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Structural characterization of immunostimulating polysaccharide from cultured mycelia of *Cordyceps militaris*

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ABSTRACT

The water soluble crude polysaccharide obtained from cultured mycelia of *Cordyceps militaris* (CPM) by hot water extraction followed by ethanol precipitation was fractionated by DEAE cellulose and Sepharose CL-6B column chromatography. This fractionation process resulted in four polysaccharide fractions that were termed CPMN Fr I, CPMN Fr II, CPMN Fr III, and CPMN Fr IV. Of these fractions, CPMN Fr III was able to upregulate the functional events mediated by activated macrophages, such as production of nitric oxide (NO) and expression of cytokines (IL-1β and TNF-α). Its structural characteristics were investigated by a combination of chemical and instrumental analyses, including methylation, reductive cleavage, acetylation, Fourier transform infrared spectroscopy (FT-IR), and gas chromatography–mass spectrometry (GC–MS). Results indicate that CPMN Fr III was a high molecular mass polysaccharide with a random coil conformation of the β-1,4-branched-β-1,6-galactoglucomannan.

1. Introduction

In recent years many natural polysaccharides and polysaccharide–protein complexes, isolated from mushrooms, have been used as therapeutic agents (Novak & Vetvicka, 2008). Among them, *Cordyceps militaris*, an entomopathogenic fungus belonging to the class Ascomycetes, has been reported to have beneficial biological activities such as hypoglycemic (Kiho, Yamane, Hui, Usui, & Ukai, 1996), hypolipidemic (Yang et al., 2000), anti-inflammatory (Won & Park, 2005), antitumor (Lin & Chiang, 2008; Park et al., 2005, 2009), anti-metastatic (Nakamura et al., 1999), immunomodulatory (Cheung et al., 2009; Kim et al., 2008), and antioxidant effect (Yu et al., 2007, 2009). The fruiting bodies of wild *C. militaris* are expensive because of host specificity and rarity in nature. Therefore, the production of adequate quantities of the fruiting bodies of wild *C. militaris* for wide spread use as a therapeutic agent is currently impractical. It takes a long time to complete the fruiting body when solid culture is used. Liquid culture has the potential to increase mycelial production in a compact space and shorter time with less chance of contamination. The production of mycelia by liquid culture is shown as a promising alternative for fruiting body (Ohta, 1990).

Many studies have demonstrated that the polysaccharides from basidiomycetes mushroom had highly beneficial therapeutic effects including (1) preventing oncogenesis after administering of peroral medications prepared from these mushrooms or their extracts, (2) direct antitumor activity against various tumors, (3) immunosynergism activity against tumors in combination with chemotherapy, and (4) preventive effects on tumor metastasis (Chihara, Maeda, Hamuro, Sasaki, & Fukuoka, 1969; Collins, Zhu, Guo, Xiao, & Chen, 2006; Ng & Wang, 2005). It has been extensively shown that the immunomodulating actions of polysaccharides are dependent on their chemical composition, molecular weight, conformation, glycosidic linkage, degree of branching, etc. (Methacanon, Madla, Kirtikara, & Prasitsil, 2005; Yadomae & Ohno, 1996). As a result of this phenomenon, several studies have been conducted to determine accurately the structures of these different polysaccharides.

The aim of this study was to better understand and characterize the structural characteristics of the polysaccharide, CPMN Fr III, which was isolated and purified from cultured mycelia of *C. militaris* by gel filtration and ion exchange chromatography. To this end, we investigated the release of NO and the production of cytokines by macrophages that were activated by this polysaccharide as part of the innate immune response. In addition, its chemical composition, molecular weight, conformation, degree of branching, and glycosidic linkage were examined.

