could be ascribed to the substituted C-3 carbons of the side chains. The ^{13}C-NMR data suggest the backbone chain of β-D-glucan is mainly composed of β-(1→6)-linked glucopyranosides rather than β-(1→3)-linked units. Therefore, CMEx-AH-β is deduced to be composed of a (1→6)-linked β-glucan having side chains branched at O-3. The spectral features of the purified fraction of CMEx are in good agreement with the results of methylation analysis and with the assignments of Q. Dong *et al.* (15).

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