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Study on macrophage activation and structural characteristics of purified polysaccharides from the liquid culture broth of *Hericium erinaceus*

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ABSTRACT

Recently, a number of bioactive molecules, including antitumor agents, have been identified in various higher basidiomycetes mushrooms. Polysaccharides are the best known and most potent mushroom derived substances that display immuno-pharmacological properties. In this study, the water soluble crude polysaccharide HEB-P, which was obtained from the liquid culture broth of *Hericium erinaceus* by ethanol precipitation, was fractionated by DEAE cellulose and Sepharose CL-6B column chromatography. This fractionation process resulted in two polysaccharide fractions that were termed HEB-NP Fr I and HEB-AP Fr I. Of the fractions, HEB-AP Fr I 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 HEB-AP Fr I was a low molecular mass polysaccharide with a laminarin-like triple helix conformation of the β-1,3-branched-β-1,2-mannan.

1. Introduction

Mushroom-derived polysaccharides can produce an antitumor effect by stimulating natural killer cells, T-cells, B-cells, and macrophage-dependent immune system responses. In the innate and adaptive immune responses, activated macrophages play an important role by producing cytokines, interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), nitric oxide (NO), and other inflammatory mediators. The production of NO, IL-1β, and TNF-α is an important part of the immune response to many inflammatory stimuli (Porcheray et al., 2005). In addition, mushroom-derived polysaccharides are known to have no toxic side effects, unlike the existing anti-cancer chemical medications. Therefore, when used as a cancer therapeutic, these polysaccharides were able to prolong the life span of cancer patient (Benzamini & Leskowitz, 1999). Most of the clinical evidence of this comes from the commercial polysaccharides lentinan, PSK (krestin), and schizophyllan. However, in addition to this there is impressive new data for polysaccharides isolated from *Phellinus linteus, Flammulina velutipes*, *Hypsizygus marmoreus*, *Agaricus blazei* and others that also hold promise for use as cancer therapeutics. As such, polysaccharides derived from mushrooms appear to have potentially beneficial immuno-pharmacological properties (Wasser, 2002). *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* possess various biological activities such as an antimicrobial effect (Kim, Pyun, Ko, & Park, 2000; Okamoto et al., 1993), antitumor activities (Kwon et al., 2003; Mizuno, Saito, Nishitoba, & Kawagishi, 1995; Mizuno, Wasa, Ito, Suzuki, & Ukai, 1992), antioxidant properties (Mau, Lin, & Song, 2002; Park, Yu, & Min, 1998), cytotoxic effect (Kawagishi, Ando, & Mizuno, 1990; Kuwahara, Morihiro, Nemoto, & Hiromatsu, 1992), hypolipidemic effect (Yang, Park, & Song, 2002), and it can promote the synthesis of the neurogrowth factor (Kawagishi et al., 1994; Lee et al., 2000). 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 immunostimulating activity and structural characteristics of the polysaccharide, HEB-AP Fr I, which was isolated and purified from the liquid culture broth of *H. erinaceus* by gel filtration and ion ex-

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change 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.

2. Materials and methods

2.1. Materials

Each polysaccharide fraction, derived from liquid culture broth of *H. erinaceus*, 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). 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 (Manassa, VA, USA). All other chemicals were of Sigma grade.