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2. Materials and methods

2.1. Materials

The strain used in this study was *C. militaris* KCTC 6064, which was cultivated for 11 days at 24 °C, 200 rpm, uncontrolled pH, and a 2% (v/v) inoculum size in modified medium containing 80 g/l glucose, 10 g/l yeast extract, 0.5 g/l $MgSO_4 \cdot 7H_2O$, and 0.5 g KH_2PO_4. After 11 days of cultivation, the culture broth was centrifuged at 5000 rpm for 20 min. Precipitated mycelia were washed three times with distilled water, and then freeze-dried (Kwon, Lee, Shin, Lee, & Hong, 2009). Dialysis tubing cellulose membranes, DEAE cellulose, Sepharose CL-6B, standard dextrans, lipopolysaccharide (LPS, *Escherichia coli* 0111:B4), laminarin, curdlan, and Congo red were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum and RPMI1640 were obtained from GIBCO (Grand Island, NY, USA). RAW264.7 macrophages were purchased from the American Type Culture Collection (Manassas, VA, USA). All other chemicals were of Sigma grade.

2.2. Extraction, fractionation and purification of water-soluble polysaccharides

Lyophilized mycelia were extracted two times with three volumes of distilled water at 121 °C for 2 h. Extracts were centrifuged at 5000*g* for 20 min and filtered through 0.45 μm Whatman filter paper to remove insoluble matter, then, freeze-dried. Polysaccharides were precipitated from resuspended extracts using 95.0% ethanol, collected by filtration through 0.45 μm Whatman filter paper, resuspended and dialyzed against distilled water for 5 days to remove low-molecular-weight compounds. The crude polysaccharides, termed CPM, was dissolved in distilled water, centrifuged at 5000*g* for 20 min, and loaded onto a DEAE cellulose (Cl^-) column (2.5 × 50 cm) to separate neutral and acidic polysaccharides. The resulting fractions were loaded onto a Sepharose CL-6B column (2.3 × 80 cm) equilibrated with 0.5 N NaCl, then eluted with the same solution to separate polysaccharides based on molecular weight. Each polysaccharide fraction, derived from cultured mycelia of *C. militaris*, contained an endotoxin level that was below the detection limit (0.0015 EU/ml) as assessed by an E-TOXATE kit (Sigma, St. Louis, MO, USA).

2.3. Cell culture

RAW264.7 cells were maintained in RPMI1640 that was supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. Cells were grown at 37 °C in a humidified 5% CO_2 incubator.

2.4. Cell viability

The effect of polysaccharides on the viability of RAW264.7 cells was determined using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in viable cells. After pre-incubating RAW264.7 cells (1×10^6 cells/ml) for 18 h, polysaccharides (1000 μg/ml) or LPS (2.5 μg/ml) was added and the mixture was incubated for an additional 24 h. Fifty microliters of the MTT stock solution (2 mg/ml) was then added to each well to attain a total reaction volume of 200 μl. After incubation for 2 h, the plate was centrifuged at 800*g* for 5 min and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl dimethylsulfoxide and the A_{540} was read on a scanning multiwell spectrophotometer.

2.5. Determination of NO production

After pre-incubation RAW264.7 cells (1×10^6 cells/ml) for 18 h, each polysaccharide (1000 μg/ml) or LPS (2.5 μg/ml) was added and the mixture was incubated for an additional 24 h. Nitrite in culture supernatants was measured by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% *N*-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μl samples. The nitrite concentration was determined at 540 nm using $NaNO_2$ as a standard.

2.6. RT-PCR

To evaluate levels of LPS or CPMN Fr III-inducible mRNA expression, total RNA from CPMN Fr III-treated or untreated RAW264.7 cells was prepared by adding TRIzol reagent (Gibco-BRL) according to the manufacturer's protocol. The total RNA solution was stored at −70 °C prior to subsequent use. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using MuLV reverse transcriptase. Total RNA (1 μg) was incubated with oligo-dT_{15} for 5 min at 70 °C, then mixed with a 5× first-strand buffer, 10 mM dNTPs, and 0.1 M DTT. The reaction mixture was further incubated for 5 min at 37 °C, then for 60 min after the addition of 2 U of MuLV reverse transcriptase. Reactions were terminated by heating for 10 min at 70 °C, and total RNA was depleted by addition of RNase H. PCR was performed with the incubation mixture (2 μl of cDNA, 4 μM forward and reverse primers [Bioneer, Seoul, Korea], a 10× buffer [10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.1% Triton X-100], 250 μM dNTPs, 25 mM $MgCl_2$, and 1 U of Taq polymerase [Promega, USA]) under the following conditions: a 45 s denaturation step at 94 °C, a 45 s annealing step between 55 and 60 °C, a 60 s extension step at 72 °C, and a 7 min final extension step at 72 °C after 30 cycles. The primers used in this experiment are indicated in Table 1. Ten microliters of PCR products were electrophoresed on a 1.2% agarose gel and visualized by ethidium bromide staining under ultraviolet light.

