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Study of Macrophage Activation and Structural Characteristics of Purified Polysaccharides from the Fruiting Body of *Hericium erinaceus*

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Most, if not all, Basidiomycetes mushrooms have biologically active polysaccharides showing potent antitumor activity with immunomodulating properties. These polysaccharides have various chemical compositions and belong primarily to the β-glucan group. In this study, the crude water-soluble polysaccharide HEF-P, which was obtained from the fruiting body of *Hericium erinaceus* by hot water extraction and ethanol precipitation, was fractionated by DEAE-cellulose and Sepharose CL-6B column chromatographies. This process resulted in four polysaccharide fractions, named HEF-NP Fr I, HEF-NP Fr II, HEF-AP Fr I, and HEF-AP Fr II. Of these fractions, HEF-AP Fr II was able to upregulate the functional events mediated by activated macrophages, such as production of nitric oxide and expression of cytokines (IL-1β and TNF-α). The molecular mass of HEF-AP Fr II was estimated by gel filtration to be 13 kDa. 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 HEF-AP Fr II is a low-molecular-mass polysaccharide with a laminarin-like triple helix conformation of a β-1,3-branched-β-1,6-glucan.

Keywords: *Hericium erinaceus*, immunostimulating polysaccharide, mushroom-derived β-glucan, macrophage activation, laminarin-like triple helix

Recently, a number of bioactive molecules including antitumor agents have been identified in various higher Basidiomycetes mushrooms. 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 [1]. As such, polysaccharides are the best-known and most potent mushroom-derived substances that display immunopharmacological properties [11, 24]. *Hericium erinaceus* belongs to the Aphyllophorales, Hydnaecae, and Hericium families and is a well-known edible and medicinal mushroom in East Asia. Many studies have demonstrated that *H. erinaceus* possesses various biological activities, which include antimicrobial [20, 37], antitumor [23, 30, 31], immunomodulatory [27], antioxidant [29, 38], cytotoxic [17, 22], and hypolipidemic [45] effects, and it can promote the synthesis of neural growth factor [18, 25]. Polysaccharides exert their antitumor effects primarily by activating various immune system responses in the host, such as complement system activation [8], macrophage-dependent immune system responses [26], and upregulation of interferon expression [5]. Various studies have been conducted to determine the mechanism by which macrophages kill tumor cells. 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 tumor necrosis factor (TNF) and nitric oxide (NO), which are harmful to cancer cells, and by regulating the processing and presentation of antigens by the immune system [28]. Immunomodulating polysaccharides are characterized by chemical composition, molecular mass, conformation, glycosidic linkage, degree of branching, *etc.* [43]. 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 HEF-AP Fr II, which

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was isolated and purified from the fruiting body of *H. erinaceus* by gel filtration and ion-exchange chromatographies. 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 mass, conformation, degree of branching, and glycosidic linkage were examined.

Materials and Methods

Materials

Dried fruiting bodies of *Hericium erinaceus* were purchased from the local market and ground in a blender. Dialysis tubing, cellulose membranes, DEAE-cellulose, Sepharose CL-6B, standard dextrans, lipopolysaccharide (LPS, *E. 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 with 10 volumes of distilled water, 10% ethanol, or 20% ethanol 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- or vacuum-dried. Polysaccharides were precipitated from resuspended extracts using 95.0% ethanol, 99.5% methanol, or 99.0% acetone, collected by filtration through 0.45 μm Whatman filter paper, and resuspended and dialyzed against distilled water for 7 days to remove low-molecular-weight compounds. After freeze-drying the solution in the dialysis tube, the relative yield of crude polysaccharides, termed HEF-P, was named. HEF-P 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 weight. 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 penicillin, 100 μg/ml 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 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, polysaccharide (1,000 μg/ml) or LPS (2.5 μg/ml) was added and the mixture was incubated for an additional 24 h.

Determination of NO Production

After pre-incubating RAW264.7 cells (1×10^6 cells/ml) for 18 h, each 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 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.

