J. Microbiol. Biotechnol. (2010), **20**(7), 1053–1060
doi: 10.4014/jmb.0910.10022
First published online 14 May 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Study of Macrophage Activation and Structural Characteristics of Purified Polysaccharide from the Fruiting Body of *Cordyceps militaris*

Lee, Jong Seok, Jeong Seok Kwon, Dong Pil Won, Jung Hyun Lee, Keun Eok Lee, Shin Young Lee, and Eock Kee Hong*

Department of Bioengineering and Technology, Kangwon National University, Chuncheon 200-701, Korea

Received: October 16, 2009 / Revised: April 19, 2010 / Accepted: April 21, 2010

***Cordyceps militaris*, an entomopathogenic fungus belonging to the class Ascomycetes, has been reported to have beneficial biological activities such as hypoglycemic, anti-inflammatory, antitumor, antimetastatic, hypolipidemic, immunomodulatory, and antioxidant effects. In this study, the crude water-soluble polysaccharide CMP, which was obtained from the fruiting body of *C. militaris* by hot water extraction and ethanol precipitation, was fractionated by DEAE-cellulose and Sepharose CL-6B column chromatographies. This process resulted in three polysaccharide fractions, termed CMP Fr I, CMP Fr II, and CMP Fr III. Of these fractions, CMP Fr II, with an average molecular mass of 127 kDa, was able to upregulate effectively the phenotypic functions of macrophages such as NO production and cytokine expression. The chemical property of the stimulatory polysaccharide, CMP Fr II, was determined based on its monosaccharide composition, which consisted of glucose (56.4%), galactose (26.4%), and mannose (17.2%). 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 indicated that CMP Fr II consisted of the (1→4) or (1→2) linked glucopyranosyl or galactopyranosyl residue with a (1→2) or (1→6) linked mannopyranosyl, glucopyranosyl, or galactopyranosyl residue as a side chain. The configuration of the β-linkage and random coil conformation of CMP Fr II were confirmed using a Fungi-Fluor kit and Congo red reagent, respectively.**

Keywords: *Cordyceps militaris*, immunostimulating polysaccharide, mushroom-derived hetero-β-glucan, macrophage activation

**Corresponding author*
Phone: +82-33-250-6275; Fax: +82-33-243-6350;
E-mail: ekhong@kangwon.ac.kr

Macrophages are a type of differentiated tissue cells that originate as blood monocytes. The cells play a major role in defending the host against infection and cancer, as they process and present antigens to the lymphocytes based on engulfing and digesting pathogens that cross the epithelial barrier [19, 31, 32]. Therefore, macrophages play a crucial role in the innate and adaptive immune responses. When the body is stimulated by pathologic stimuli or injury, phagocytosis is the first step in the macrophages' response to pathogens. In addition, macrophages can defend against pathogen invasion by secreting proinflammatory cytokines [*e.g.*, tumor necrosis factor (TNF)-α and interleukin (IL)-1] and releasing cytotoxic and inflammatory molecules [*e.g.*, nitric oxide (NO), reactive oxygen species (ROS)] [26, 29]. Since macrophages act as regulatory and effector cells in the immune system, understanding the activation of macrophages is expected to aid in the development of therapeutics to treat microbial pathogens and cancers [24].

Recently, a number of bioactive molecules including antitumor agents have been identified in various higher Basidiomycetes mushrooms [44]. Unlike existing chemical anticancer agents, polysaccharides are known to have no toxic side effects. When used as cancer therapeutics, these polysaccharides are able to prolong the life span of cancer patients [2]. As such, polysaccharides are the best-known and most potent mushroom-derived substances that display immunopharmacological properties [34]. *Cordyceps militaris*, an entomopathogenic fungus belonging to the class Ascomycetes, has been reported to have beneficial biological activities such as hypoglycemic, anti-inflammatory, antitumor, antimetastasis, hypolipidemic, immunomodulatory, and antioxidant effects [23 30, 38, 47–50]. Many studies have demonstrated that the polysaccharides from the Basidiomycetes mushroom has highly beneficial therapeutic effects including (1) preventing oncogenesis after administering of peroral medications that were 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 [8, 11, 33]. Immunomodulating polysaccharides are characterized by chemical composition, molecular mass, conformation, glycosidic linkage, degree of branching, *etc*. [45]. Biologically active polysaccharides are widespread among mushrooms, and most of them have unique structures in different species. 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 immunostimulating activity and structural characteristics of the polysaccharide CMP Fr II, which was isolated and purified from the fruiting body of *C. militaris* by gel filtration and ion-exchange chromatographies. To this end, we investigated the functional events such as the release of NO and the production of cytokines mediated by macrophages that were activated by this polysaccharide as part of the innate immune response. In addition, its chemical composition, molecular mass, conformation, and glycosidic linkage were examined.