2.7. TNF-α production

The ability of CPMN Fr III to induce production of TNF-α in RAW264.7 cells was determined by dissolving the polysaccharide in the culture medium. Supernatants were harvested and the concentration of TNF-α was determined using an ELISA kit (Biosource International, Camarillo, CA, USA), according to the manufacturer's instructions.

2.8. Analysis of chemical properties

The total sugar content of each polysaccharide was determined using the phenol–sulfuric acid method (Chaplin & Kennedy, 1986),

Table 1
Primer sequences of genes investigated by RT-PCR analysis.

Gene	Primer sequences
IL-1β	
F[a]	5′-CAGATGAGGACATGAGCACC-3′
R[b]	5′-CACCTCAAACTCAGACGTCTC-3′
TNF-α	
F[a]	5′-TTGACCTCAGCGCTGAGTTG-3′
R[b]	5′-CCTGTAGCCCACGTCGTAGC-3′
GAPDH	
F[a]	5′-CACTCACGGCAAATTCAACGGCAC-3′
R[b]	5′-GACTCCACGACATACTCAGCAC-3′

[a] Forward.
[b] Reverse.

the total protein concentration was determined using the Bradford method (Bradford, 1976), the hexosamine content was evaluated using the Elson–Morgan method (Dische, 1962), and the uronic acid content was assessed using the Blumenkrantz method (Blumenkrantz & Asboe-Hansen, 1973).

2.9. Analysis of monosaccharide composition

Monosaccharide composition and ratios were determined by first hydrolyzing the polysaccharide with 2 M trifluoroacetic acid (TFA) in a sealed tube at 100 °C for 4 h. Acid was removed by repeated evaporation using a vacuum distillation device. The hydrolysate was then dissolved in 1.0 ml of distilled water and filtered through a 0.2 μm PTFE membrane. The aqueous hydrolysate was analyzed by reverse-phase HPLC using an ED50 electrochemical detector (Dionex, Sunnyvale, CA, USA) under the following conditions: column: CarboPac PA10 Analytical Column (4 × 240 mm); solvent: A, deionized water, B, 200 mM NaOH; program: 0–20 min (B conc. 8%), 20–40 min (B conc. 25%), 40–70 min (B conc. 8%); flow rate: 0.9 ml/min; temp.: 30 °C. Glucose, galactose, mannose, and fucose were used as monosaccharide standards.

2.10. Determination of molecular weight

The molecular weight of the polysaccharide fractions was determined by gel filtration using a Sepharose CL-6B packed column. A standard curve was prepared based on the elution volume and the molecular weight. Standard dextrans (MW: 670, 410, 150, and 25 kDa) were used for calibration.

2.11. Analysis of helix-coil transition

The conformational structure of the polysaccharides in solution was determined by characterizing Congo red–polysaccharide complexes. The transition from a triple-helical arrangement to the single-stranded conformation was examined by measuring the λ_{max} of Congo red–polysaccharide solutions at NaOH concentrations ranging from 0.01 to 0.5 N. Polysaccharide aqueous solutions (1 mg/ml) containing 100 μl of 0.5 mg/ml Congo red were treated with different concentrations of NaOH. Visible absorption spectra were recorded with a UV/vis spectrophotometer at each alkali concentration (Ogawa & Hatano, 1978; Ogawa, Tsurugi, & Watanabe, 1973).

2.12. Identification of anomeric configuration

To ascertain the presence or absence of the α or β configuration in each polysaccharide, β-linked polysaccharides were detected using a Fungi-Fluor Kit (Polysciences, Warrington, PA, USA). Each sample was dissolved in distilled water and the solution was placed on a slide and dried in an oven. Following the addition of methanol, each sample dried for an additional 20 min. Fungi-Fluor Solution A (cellufluor, water, and potassium hydroxide) was used as a dye. A few drops were added to each sample and the mixtures were incubated for 3 min. After washing with distilled water, the fluorescence level was determined using a UV Illuminator (Vilber Lourmat Inc., France).