2.2. Liquid culture of H. erinaceus

The seed culture was cultivated for 4 days in 100 ml of the YMK media, which contained 20 g/L glucose, 5.0 g/L yeast extract, 2.0 g/L KH_2PO_4, and 1.0 g/L $MgSO_4{\cdot}7H_2O$, in a 250 ml flask that was inoculated with 10 ml of an activated stock solution frozen at −70 °C. For mycelial growth, the mycelia were homogenized with a Heidolph DIAX 600 homogenizer (VWR International, West Chester PA, USA) and 10% of the seed culture broth was inoculated into 100 ml of the GYA media, which contained 20 g/L glucose, 10 g/L yeast extract, and 2.0 g/L ascorbic acid, in a 250 ml flask. They were then cultivated for 7 days at 23 °C in a shaking incubator that was set at 200 rpm (Vision Scientific Co., Ltd., Buchun, Korea). Fermentation was carried out in a 5 L bioreactor (Korea Fermentor Co., Seoul, Korea). The mycelia were cultivated for 9 days at 23 °C with 1.0 vvm, uncontrolled initial pH, and a 10% inoculum size in GYA medium. The working volume in the 5 L bioreactor was 3.0 L. After 9 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 dried for 24 h at 60 °C. The exo-polysaccharides (EPS) derived from the liquid culture broth were prepared by the ethanol precipitation method, filtered with 0.45 μm Whatman filter paper, and then dried in a drying oven to a constant weight. The dry weight of mycelia and EPS was quantified by subtracting the dry weight of the filter paper from the total weight. The residual glucose in the cultured broth was determined using a glucose assay kit (Sigma Diagnostics, St. Louis, MO, USA) and glucose analyzer (YSI Inc., Yellow Springs, OH, USA) according to the manufacturer's instructions.

2.3. Fractionation and purification of water-soluble polysaccharides

The crude polysaccharides, termed HEB-P, 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.

2.4. 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.

2.5. 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 centrifuge 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.

2.6. 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.

2.7. RT-PCR

To evaluate levels of LPS-inducible mRNA expression, total RNA from HEB-AP Fr I-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.

2.8. TNF-α production

The ability of HEB-AP Fr I 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.

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.

2.9. Analysis of chemical properties

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

2.10. 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.

2.11. 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 kDa, 410 kDa, 150 kDa, 25 kDa, and 12 kDa) were used for calibration.

2.12. 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).

2.13. 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, 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).

2.14. Methylation of HEB-AP Fr I

HEB-AP Fr I 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.

2.15. Determination of glycosidic linkage

Permethylated HEB-AP Fr I 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.

2.16. 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. Exo-polysaccharide production by fermentation

The mycelial growth of *H. erinaceus* in a 5 L bioreactor continually increased up to the 9th day of culture, at which point the mycelial growth was highest (14.021 g/L). The polysaccharide production was 0.994 g/L by the 7th day of culture (Fig. 1).

3.2. 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 (HEB-NP) and the acidic fraction (HEB-AP) obtained from the crude polysaccharide extract HEB-P was 0.140 g/g and 0.651 g/g, respectively (Fig. 2A). The molecular distribution of HEB-NP and HEB-AP was investigated using gel filtration chromatography with a Sepharose CL-6B column, resulting in two polysaccharide fractions, namely HEB-NP Fr I (0.880 g/g) and HEB-AP Fr I (0.900 g/g) (Fig. 2B and C).

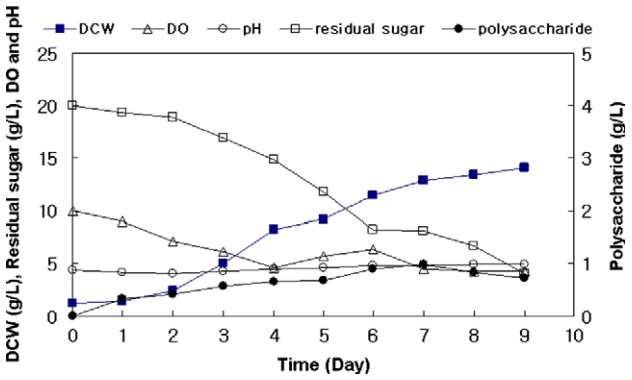

Fig. 1. The fermentation patterns of *Hericium erinaceus* in a 5 L bioreactor at 23 °C with 1.0 vvm, 200 rpm, uncontrolled initial pH, and a 10% inoculum size in GYA medium. The working volume in the 5 L bioreactor was 3.0 L.

3.3. Macrophage activation by polysaccharides

To examine whether polysaccharides purified from the liquid culture broth of *H. erinaceus* 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, and HEB-AP Fr I triggered production of the most NO among the polysaccharides (Fig. 3A). To address whether HEB-AP Fr I 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 HEB-AP Fr I strongly triggers the expression of proinflammatory cytokines TNF-α and interleukin-1β (IL-1β) (Fig. 3B and C).