RT–PCR

To evaluate levels of LPS-inducible mRNA expression, total RNA from HEF-AP Fr II-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, 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 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. PCR products (10 μl) were electrophoresed on a 1.2% agarose gel and visualized by ethidium bromide staining under ultraviolet light.

TNF-α Production

The ability of HEF-AP-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.

Analysis of Chemical Properties

The total sugar content of each polysaccharide was determined using the phenol–sulfuric acid method [4], the total protein concentration was determined using the Bradford method [3], the hexosamine content was evaluated using the Elson–Morgan method [9], and the

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

Gene		Primer sequences
IL-1β	F	5'-CAGATGAGGACATGAGCACC-3'
	R	5'-CACCTCAAACTCAGACGTCTC-3'
TNF-α	F	5'-TTGACCTCAGCGCTGAGTTG-3'
	R	5'-CCTGTAGCCCACGTCGTAGC-3'
GAPDH	F	5'-CACTCACGGCAAATTCAACGGCAC-3'
	R	5'-GACTCCACGACATACTCAGCAC-3'

F, forward; R, reverse.

uronic acid content was assessed using the Blumenkrantz method [2].

Analysis of Monosaccharide Composition

Monosaccharide composition and ratios were determined by first hydrolyzing the polysaccharide with 2 M trifluoracetic 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, U.S.A.) under the following conditions: column: CarboPac PA10 Analytical Column (4 mm×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.

Determination of Molecular Mass

The molecular mass 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 mass. Standard dextrans (M: 670 kDa, 410 kDa, 150 kDa, 25 kDa, and 12 kDa) were used for calibration.

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 [32, 33].

Identification of Anomeric Configuration

To ascertain the presence or absence of the α or β configuration in each polysaccharide, the FT–IR spectrum was obtained with an Excalibur Series Spectrometer (Bio-Rad), and 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 HEF-AP Fr II

HEF-AP Fr II was methylated according to the method developed by Ciucanu and Kerek [6], using powdered NaOH in Me_2SO–MeI. Methylation was confirmed by measuring the FT–IR spectrum.

Determination of Glycosidic Linkage

Permethylated HEF-AP 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 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.

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

Comparison of Extraction Yield from Milled Fruiting Body

Three different solvents (distilled water and 10% and 20% ethanol) and two different drying methods (freeze drying and vacuum drying) were used to examine the efficiency of extracting solids from the milled fruiting body of *Hericium erinaceus*. The highest yield was obtained with distilled water and the freeze drying method (data not shown). To determine the optimal solvent and volume ratio for precipitation of crude polysaccharides, the volumes of 95% ethanol, 99.5% methanol, and 99% acetone were varied from 2- to 4-fold. Among the three solvents, ethanol gave the highest relative yield. The yield of crude polysaccharides increased at higher ethanol volume ratios and reached a maximum value at a 3-fold increase (data not shown). When dialysis time was varied, it was shown that the low-molecular-mass compounds were completely removed after 5 days (data not shown). Based on the above experimental results, the maximal yield of crude polysaccharides extracted from milled fruiting bodies was determined to be 18.59%.

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 (HEF-NP) and the acidic fraction (HEF-AP) obtained from the crude polysaccharide extract HEF-P was 0.404 g/g and 0.352 g/g, respectively (Fig. 1A). The molecular distribution of HEF-NP and HEF-AP was investigated using gel filtration chromatography with a Sepharose CL-6B column, resulting in four polysaccharide fractions: HEF-NP Fr I (0.122 g/g), HEF-NP Fr II (0.522 g/g), HEF-AP Fr I (0.185 g/g), and