2.13. Methylation of CPMN Fr III

CPMN Fr III was methylated according to the method developed by Ciucanu and Kerek, using powdered NaOH in Me_2SO–MeI (Ciucanu & Kerek, 1984). Methylation was confirmed by measuring the FT-IR spectrum.

2.14. Determination of glycosidic linkage

Permethylated CPMN Fr III was extracted in dichloromethane and reductive cleavage was performed using a combination of trimethylsilyl methanesulfonate and trifluoride etherate as the catalyst as previously described (Rolf & Gray, 1982). The reaction was allowed to proceed for 8–12 h at room temperature, then was quenched by addition of sodium bicarbonate. The organic layer was separated with a syringe and products were isolated and acetylated. Glycosidic linkage was analyzed by GC–MS on a Micromass apparatus (Waters Corp., Milford, MA, USA) equipped with an HP-5MS column and a temperature program of 120–180 °C at 5 °C/min and 180–250 °C at 2 °C/min). Mass spectra were obtained at an ion energy of 70 eV, a current intensity of 500 μA and temperature of 250 °C.

2.15. Statistical analysis

A Student's *t*-test and a one-way ANOVA were used to determine the statistical significance of the differences between the values determined for the various experimental and control groups. Data are expressed as means ± standard errors (SEM) and the results are taken from at least three independent experiments performed in triplicate. *P* values of 0.05 or less were considered to be statistically significant.

3. Results

3.1. Purification and fractionation

In the first stage of purification and fractionation, ion exchange chromatography through a DEAE-cellulose column was used to separate neutral polysaccharides from acidic fractions. The yield of the neutral fraction (CPMN) and the acidic fraction (CPMA) obtained from the crude polysaccharide extract CPM was 0.668 g/g and 0.052 g/g, respectively (Fig. 1A). The molecular distribution of CPMN was investigated using gel filtration chromatography with a Sepharose CL-6B column, resulting in four polysaccharide fractions, namely CPMN Fr I (0.018 g/g), CPMN Fr II (0.125 g/g), CPMN Fr III (0.408 g/g), and CPMN Fr IV (0.049 g/g) (Fig. 1B).

3.2. Macrophage activation by polysaccharides

To examine whether polysaccharides purified from cultured mycelia of *C. militaris* were able to stimulate the functional activation of macrophages, macrophage-like RAW264.7 cells were incubated with 1000 μg/ml of each polysaccharide and NO production was measured and compared to the amount produced by the untreated control group. Polysaccharide-treated cells produced larger amounts of NO than untreated cells (Fig. 2A). To address whether CPMN Fr III elicits innate immune responses in macrophages, RT-PCR and ELISA assays were used to examine induction of transcriptional gene upregulation and increased expression of proinflammatory cytokines. These experiments showed that CPMN Fr III strongly triggers the expression of proinflammatory cytokines TNF-α and interleukin-1β (IL-1β) (Fig. 2B and C).

3.3. Chemical properties and monosaccharide composition

The total sugar content of CPMN Fr III was 92.34%. Its major sugar constituents are mannose (72.22%), galactose (18.61%) and glucose (9.17%) (Table 2 and Fig. S1). The contents of proteins, hexosamine and uronic acid of this polysaccharide are 0.21%, 0.12% and 0.33%, respectively (Table 2).