Materials and Methods

Materials

Dried fruiting bodies of *Cordyceps militaris* were purchased from the local market and ground in a blender. 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, U.S.A.). Fetal bovine serum and RPMI1640 were obtained from GIBCO (Grand Island, NY, U.S.A.). RAW264.7 macrophages were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). All other chemicals were of Sigma grade.

Extraction, Fractionation, and Purification of Water-Soluble Polysaccharides

A milled mushroom (100 g) was extracted two times with 3 volumes of distilled water at 121°C for 2 h. Extracts were centrifuged at 10,000 ×*g* for 20 min and filtered through 0.45-μm Whatman filter paper to remove insoluble matter, and then freeze-dried. Polysaccharides were precipitated from resuspended extracts using 95% ethanol, collected by filtration through 0.45-μm Whatman filter paper, resuspended, and dialyzed against distilled water for 5 days to remove low-molecular-mass compounds. The CMP was then dissolved in distilled water, centrifuged at 5,000 ×*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, and then eluted with the same solution to separate polysaccharides based on molecular mass. The endotoxin level in each polysaccharide fraction was assessed using an E-TOXATE kit (Sigma, St. Louis, MO, U.S.A.) and was found to be below the limit of detection (0.0015 EU/ml) (data not shown).

Cell Culture

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

Cell Viability

The effect of polysaccharides on the viability of the 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 [6]. After pre-incubating RAW264.7 cells (1×10^6 cells/ml) for 18 h, polysaccharide (1,000 μg/ml) or LPS (2.5 μg/ml) was added and the mixture was incubated for an additional 24 h, as reported previously [27]. Fifty μl 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 of dimethylsulfoxide and the color generated was determined by measuring the optical density at 540 nm on a scanning multiwell spectrophotometer.

Determination of NO Production

After pre-incubating the RAW264.7 cells (1×10^6 cells/ml) for 18 h, polysaccharide (1,000 μg/ml) or LPS (2.5 μg/ml) was added and the mixture was incubated for an additional 24 h. Nitrite in the culture supernatants was measured by adding 100 μl of the Griess reagent (1% sulfanilamide and 0.1% *N*-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μl of the samples. The nitrite concentration was determined at 540 nm using $NaNO_2$ as a standard.

RT–PCR

To evaluate levels of CMP Fr II-inducible mRNA expression, the total RNA from CMP Fr II-treated or untreated RAW264.7 cells was prepared by adding the 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, and 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, and then for 60 min after the addition of 2 units of MuLV reverse transcriptase. Reactions were terminated by

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.

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 unit of *Taq* polymerase (Promega, U.S.A.)] 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 μl of PCR products was electrophoresed on a 1.2% agarose gel and visualized by ethidium bromide staining under ultraviolet light.

TNF-α Production

The ability of CMP Fr II 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, U.S.A.) according to the manufacturer's instructions.

Determination of Molecular Mass

The molecular mass of the polysaccharide fraction 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 mass. Standard dextrans (MW: 670 kDa, 410 kDa, 150 kDa, and 25 kDa) were used for calibration.

Analysis of Chemical Properties

The total sugar content of polysaccharide was determined using the phenol–sulfuric acid method [7], the total protein concentration was determined using the Bradford method [5], the hexosamine content was evaluated using the Elson–Morgan method [15], and the uronic acid content was assessed using the Blumenkrantz and Asboe-Hansen method [4].

Analysis of Monosaccharide Composition

The 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 reversed-phase HPLC using an ED 50 electrochemical detector (Dionex, Sunnyvale, CA, U.S.A.) under the following conditions: column: CarboPac PA10 Analytical Column (4 mm×240 mm, 10 μm diameter, Dionex); solvent: A, deionized water, and B, 200 mM NaOH; program: 0–20 min (8% B), 20–40 min (25% B), 40–70 min (8% B); flow rate: 0.9 ml/min; column oven temp.: 30°C. Glucose, galactose, mannose, and fucose were used as monosaccharide standards.

Analysis of Helix–Coil Transition

The conformational structure of the polysaccharides in solution was determined by characterizing the 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 (Milton Roy Co., Rochester, NY, U.S.A.) at each alkali concentration [35, 36].

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, U.S.A.). 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 was 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).

Methylation of CMP Fr II

CMP Fr II was methylated according to the method developed by Ciucanu and Kerek [10], using powdered NaOH in Me_2SO–MeI. Methylation was confirmed by measuring the FT–IR spectrum.

Determination of Glycosidic Linkage

Permethylated CMP Fr II was extracted in dichloromethane and reductive cleavage was performed using a combination of trimethylsilyl methanesulfonate and trifluoride etherate as the catalyst as previously described [41]. The reaction was allowed to proceed for 8–12 h at room temperature, and 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, U.S.A.) equipped with an HP-5MS column (Agilent Technologies, Wilmington, DE, U.S.A.) and a temperature program of 120–180°C at 5°C/min and 180–250°C at 2°C/min. The mass conditions were set as follows: ionization mode with EI, ionization energy of 70 eV, a current intensity of 500 μA, and ion source temperature at 250°C.

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.