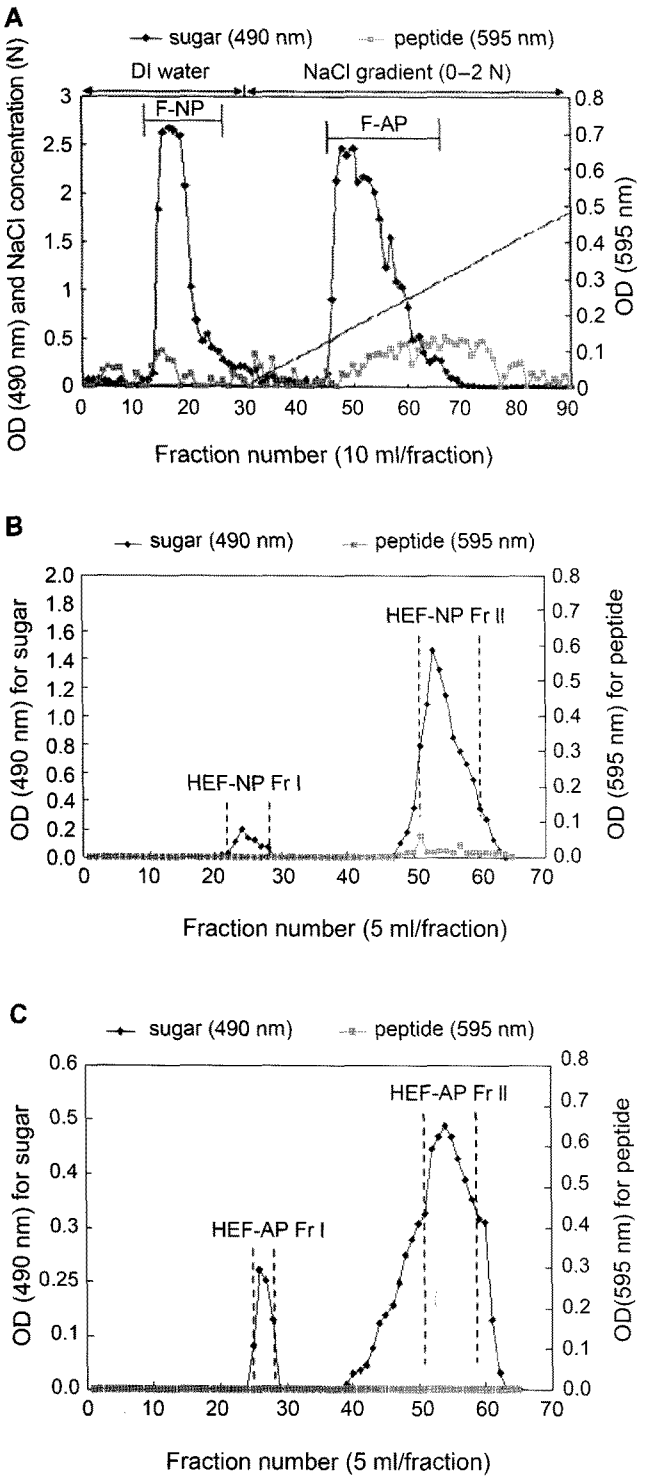


Fig. 1. Isolation and purification of polysaccharides extracted from the fruiting body of *Hericium erinaceus*.
A. Ion-exchange chromatogram of the crude polysaccharides, HEF-P, on a DEAE cellulose column. **B**. Gel filtration chromatogram of the neutral polysaccharide fraction, HEF-NP, on a Sepharose CL-6B column (fraction number of ion-exchange chromatography: 13–25). **C**. Gel filtration chromatogram of the acidic polysaccharide fraction, HEF-AP, on a Sepharose CL-6B column (fraction number of ion-exchange chromatography: 45–69).

HEF-AP Fr II (0.354 g/g) (Figs. 1B and 1C). With the exception of HEF-NP Fr II, these fractions did not contain protein, and all non-bound peptides were removed during ion-exchange chromatography and dialysis.