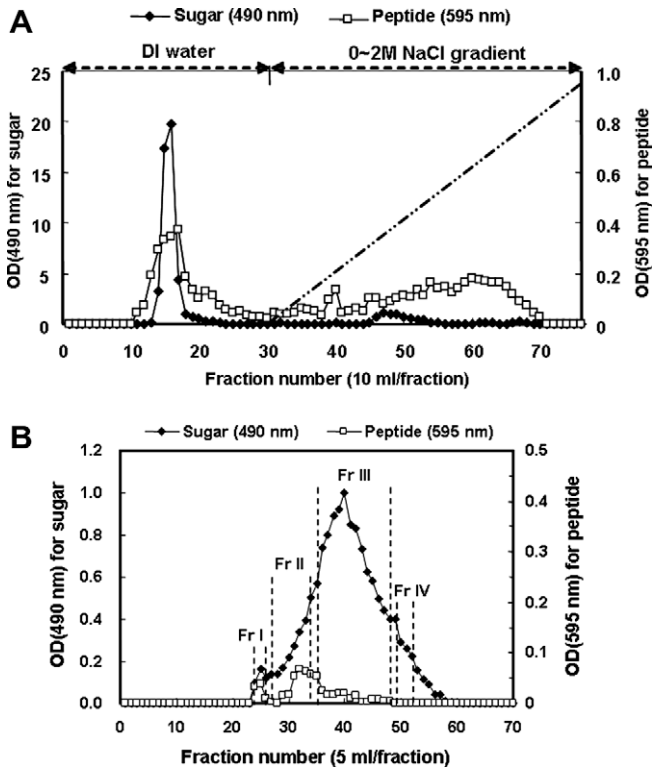

Fig. 1. Isolation and purification of polysaccharides extracted from cultured mycelia of *Cordyceps militaris*. (A) Ion exchange chromatogram of the crude polysaccharides, CPM, on a DEAE-cellulose column. (B) Gel filtration chromatogram of the neutral polysaccharide fraction, CPMN, on a Sepharose CL-6B column (fraction number of ion exchange chromatography: 14–18).

3.4. Homogeneity and molecular weight

The homogeneity of CPMN Fr III was confirmed by refractionation through gel filtration chromatography using a Sepharose CL-6B packed column (Fig. 3A). The molecular weight of this fraction was then determined by gel filtration chromatography to be 210 kDa using dextrans as standards (Fig. 3B).

3.5. Identification of helix-coil transition

A shift in the visible absorption maximum of Congo red is induced by the presence of polysaccharides and can thus be used to provide conformational information. The absorption maximum of dextran, which has a random coil conformation, was around 450 nm (Fig. 4). Curdlan exhibits a triple-helical conformation, which was demonstrated by the shift in the absorption maximum at 0.24 M NaOH. However, the absorption maximum of laminarin, which has a different triple-helical conformation, was around 550 nm. Based on this analysis, CPMN Fr III was found to exhibit a random coil conformation similar to that of dextran.

3.6. Identification of anomeric configuration

To ascertain the presence or absence of the α or β configuration in CPMN Fr III, the Fungi-Fluor Kit was used. The Fungi-Fluor staining solution, cellufluor, binds nonspecifically to β-linked polysaccharides, thus enabling their rapid detection. While dextran, which is an α-glucan, did not exhibit fluorescence in the presence of cellufluor, a signal was clearly observed for curdlan, which is a β-glucan. CPMN Fr III displayed a fluorescence signal very similar to that of curdlan, indicating that it is a β-linked polysaccharide (Fig. 5).

3.7. Glycosidic linkage of the polysaccharide

CPMN Fr III exhibited an IR absorption spectrum characteristic of a polysaccharide, with bands at 1080 cm^{-1} (C═O), 2800–2900 cm^{-1} (C—H), and 3400 cm^{-1} (O—H). Glycosidic linkage analysis of permethylated CPMN Fr III was performed by the reductive cleavage method. The polysaccharide was shown to be fully methylated, as indicated by the disappearance of the band at 3400 cm^{-1}