RESULTS

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 obtained from the crude polysaccharide extract CMP was 0.654 g/g (Fig. 1A). The molecular distribution of the neutral fraction was investigated using gel filtration chromatography with a Sepharose CL-6B column, resulting in three polysaccharide fractions: namely, CMP Fr I (0.037 g/g), CMP Fr II (0.167 g/g), and CMP Fr III (0.387 g/g) (Fig. 1B).

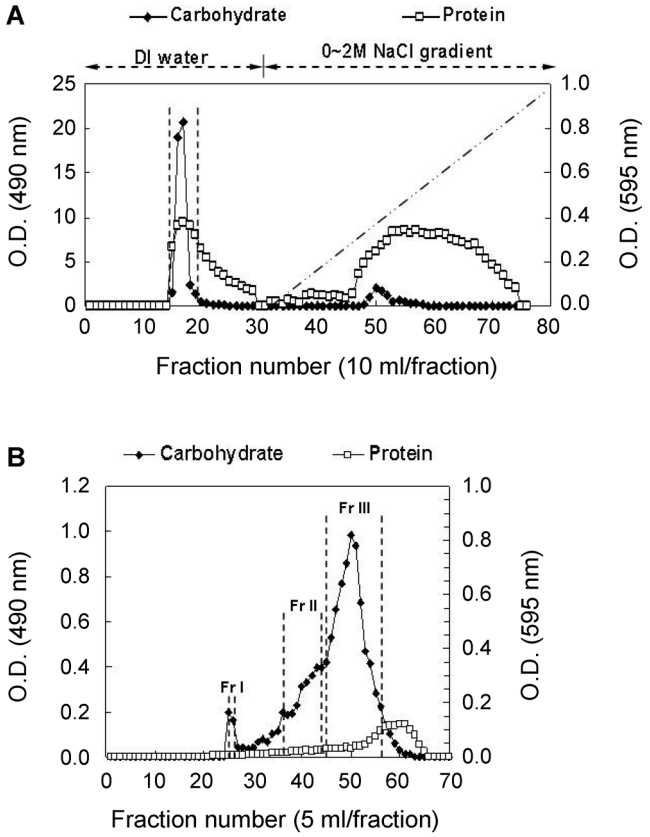

Fig. 1. Isolation and purification of polysaccharides extracted from the fruiting body of *Cordyceps militaris*.
A. Ion-exchange chromatogram of the crude polysaccharides, CMP, on a DEAE-cellulose column. **B**. Gel filtration chromatogram of the neutral polysaccharide fraction on a Sepharose CL-6B column (fraction number of ion-exchange chromatography: 15–19).

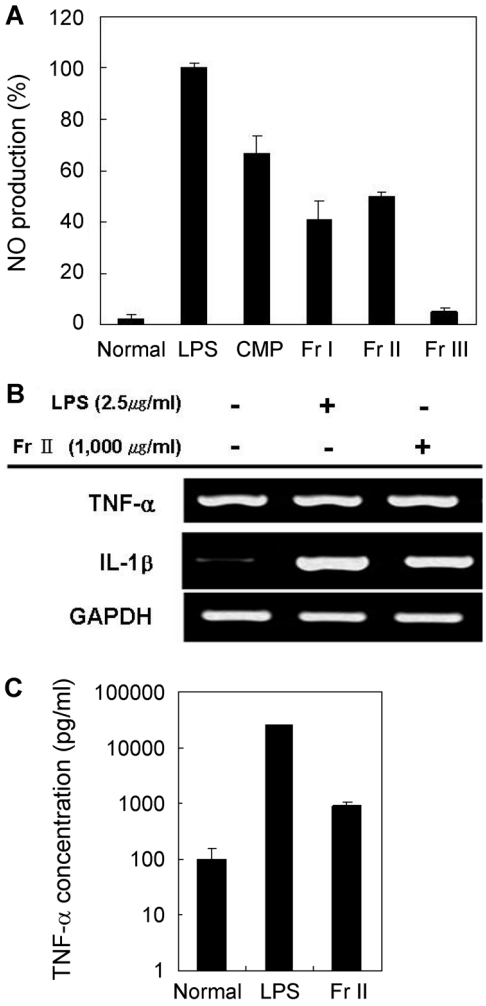

Fig. 2. Immunostimulating effects of polysaccharide CMP Fr II purified by DEAE-cellulose and Sepharose CL-6B column chromatographies.
A. Effect of purified polysaccharides on NO synthesis in macrophage-like cells. RAW264.7 cells (1×10^6 cells/ml) were stimulated by each polysaccharide fraction (1,000 μg/ml) for 24 h. Supernatants were collected and NO concentration was determined using the Griess reagent, as described in Materials and Methods. **B**. Effect of CMP Fr II on the expression of cytokines in RAW264.7 cells. RAW264.7 cells (1×10^7 cells/ml) were incubated with CMP Fr II (1,000 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**. Effect of CMP Fr II on TNF-α production. RAW264.7 cells (1×10^6 cells/ml) were stimulated by CMP Fr II (1,000 μg/ml) or LPS (2.5 μg/ml) for 6 h. Supernatants were collected and the TNF-α concentration was determined by ELISA, as described in Materials and Methods. Data (**A** and **C**) represent the mean ± SEM of three independent observations performed in triplicate.