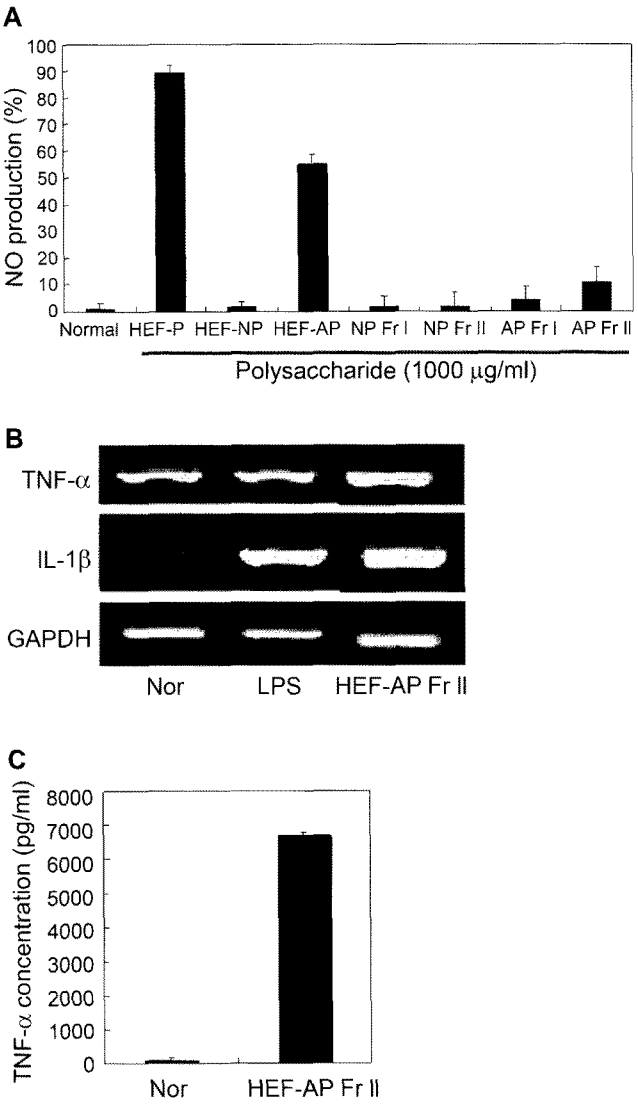


Fig. 2. Immunostimulating effects of polysaccharide HEF-AP Fr II purified by DEAE-cellulose and Sepharose CL-6B.
A. The effect of purified polysaccharides on NO synthesis in murine macrophage-like cells. RAW264.7 cells (1×10^6 cells/ml) were stimulated by each polysaccharide fraction (1,000 μg/ml) for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

Macrophage Activation by Polysaccharides

To address whether purified polysaccharides from the fruiting body of *H. erinaceus* 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 HEF-AP Fr II triggered production of the most NO among the purifed polysaccharides (Fig. 2A). To determine whether HEF-AP Fr II 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 HEF-AP Fr II strongly triggers the expression of proinflammatory cytokines TNF-α and interleukin-1β (IL-1β) (Figs. 2B and 2C).

Chemical Properties and Monosaccharide Composition

The total sugar content of HEF-AP Fr II is 89.67%. Its major sugar constituents are glucose (91.11%), galactose (6.09%), and mannose (2.80%) (Fig. S1). Proteins were not detected in this polysaccharide. The hexosamine and uronic acid content of HEF-AP Fr II are 1.33 and 4.00%, respectively (Table 2).

Homogeneity and Molecular Mass

The homogeneity of HEF-AP 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 chromatography to be 13 kDa using dextrans as standards (Fig. 3B).

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 N NaOH. However, the absorption maximum of laminarin, which has a different triple helical conformation, was around 580 nm and did not change. Based on this analysis, HEF-AP Fr II was found to exhibit a triple helical conformation similar to that of laminarin.