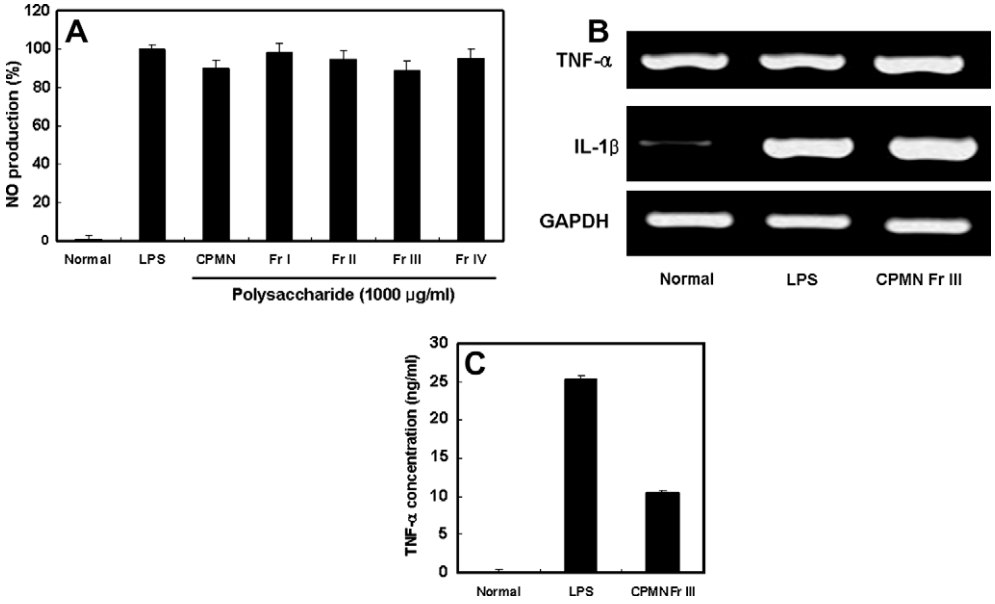

Fig. 2. Immunostimulating effects of polysaccharide, CPMN Fr III, purified by DEAE cellulose and Sepharose CL-6B chromatography. (A) Effect of purified polysaccharides on NO synthesis in murine macrophage-like cells. RAW264.7 cells (1×10^6 cells/ml) were stimulated by each polysaccharide fraction (1000 μg/ml) for 24 h. Supernatants were collected and NO concentration was determined using the Griess reagent, as described in Section 2. (B) The effect of CPMN Fr III on the expression of cytokines. RAW264.7 cells (1×10^7 cells/ml) were incubated with CPMN Fr III (1000 μg/ml) or LPS (2.5 μg/ml) for 6 h. Cytokine mRNA levels were determined by semiquantitative RT-PCR. The results shown are from one of three experiments performed. (C) The effect of CPMN Fr III on TNF-α production. RAW264.7 cells (1×10^6 cells/ml) were stimulated by CPMN Fr III (1000 μg/ml) for 6 h. Supernatants were collected and TNF-α concentration was determined by ELISA, as described in Section 2. Data (A and C) represent means ± SEM of three independent experiments performed in triplicate.

Table 2
Proximate composition and monosaccharide composition of purified polysaccharide, CPMN Fr III, from cultured mycelia of *Cordyceps militaris* (%, dry basis).

Polysaccharide	Protein	Hexosamine	Uronic acid	Total sugar	Component sugar (molar %)			
					Glc	Gal	Man	Fuc
CPMN Fr III	0.21	0.12	0.33	92.34	9.17	18.61	72.22	ND[a]

%, dry basis.
[a] Not detected.

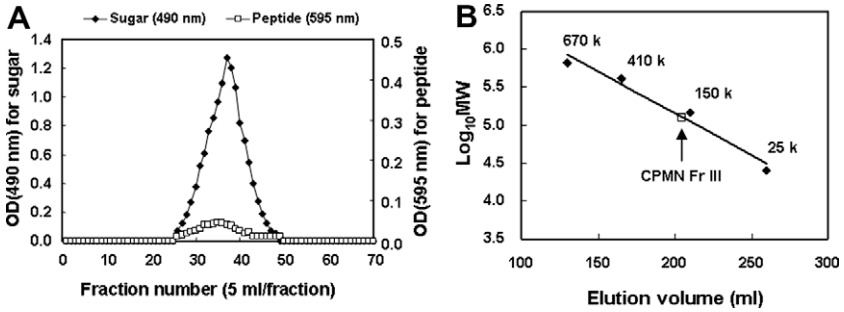

Fig. 3. Average molecular weight of CPMN Fr III. (A) Elution profile of polysaccharide refractionated by gel filtration with Sepharose CL-6B. (B) Molecular weights of standard dextrans and CPMN Fr III determined by Sepharose CL-6B gel filtration chromatography.