Macrophage Activation by Polysaccharide

To address whether purified polysaccharides derived from the fruiting body of C. *militaris* were able to stimulate the functional activation of macrophages, macrophage-like RAW264.7 cells were incubated with 1,000 μg/ml of each polysaccharide, and NO production was measured and compared with the amount produced by the untreated control group. Polysaccharide-treated cells produced larger amounts of NO than untreated cells, and CMP Fr II triggered production of the most NO among the polysaccharides (Fig. 2A). To address whether CMP Fr II elicits innate immune responses in macrophages, RT–PCR and ELISA assays were used to examine the induction of transcriptional gene upregulation and increased expression of proinflammatory cytokines. These experiments showed that CMP Fr II triggers the expression of proinflammatory cytokines TNF-α and interleukin-1β (IL-1β) (Fig. 2B and 2C). Therefore, we continued further experiments to understand the structural characteristics by which CMP Fr II stimulates the cellular activities of macrophages.

Homogeneity and Molecular Mass

The homogeneity of CMP Fr II was confirmed by refractionation through gel filtration chromatography using a Sepharose CL-6B packed column (Fig. 3A). The molecular mass of this fraction was then determined by gel filtration

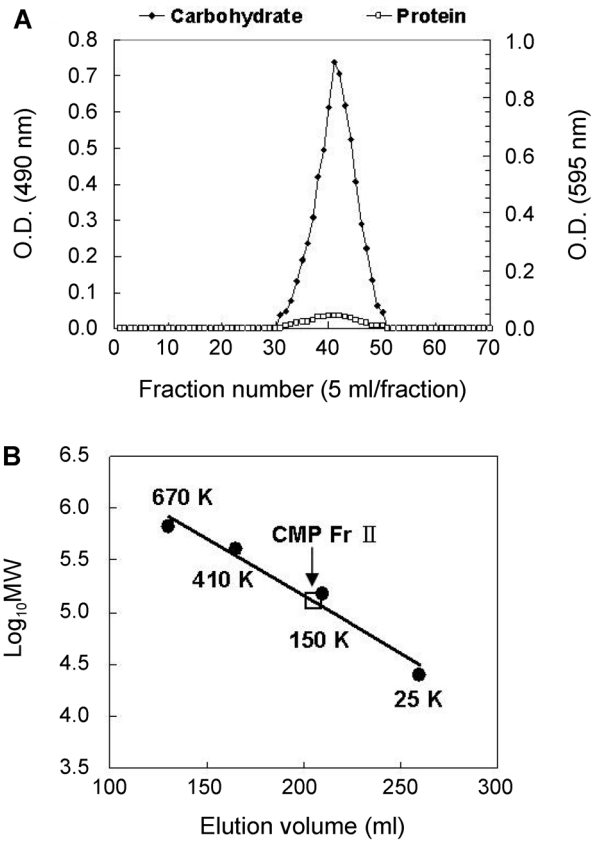

Fig. 3. Average molecular mass of CMP Fr II.
A. Elution profile of polysaccharide refractionated by gel filtration with Sepharose CL-6B (fraction number of gel filtration chromatography: 36–44). **B**. Molecular masses of standard dextrans and CMP Fr II, determined by Sepharose CL-6B gel filtration chromatography.

chromatography to be 127 kDa, using dextrans as standards (Fig. 3B).

Chemical Properties and Monosaccharide Composition

The total sugar content of the CMP Fr II was 89.48%. Its major sugar constituents are glucose (56.4%), galactose (26.4%), and mannose (17.2%) as the major sugar constituents (Supplemental Fig. 1). The contents of proteins, hexosamine, and uronic acid of this polysaccharide are 0.90%, 0.45%, and 0.17%, respectively (Table 2).

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

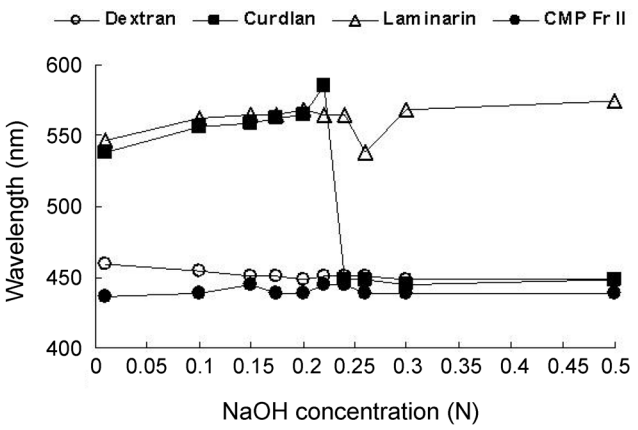

Fig. 4. Helix–coil transition analysis of CMP Fr II and standard polymer according to the absorption maximum of the Congo red–polysaccharide complex at various concentrations of NaOH (for more details, see Materials and Methods).

be used to provide conformational information. The absorption maximum of dextran, 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 N NaOH. However, the absorption maximum of laminarin, which has a different triple helical conformation, was around 550 nm. Based on this analysis, CMP Fr II was found to exhibit a random coil conformation similar to that of dextran.