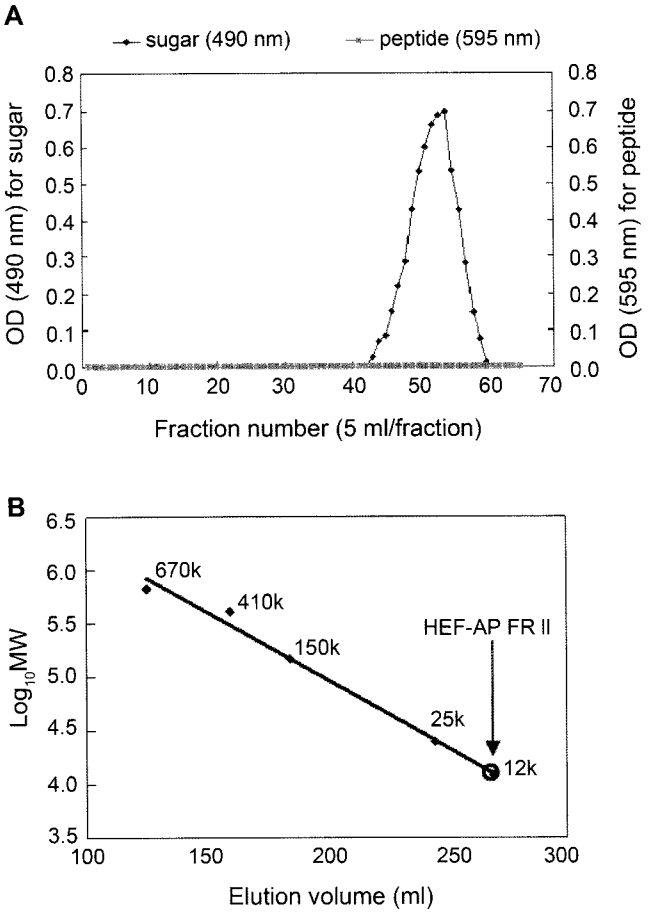


Fig. 3. Average molecular mass of HEF-AP Fr II.
A. Elution profile of polysaccharide refractionated by gel filtration with Sepharose CL-6B. **B.** Molecular masses of standard dextrans and HEF-AP Fr II determined by Sepharose CL-6B gel filtration chromatography.

Identification of Anomeric Configuration

To ascertain the presence or absence of the α or β configuration in each polysaccharide, the FT–IR spectrum was obtained and the Fungi-Fluor Kit was used. Curdlan, laminarin, and HEF-AP Fr II exhibited an IR absorption band at 890 cm^{-1}, characteristic of a β-linked polymer (Fig. 5A). The Fungi-Fluor staining solution, cellufluor, binds nonspecifically to β-linked polysaccharides, thus enabling their rapid detection. Whereas dextran, which is an α-

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

Polysaccharide	Protein	Hexosamine	Uronic acid	Total sugar	Component sugar (molar %)			
					Glc	Gal	Man	Fuc
HEF-AP Fr II	ND	1.33	4.0	89.67	91.11	6.09	2.80	ND

ND: not detected

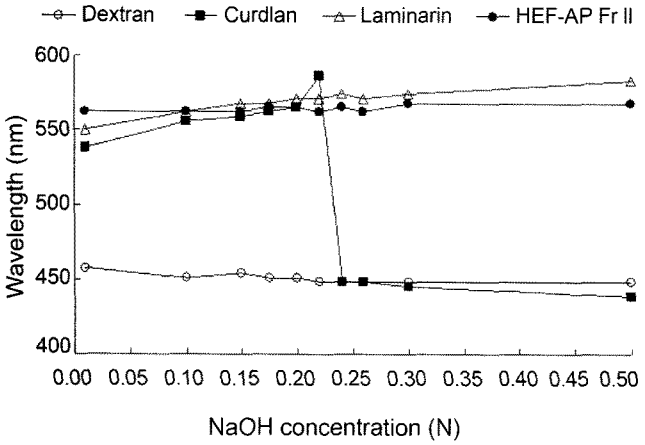


Fig. 4. Helix–coil transition analysis of HEF-AP Fr II and standard polymer according to the absorption maximum of the Congo red–polysaccharide complex at various concentrations of NaOH. For more details, see Materials and Methods.