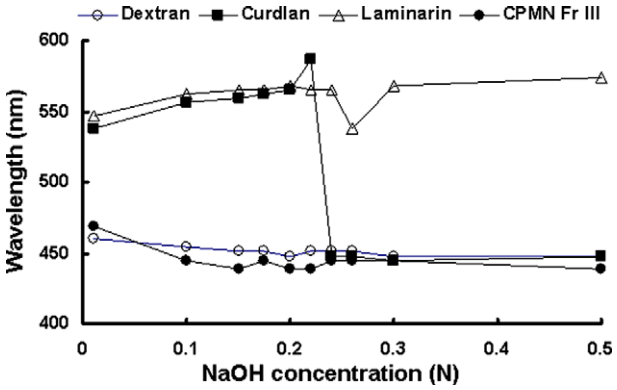

Fig. 4. Helix-coil transition analysis of CPMN Fr III and standard polymers according to the absorption maximum of the Congo red–polysaccharide complex at various concentrations of NaOH. For more details, see Section 2.

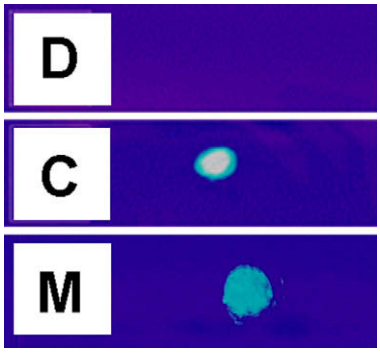

Fig. 5. Identification of the anomeric configuration of CPMN Fr III and standard polymers. Visualization of β-linked polysaccharides using the Fungi-Fluor kit. D. Dextran; C. Curdlan; M. CPMN Fr III.

characteristic of a carbohydrate ring (Fig. S2). Following reductive cleavage, CPMN Fr III was found to be hydrolyzed to its monosaccharide components, as indicated by comparing the GC traces of the polysaccharide hydrolysate to those of monosaccharide standards. The data summarized in Table 3 (see also Fig. S3) indicate that CPMN Fr III has a backbone of (1 → 6)-linked D-mannopyranosyl and (1 → 6)-linked D-glucopyranosyl residue. The branches were mainly composed of (1 → 4)-linked D-mannopyranosyl residue, and terminated with D-galactopyranosyl residues and D-mannopyranosyl residues, with a degree of branching (DB) of 0.33.

4. Discussion

Immunostimulation itself is regarded as one of the important strategies to improve the body's defense mechanism in elderly people as well as in cancer patient. There is a significant amount of experimental evidence suggesting that polysaccharides from mushrooms enhance the host immune system by stimulating natural killer cells, T-cells, B-cells, and macrophage-dependent immune system response (Dalmo & Boqwald, 2008; Dennert & Tucker, 1973). Polysaccharides obtained from different natural sources represent a structurally diverse class of macromolecules and exert their antitumor action mostly by activating various immune system responses (Schepetkin & Quinn, 2006). In an indirect manner, activated macrophages play an important role in antitumor activity by secreting secondary compounds, such as proinflammatory cytokines [e.g., TNF-α and IL-1] and releasing cytotoxic and inflammatory molecules [e.g., NO and ROS], which are harmful to cancer cells, and by regulating the immune system to process and present antigens (Medzhitov & Janeway, 2000). In the present study, CPMN Fr III, which was obtained from cultured mycelia of *C. militaris* by hot water extraction, ethanol precipitation and fractionation by DEAE cellulose and Sepharose CL-6B column chromatography, was found to very effectively upregulate cytokine expression (TNF-α and IL-1β) and NO release indicating that it was able to induce the functional activation of macrophages (Fig. 2). Polysaccharides, polymers of monosaccharide residues joined to each other by glycosidic linkages, belong to a structurally diverse class of macromolecules. Because they have the greatest potential for structural variability relative to other biopolymers, polysaccharides have the highest capacity for carrying biological information. As a result of this phenomenon, it is highly important to determine the accurate structures of polysaccharides. Polysaccharides differ greatly in their chemi-

Table 3
Identification and linkage analysis of partially methylated alditol acetates of the purified polysaccharide, CPMN Fr III, isolated from cultured mycelia of *Cordyceps militaris*.