Identification of Anomeric Configuration

To ascertain the presence or absence of the α or β configuration in CMP Fr II, the Fungi-Fluor Kit was used. The Fungi-Fluor staining solution, cellufluor, binds nonspecifically to β-linked polysaccharides, thus enabling their rapid detection. Whereas 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. CMP Fr II displayed a fluorescence signal very similar to that of curdlan, indicating that it is a β-linked polysaccharide (Fig. 5).

Glycosidic Linkage of CMP Fr II

CMP Fr II exhibited an IR absorption spectrum characteristic of a polysaccharide, with bands at 1,080 cm^{-1} (C=O), 2,800 cm^{-1}–2,900 cm^{-1} (C–H), and 3,400 cm^{-1} (O–H). Glycosidic linkage analysis of permethylated CMP Fr II

Table 2. Proximate composition and monosaccharide composition of CMP Fr II. (%, dry basis)

Polysaccharide	Protein	Hexosamine	Uronic acid	Total sugar	Component sugar (molar %)[a]			
					Glc	Gal	Man	Fuc
CMP Fr II	0.90	0.45	0.17	89.48	56.4	26.4	17.2	ND[b]

[a]Glc, glucose; Gal, galactose; Man, mannose; Fuc, fucose.
[b]Not detected.

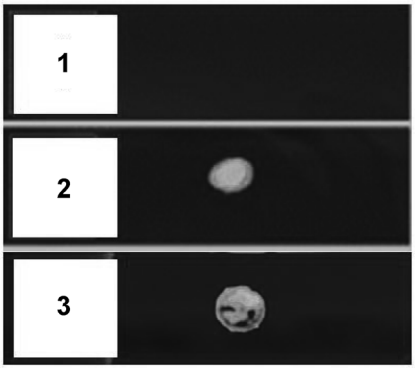

Fig. 5. Identification of the anomeric configuration of CMP Fr II and standard polymers.
Visualization of β-linked polysaccharides using the Fungi-Fluor kit. **1**, Dextran; **2**, Curdlan; **3**, CMP Fr II.

was performed by the reductive cleavage method. The polysaccharide was shown to be fully methylated, as indicated by the disappearance of the band at 3,400 cm^{-1}, characteristic of a carbohydrate ring (Supplemental Fig. 2). Following reductive cleavage, CMP Fr II was found to be hydrolyzed to its monosaccharide components, as indicated by comparing the GC traces of the polysaccharide hydrolysate with those of the monosaccharide standards. The data summarized in Table 3 (Supplemental Fig. 3) indicate that the principal component of CMP Fr II is a (1→4) or (1→2) linked glucopyranosyl or galactopyranosyl residue with a (1→2) or (1→6) linked mannopyranosyl, glucopyranosyl, or galactopyranosyl residue as a side chain.

DISCUSSION

Immunostimulation is regarded as one of the important strategies to improve the body's defense mechanism in elderly people as well as in cancer patients. 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 responses [13]. Polysaccharides exert their antitumor effects primarily by activating various immune system responses in the host, such as complement system activation, macrophage-dependent immune system responses, and upregulation of interferon expression [14, 20, 28]. Macrophages are the first line of defense among innate immune responses against microbial infection and cancer [3]. Activated macrophages recognize and kill tumor cells in a direct manner. However, they also play an indirect 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 processing and presentation of antigens by the immune system [1, 29]. In the present study, CMP Fr II, which was obtained from the fruiting body of *Cordyceps militaris* by hot water extraction, ethanol precipitation, and fractionation by DEAE-cellulose and Sepharose CL-6B column chromatographies, significantly upregulated the innate immune functions of macrophages, such as NO production (Fig. 2A) and cytokine production (Fig. 2B and 2C), indicating that it is able to induce the functional activation of macrophages. The production of proinflammatory cytokine and cytotoxic molecules is an important part of the immune responses to many inflammatory stimuli [39]. The role of NO in the antitumor action of murine macrophages is well-established [12, 22]. NO is synthesized enzymatically from L-arginine by NOS in activated macrophages. The toxic effects of NO and its derivatives on target cells are due to several known mechanisms, which include (1) inactivation of iron–sulfur cluster-containing enzymes through loss of iron from cells; (2) inhibition of DNA-binding activity of zinc finger-type transcriptional factors by inducing the release of zinc from zinc-containing proteins, which ultimately induces disulfide formation; and (3) destruction of mitochondrial membrane potential by influencing the activity of ion channels [16, 25, 40]. TNF-α, the most important mediator directly involved in the killing of tumor cells, affects the production of reactive oxygen species in mitochondria, which results in plasma membrane permeabilization, expression of inducible nitric oxide synthase, DNA strand breaks, and induction of serine protease activity [18, 37, 42].