3.4. Chemical properties and monosaccharide composition

The total sugar content of HEB-AP Fr I was 93.50%. Its major sugar constituents are mannose (Fig. S1). Proteins were not detected in this polysaccharide. The hexosamine and uronic acid content of HEB-AP Fr I are 0.46% and 2.70%, respectively (Table 2 and Fig. S1).

3.5. Homogeneity and molecular weight

The homogeneity of HEB-AP Fr I was confirmed by refractionation through gel filtration chromatography using a Sepharose CL-6B packed column (Fig. 4A). The molecular weight of this fraction was then determined by gel filtration chromatography to be 46 kDa using dextrans as standards (Fig. 4B).

3.6. 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. 5). 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 580 nm and did not change. Based on this analysis, HEB-AP Fr I was found to exhibit a triple-helical conformation similar to that of laminarin.

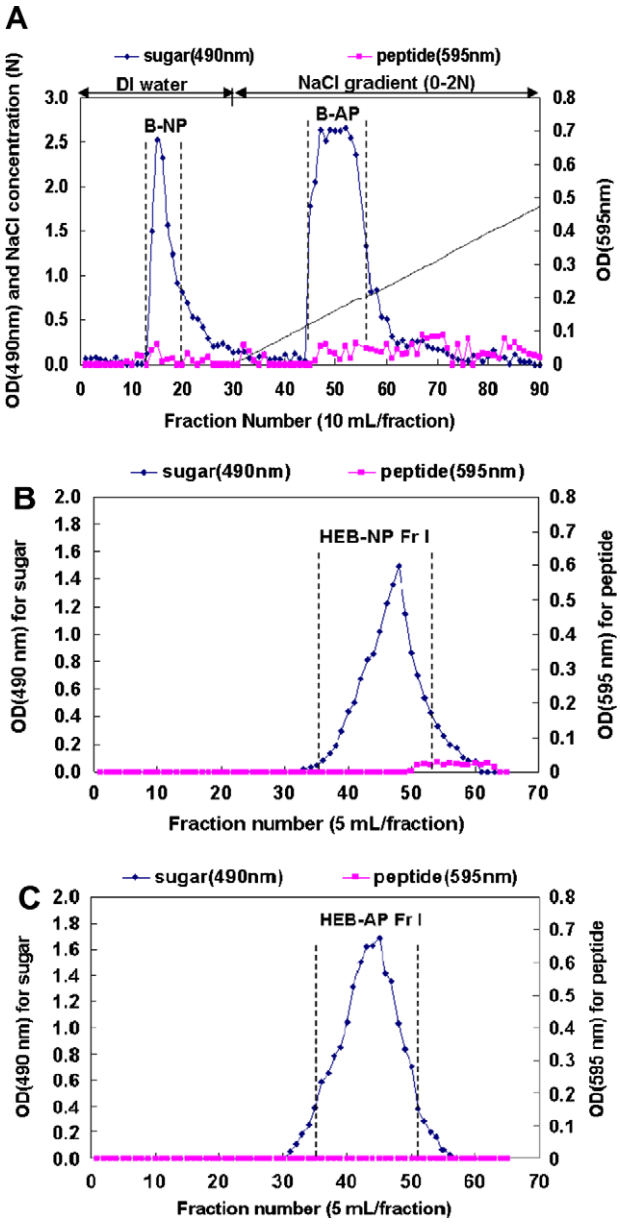

Fig. 2. Isolation and purification of polysaccharides extracted from the liquid culture broth of *Hericium erinaceus*. (A) Ion exchange chromatogram of the crude polysaccharides, HEB-P, on a DEAE-cellulose column. (B) Gel filtration chromatogram of the neutral polysaccharide fraction, HEB-NP, on a Sepharose CL-6B column (fraction number of ion exchange chromatography: 14–20). (C) Gel filtration chromatogram of the acidic polysaccharide fraction, HEB-AP, on a Sepharose CL-6B column (fraction number of ion exchange chromatography: 45–56).