Polysaccharide	Alditol acetate derivative	Type of linkage	Relative molar ratio
CPMN Fr III	1,5-Anhydro-2,3,4,6-tetra-*O*-methyl-D-galactitol	Terminal Gal*p*	0.290
	1,5-Anhydro-2,3,4,6-tetra-*O*-methyl-D-mannitol	Terminal Man*p*	0.100
	1,5-Anhydro-4-*O*-acetyl-2,3,6-tri-*O*-methyl-D-mannitol	→4)-Man*p*-(1→	0.153
	1,5-Anhydro-6-*O*-acetyl-2,3,4-tri-*O*-methyl-D-mannitol	→6)-Man*p*-(1→	1.000
	1,5-Anhydro-3-*O*-acetyl-2,4,6-tri-*O*-methyl-D-mannitol	→3)-Man*p*-(1→	0.100
	1,5-Anhydro-4,6-di-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol	→4,6)-Glc*p*-(1→	0.681

cal composition, molecular weight, conformation, glycosidic linkage, degree of branching, etc. (Methacanon et al., 2005; Yadomae & Ohno, 1996). Recently, the structural characterizations of several bioactive polysaccharides obtained from *Cordyceps* spp. were reported (Wu, Sun, & Pan, 2006; Xiao et al., 2006; Yu, Wang, Zhang, Zhou, & Zhao, 2004; Yu et al., 2007, 2009). In the present study, CPMN Fr III, which was found to act as an immunostimulant through the activation of macrophages, was a β-1,4-branched-β-1,6-galatolgucomannan that had a molecular weight of 210 kDa (Table 3 and Fig. 3). Polysaccharides from the liquid culture of *Grifola frondosa* were heteromannan, heterofucans, and heteroxylans, or complexed with proteins and were not found in the fruiting body of this mushroom. However, it is worth noting that the polysaccharide structure produced from cultured mycelia may depend on the composition of the nutrient medium used for cultivation (Zhuang et al., 1994). Molecular weight has long been recognized as a critical parameter in the antigenicity of a molecule. Most polysaccharides with medicinal properties are high molecules above 100 kDa of molecular weight (Kabat & Bezer, 1958). Similarly, CPMN Fr III is high-molecular-weight (210 kDa) polysaccharide with immunostimulant properties (Fig. 3). In contrast, a low-molecular-weight (20 kDa) fraction from the fruiting body of *Agaricus blazei* was found to exhibit tumor-specific cytocidal and immunopotentiating effects (Fujimiya, Suzuki, Katakura, & Ebina, 1999). In addition, acidic hydrolysate fractions, with molecular weights ranging from 53 to 1 kDa, from the fruiting body of *Tremella fuciformis*, induced human monocytes to produce interleukin-6 as efficiently as the non-hydrolyzed fraction (Gao, Jiang, Chen, Jensen, & Seljelid, 1996). It has been shown that a triple-helical tertiary conformation of medicinal mushroom-derived polysaccharide was important for their immune-stimulating activity indicating that polysaccharide-mediated immuno-pharmacological activities were dependent on the helical conformation (Yanaki, Ito, & Tabata, 1986). Interestingly, unlike other medicinal mushroom-derived β-polymers, CPMN Fr III has a random coil conformation but not a triple helix conformation (Fig. 4). There are some data suggesting that polysaccharides with no triple-helical conformation show great antitumor activity. The antitumor activity of a (1 → 3)-β-glucan with a high molecular weight (670 kDa) isolated from *Glomerella cingulata* appears to be independent of the presence of ordered structures (Gomaa, Kraus, Rosskopf, Roper, & Franz, 1992). Polysaccharides from *Pythium aphanidermatum* with molecular weights of 10 kDa and 20 kDa, respectively, have antitumor activity but no ordered structure (Blaschek, Kasbauer, Kraus, & Franz, 1992). Various *Phytophthora* species-derived β-type polymers with no helical conformation were active against sarcoma 180 (Kraus, Blaschek, Schutz, & Franz, 1992).

In conclusion, CPMN Fr III, a high molecular mass polysaccharide with a random coil conformation of the β-1,4-branched-β-1,6-galactoglucomannan, was a potent murine macrophage stimulator. To address the correlation between structure and the immunostimulating activities of this polysaccharide, mechanism studies in terms of macrophage activation signaling pathways will be the subject of further investigations.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2010.01.017.

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