Table 3. Identification and linkage analysis of partially methylated alditol acetates of CMP Fr II.

Polysaccharide	Alditol acetate derivative	Type of linkage	Relative molar ratio[a]
CMP Fr II	1,5-Anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol	Terminal Glc*p*	0.161
	1,5-Anhydro-2,3,4,6-tetra-*O*-methyl-D-galactitol	Terminal Gal*p*	0.133
	1,5-Anhydro-2,3,4,6-tetra-*O*-methyl-D-mannitol	Terminal Man*p*	0.085
	1,5-Anhydro-2-*O*-acetyl-3,4,6-tri-*O*-methyl-D-mannitol	→2)-Man*p*-(1→	0.183
	1,5-Anhydro-2-*O*-acetyl-3,4,6-tri-*O*-methyl-D-galactitol	→2)-Gal*p*-(1→	0.142
	1,5-Anhydro-4-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol	→4)-Gal*p*-(1→	0.321
	1,5-Anhydro-4,6-di-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol	→4,6)-Glc*p*-(1→	0.600

[a]Calculated from peak areas measured as alditol acetates by GC–MS.

Recently, TNF-related apoptosis-inducing ligand (TRAIL) and the Fas-ligand (FasL), another member of the TNF family, were shown to be related to tumor cell death [17]. Polysaccharides, which are polymers of monosaccharide residues joined 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 [43]. In light of this phenomenon, it is highly important to accurately determine polysaccharide structure. Polysaccharides differ greatly in their chemical composition, molecular mass, conformation, glycosidic linkage, degree of branching, *etc*. [45]. Mushrooms, which have been known for thousands of years in the Eastern countries, contain biologically active polysaccharides that mostly belong to the group of β-glucans. For example, the first three major drugs, which were developed from medicinal mushrooms, were polysaccharides, specifically β-glucans: Krestin from the cultured mycelial biomass of *Trametes versicolor*, lentinan from fruiting bodies of *Lentinus edodes*, and schizophyllan from the liquid-cultured broth product of *Schizophyllum commune* [34]. However, obvious variations in antitumor polysaccharides have also been noted. Interestingly, three immunostimulating hetero-β-glucans were extracted from fruiting bodies of *Agaricus blazei* [9]. Similarly, CMP Fr II was found to be a β-(1→4)-(1→2)-heteroglucan with the ability to stimulate macrophages (Table 3 and Fig. 5). Taken together, polysaccharides with medicinal properties may have other chemical structures, such as hetero-β-glucans, hetero-glycan, α-manno-β-glucan, and heteroglycan–protein complexes [43]. The molecular mass has long been recognized as a critical parameter in the antigenicity of molecule. Most polysaccharides with medicinal properties are large molecules of above 100 kDa of molecular mass [21]. Similarly, CMP Fr II is high-molecular-mass (126 kDa) polysaccharide with immunostimulant properties (Fig. 3). Unlike other medicinal mushroom-derived β-glucans, CMP Fr II has a random coil conformation but not a triple helix conformation (Fig. 4), which has been shown to be important for immune-stimulating activity [46].

In conclusion, CMP Fr II, a high-molecular-mass polysaccharide with a random coil conformation of a hetero-β-glucan, is a potent murine macrophage stimulator. The mechanisms of activation of macrophage signaling pathways will be the subject of further investigations.