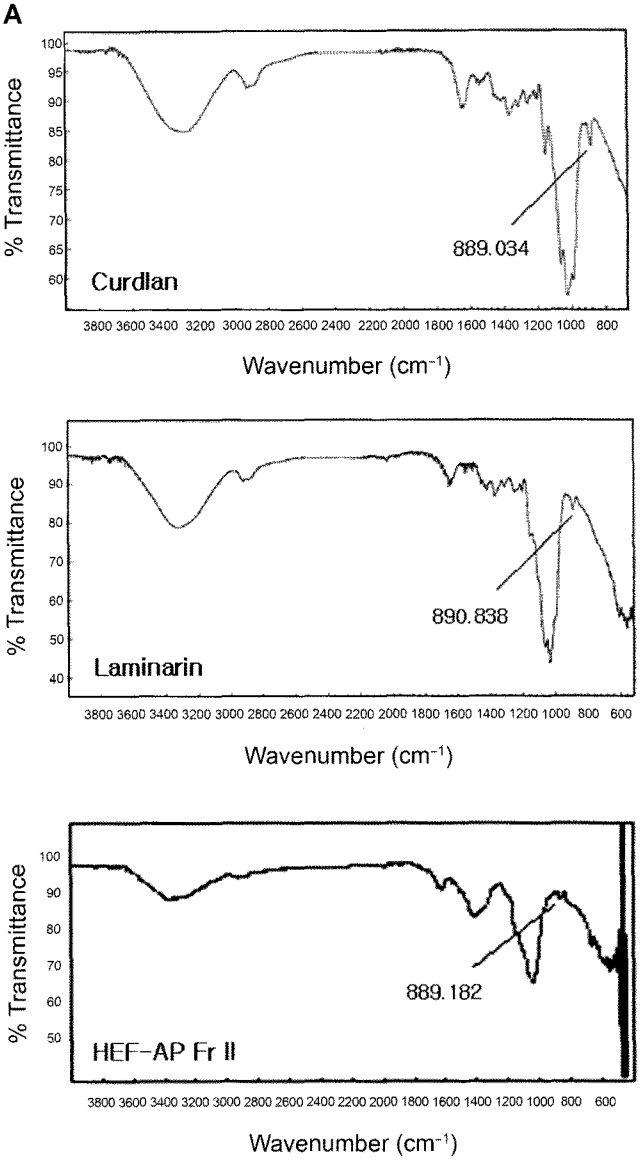
glucan, did not exhibit fluorescence in the presence of cellufluor, a signal was clearly observed for curdlan, which is a β-glucan. HEF-AP Fr II displayed a fluorescence signal very similar to that of curdlan, indicating that it is a β-linked polysaccharide (Fig. 5B).

Glycosidic Linkage of HEF-AP Fr II

HEF-AP Fr II exhibited an IR absorption spectrum characteristic of a polysaccharide, with bands at 1,080 cm^{-1} (C=O), 2,800–2,900 cm^{-1} (C-H), and 3,400 cm^{-1} (O-H). Glycosidic linkage analysis of permethylated HEF-AP 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 (Fig. S2). Following reductive cleavage, HEF-AP Fr II was found to be hydrolyzed to its monosaccharide components, as indicated by comparing the GC traces of the polysaccharide hydrolysate to those of the monosaccharide standards. The data summarized in Table 3 (see also Fig. S3) indicate that the principal component of HEF-AP Fr II is a (1→6)-linked glucopyranosyl residue with a (1→3)-linked glucopyranosyl side chain, with a degree of branching (DB) of 0.2.

Discussion

Mushroom-derived polysaccharides do not attack cancer cells directly, but rather produce their antitumor action by stimulating natural killer cells, T-cells, B-cells, and macrophage-dependent immune system responses. Activated macrophages play an important role in innate and adaptive immune responses by producing cytokines such as IL-1β and TNF-α, NO, and other inflammatory mediators. The