REFERENCES

1. Allen, L. A. and A. Aderem. 1996. Mechanisms of phagocytosis. *Curr. Opin. Immunol*. **8:** 36–40.
2. Benjamini, E. and S. Leskowitz. 1991. *Immunology: A Short Course*, pp. 51–58. Wiley-Liss Inc.
3. Beutler, B. 2004. Innate immunity: An overview. *Mol. Immunol*. **40:** 845–859.
4. Blumenkrantz, N. and G. Asboe-Hansen. 1973. New method for quantitative determination of uronic acids. *Anal. Biochem*. **54:** 484–489.
5. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **72:** 248–254.
6. Carmichael, J., W. G. DeGraff, A. F. Gazdar, J. D. Minna, and J. B. Mitchell. 1987. Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. *Cancer Res*. **47:** 936–942.
7. Chaplin, M. F. and J. F. Kennedy (eds.). 1986. *Carbohydrate Analysis. A Practical Approach,* pp. 3. Oxford IRL Press.
8. Chihara, G., Y. Maeda, J. Hamuro, T. Sasaki, and F. Fukuoka. 1969. Inhibition of mouse sarcoma 180 by polysaccharides from *Lentinus edodes* (Berk.) sing. *Nature* **222:** 687–688.
9. Cho, S. M., J. S. Park, K. P. Kim, D. Y. Cha, H. M. Kim, and I. D. Yoo. 1999. Chemical features and purification of immunostimulating polysaccharides from the fruit bodies of *Agaricus blazei*. *Kor. J. Mycol*. **27:** 170–174.
10. Ciucanu, I. and F. Kerek. 1984. A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res*. **131:** 209–217.
11. Collins, L., T. Zhu, J. Guo, Z. J. Xiao, and C. Y. Chen. 2006. *Phellinus linteus* sensitizes apoptosis induced by doxorubicin in prostate cancer. *Br. J. Cancer* **95:** 282–288.
12. Cui, S., J. S. Reichner, R. B. Mateo, and J. E. Albina. 1994. Activated murine macrophages induce apoptosis in tumor cells through nitric oxide-dependent or -independent mechanisms. *Cancer Res*. **54:** 2462–2467.
13. Dalmo, R. A. and J. Boqwald. 2008. Beta-glucans as conductors of immune symphonies. *Fish Shellfish Immunol*. **25:** 384–396.
14. Dennert, G. and D. Tucker. 1973. Antitumor polysaccharide lentinan. A T cell adjuvant. *J. Natl. Cancer Inst*. **51:** 1727–1729.
15. Dische, Z. 1962. Color reactions of hexosamines, pp. 507–512. *In: Methods in Carbohydrate Chemistry I.* Academic Press.
16. Drapier, J. C. and J. B. Hibbs Jr. 1988. Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron–sulfur enzymes in the macrophage effector cells. *J. Immunol*. **140:** 2829–2838.
17. Ghobrial, I. M., T. E. Witziq, and A. A. Adjei. 2005. Targeting apoptosis pathways in cancer therapy. *CA Cancer J. Clin*. **55:** 178–194.
18. Goossens, V., J. Grooten, K. De Vos, and W. Fiers. 1995. Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. *Proc. Natl. Acad. Sci. U.S.A*. **92:** 8115–8119.
19. Gordon, S. B. and R. C. Read. 2002. Macrophage defences against respiratory tract infections. *Br. Med. Bull*. **61:** 45–61.
20. Hamuro, J. and G. Chihara. 1985. Lentinan, a T-cell oriented immunopotentiator: Its experimental and clinical applications and possible mechanism of immune modulation, pp. 409–436. *In* R. L. Fenichel and M. A. Chirigos (eds.). *Immunomodulation Agents and Their Mechanisms*. Dekker, New York.
21. Kabat, E. A. and A. E. Bezer. 1958. The effect of variation in molecular weight on the antigenicity of dextran in man. *Arch. Biochem. Biophys*. **78:** 306–318.

22. Keller, R., M. Geiges, and R. Keist. 1990. L-Arginine-dependent reactive nitrogen intermediates as mediators of tumor cell killing by activated macrophages. *Cancer Res*. **50:** 1421–1425.
23. Kiho, T., A. Yamane, J. Hui, S. Usui, and S. Ukai. 1996. Polysaccharides in fungi. XXXVI. Hypoglycemic activity of polysaccharide (CF-F30) from the cultural mycelium of *Cordyceps sinensis* and its effect on glucose metabolism in mouse liver. *Biol. Pharm. Bull*. **19:** 294–296.
24. Klimp, A. H., E. G. E. de Vries, G. L. Scherphof, and T. Daemen. 2002. A potential role of macrophage activation in the treatment of cancer. *Crit. Rev. Oncol. Hematol.* **44:** 143–161.
25. Kroncke, K. D., K. Fehsel, T. Schmidt, F. T. Zenke, I. Dasting, J. R. Wesener, H. Bettermann, K. D. Breunig, and V. Kolb-Bachofen. 1994. Nitric oxide destroys zinc–sulfur clusters inducing zinc release from metallothionein and inhibition of the zinc finger-type yeast transcription activator LAC9. *Biochem. Biophys. Res. Commun.* **200:** 1105–1110.
26. Lee, H. H., J. S. Lee, J. Y. Cho, Y. E. Kim, and E. K. Hong. 2009. Study on immunostimulating activity of macrophage treated with purified polysaccharides from liquid culture and fruiting body of *Lentinus edodes*. *J. Microbiol. Biotechnol*. **19:** 566–572.
27. Lee, J. S., J. Y. Cho, and E. K. Hong. 2009. Study on macrophage activation and structural characteristics of purified polysaccharides from the liquid culture broth of *Hericium erinaceus*. *Carbohydr. Polym*. **78:** 162–168.
28. Lee, J. S., K. M. Min, J. Y. Cho, and E. K. Hong. 2009. Study of macrophage activation and structural characteristics of purified polysaccharides from the fruiting body of *Hericium erinaceus*. *J. Microbiol. Biotechnol*. **19:** 951–959.
29. Medzhitov, R. and C. Janeway. 2000. Innate immune recognition: Mechanisms and pathways. *Immunol. Rev*. **173:** 89–97.
30. Nakamura, K., Y. Yamaguchi, S. Kagota, Y. M. Kwon, K. Shinozuka, and M. Kunitomo. 1999. Inhibitory effects of *Cordyceps sinensis* on spontaneous liver metastasis of Lewis lung carcinoma and B16 melanoma cells in syngeneic mice. *Jpn. J. Pharmacol*. **79:** 335–341.
31. Nardin, A. and J. P. Abastado. 2008. Macrophages and cancer. *Front. Biosci*. **13:** 3494–3505.
32. Nathan, C. F., S. C. Silrerstein, L. D. Bruker, and Z. A. Lohn. 1979. Extracellular cytolysis by activated macrophages and granulocytes. *J. Exp. Med*. **149:** 100–113.
33. Ng, T. B. and H. X. Wang. 2005. Pharmacological actions of *Cordyceps*, a prized folk medicine. *J. Pharm. Pharmacol*. **57:** 1509–1520.
34. Novak, M. and V. Vetvicka. 2008. Beta-glucans, history, and the present: Immunomodulatory aspects and mechanisms of action. *J. Immunotoxicol*. **5:** 47–57.
35. Ogawa, K. and M. Hatano. 1978. Circular dichroism of the complex of a (1→3)-β-D-glucan with Congo red. *Carbohydr. Res*. **67:** 527–535.
36. Ogawa, K., J. Tsurugi, and T. Watanabe. 1973. The dependence of the conformation of a (1→3)-β-D-glucan on chain-length in alkaline solution. *Carbohydr. Res*. **29:** 397–403.
37. Ogura, T., M. Tatemichi, and H. Esumi. 1997. TNF-alpha mediates inducible nitric oxide synthase expression in human neuroblastoma cell line by cisplatin. *Biochem. Biophys. Res. Commun*. **233:** 788–791.
38. Park, C., S. H. Hong, J. Y. Lee, G. Y. Kim, B. T. Choi, Y. T. Lee, *et al*. 2005. Growth inhibition of U937 leukemia cells by aqueous extract of *Cordyceps militaris* through induction of apoptosis. *Oncol. Rep*. **13:** 1211–1216.
39. Porcheray, F., S. Viaud, A. C. Rimaniol, C. Leone, B. Samah, N. Dereuddre-Bosquet, D. Dormont, and G. Gras. 2005. Macrophage activation switching: An asset for the resolution of inflammation. *Clin. Exp. Immunol*. **142:** 481–489.
40. Richter, C., V. Gogvadze, R. Schlapbach, M. Schweizer, and J. Schlegel. 1994. Nitric oxide kills hepatocytes by mobilizing mitochondrial calcium. *Biochem. Biophys. Res. Commun*. **205:** 1143–1150.
41. Rolf, D. and G. R. Gray. 1982. Reductive cleavage of glycosides. *J. Am. Chem. Soc*. **104:** 3539–3541.
42. Suffys, P., R. Beyaert, F. Van Roy, and W. Fiers. 1988. Involvement of a serine protease in tumor-necrosis-factor-mediated cytotoxicity. *Eur. J. Biochem*. **178:** 257–265.
43. Vetvicka, V. and J. Vetvickova. 2007. Physiological effects of different types of beta-glucan. *Biomed. Pap. Med. Fac. Univ. Palacky Olomouc. Czech. Repub*. **151:** 225–231.
44. Wasser, S. P. 2002. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Appl. Microbiol. Biotechnol*. **60:** 258–274.
45. Yadomae, T. and N. Ohno. 1996. Structure–activity relationship of immuno-modulating (1-3)-β-D-glucans. *Recent Res. Dev. Chem. Pharm. Sci*. **1:** 23–33.
46. Yanaki, T., W. Ito, and K. Tabata. 1986. Correlation between antitumor activity of schizophyllan and its triple helix. *Agric. Biol. Chem*. **509:** 2415–2426.
47. Yang, B. K., J. Y. Ha, S. C. Jeong, S. Das, J. W. Yun, Y. S. Lee, J. W. Choi, and C. H. Song. 2000. Production of exo-polymers by submerged mycelial culture of *Cordyceps militaris* and its hypolipidemic effect. *J. Microbiol. Biotechnol*. **10:** 784–788.
48. Yang, L. Y., A. Chen, Y. C. Kuo, and C. Y. Lin. 1999. Efficacy of a pure compound H1-A extracted from *Cordyceps sinensis* on autoimmune disease of MRL lpr/lpr mice. *J. Lab. Clin. Med*. **134:** 492–500.
49. Yu, R., L. Song, Y. Zhao, W. Bin, L. Wang, H. Zhang, Y. Wu, Y. Ye, and X. Yao. 2004. Isolation and biological properties of polysaccharide CPS-1 from cultured *Cordyceps militaris*. *Fitoterapia* **75:** 465–472.
50. Yu, R., W. Yang, L. Song, C. Yan, Z. Zhang, and Y. Zhao. 2007. Structural characterization and antioxidant activity of a polysaccharide from the fruiting bodies of cultured *Cordyceps militaris*. *Carbohydr. Polym*. **70:** 430–436.