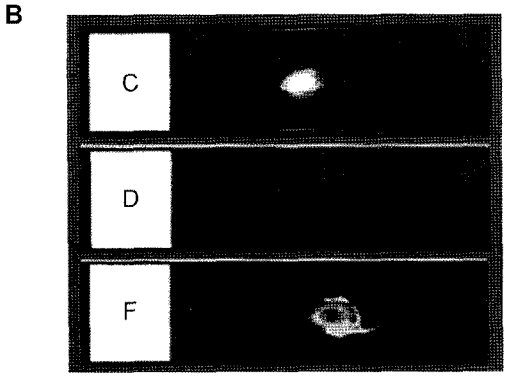


Fig. 5. Identification of anomeric configuration of HEF-AP Fr II and standard polymers.
A. FT-IR analysis of HEF-AP Fr II and standard polymer showing identical spectra. **B.** Visualization of β-linked polysaccharides using the Fungi-Fluor kit. C: curdlan; D: dextran; F: HEF-AP Fr II.

Table 3. Identification and linkage analysis of partially methylated alditol acetates of HEF-AP Fr II.

Polysaccharide	Alditol acetate derivative	Type of linkage	Relative molar ratio
HEF-AP Fr II	1,5-Anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol	Terminal Glc*p*	0.22
	1,5-Anhydro-6-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol	→6)-Glc*p*-(1→	1
	1,5-Anhydro-3-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol	→3)-Glc*p*-(1→	0.46
	1,5-Anhydro-3,6-di-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol	→3,6)-Glc*p*-(1→	0.21

production of these mediators is an important part of the immune response to many inflammatory stimuli [39]. The role of NO in the antitumor action of murine macrophages is well established [7, 19]. The toxic effects of NO and its derivatives on target cells are due to several known mechanisms, which include (i) inactivation of iron–sulfur cluster-containing enzymes through loss of iron from cells; (ii) 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 (iii) destruction of mitochondrial membrane potential by influencing the activity of ion channels [10, 21, 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 [15, 34, 41]. In the present study, HEF-AP Fr II, which was obtained from the fruiting body of *Hericium erinaceus* by hot water extraction, ethanol precipitation, and fractionation by DEAE-cellulose and Sepharose CL-6B column chromatographies, significantly upregulated the expression of cytokines (TNF-α and IL-1β) and release of NO, indicating that it is able to induce the functional activation of macrophages (Fig. 2). 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 [35]. 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.* [43]. The predominant antitumor glucans isolated from mushrooms thus far have a β-1,3-linked main chain [13]. For example, a β-1,6-branched-β-1,3-glucan isolated from the fruiting body of *Grifola frondosa* inhibited the growth of an allogenic tumor in mice through activation of cellular immunity [36]. In contrast, HEF-AP Fr II was found to be a β-1,3-branched-β-1,6-glucan with the ability to stimulate macrophages (Table 3 and Fig. 3). In addition, the mushroom *Agaricus blazei* contains an antitumor glucan with an α-1,4-linked backbone and β-1,6 branching [13]. Taken together, these results indicate that β-1,3- and β-1,6-linkage types are important in antitumor activity [36]. Molecular mass has long been recognized as a critical parameter in the antigenicity of a molecule [16]. Interestingly, a low-molecular-mass (20 kDa) fraction from the fruiting body of *A. blazei* was found to exhibit tumor-specific cytocidal and immunopotentiating effects [12]. In addition, it was reported that acidic hydrolysate fractions from the fruiting body of *T. fuciformis*, with molecular mass ranging from 1 to 53 kDa, induced human monocytes to produce interleukin-6 as efficiently as the non-hydrolyzed fraction [14]. Similarly, HEF-AP Fr II is a low-molecular-mass (13 kDa) polysaccharide with immunostimulant properties. Like other medicinal mushroom-derived β-glucans, HEF-AP Fr II has a triple helix conformation (Fig. 4), which has been shown to be important for immune-stimulating activity [44].

In conclusion, HEF-AP Fr II, a low-molecular-mass polysaccharide with a laminarin-like triple helix conformation of a β-1,3-branched-β-1,6-glucan, is a potent murine macrophage stimulator. The mechanisms of activation of macrophage signaling pathways will be the subject of further investigations.

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