3.7. 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 HEB-AP Fr I exhibited an IR absorption band at 890 cm^{-1} characteristic of a β-linked polymer (Fig. 6A). 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. HEB-AP Fr I displayed a fluorescence signal very similar to that of curdlan, indicating that it is a β-linked polysaccharide (Fig. 6B).

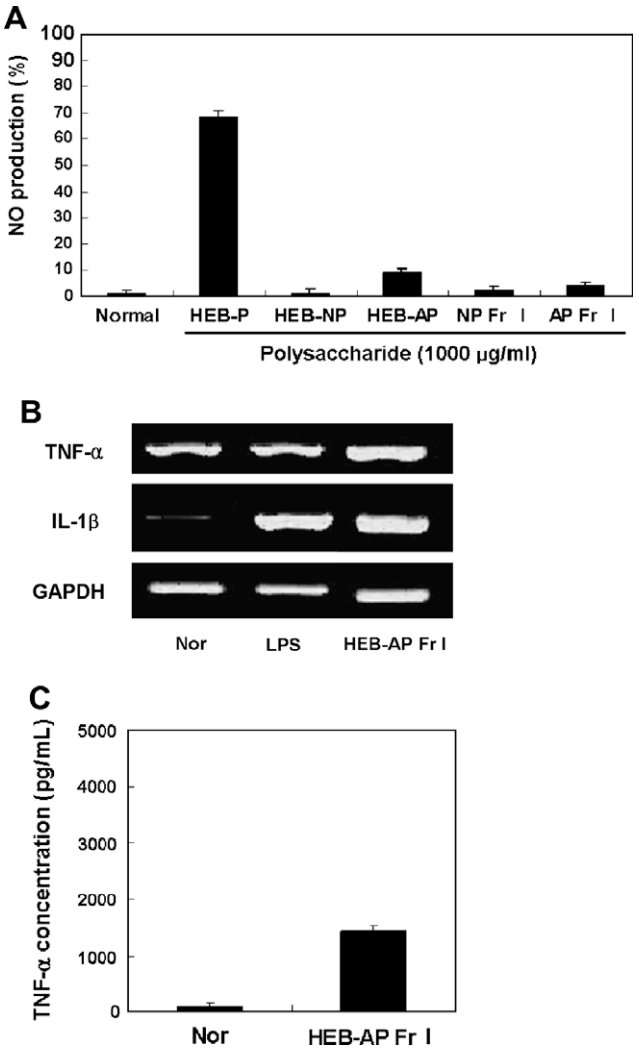

Fig. 3. Immunostimulating effects of polysaccharide, HEB-AP Fr I, purified by DEAE cellulose and Sepharose CL-6B chromatography. (A) Effect of purified polysaccharides on NO synthesis in murine macrophage-like cells. RAW264.7 cells (1×10^6 cells/ml) were stimulated by each polysaccharide fraction (1000 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 HEB-AP Fr I on the expression of cytokines. RAW264.7 cells (1×10^7 cells/ml) were incubated with HEB-AP Fr I (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 HEB-AP Fr I on TNF-α production. RAW264.7 cells (1×10^6 cells/ml) were stimulated by HEB-AP Fr I (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 mean ± SEM of three independent experiments performed in triplicate.

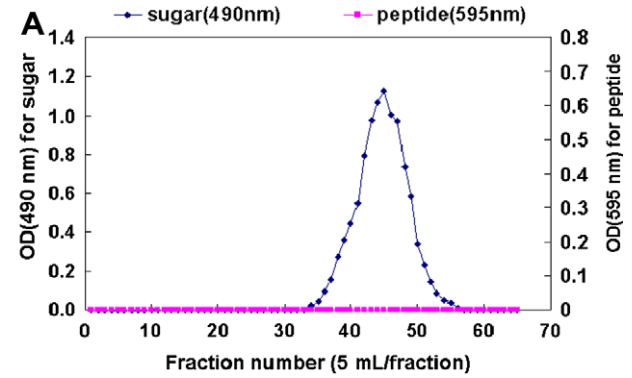

Fig. 4. Average molecular weight of HEB-AP Fr I. (A) Elution profile of polysaccharide refractionated by gel filtration with Sepharose CL-6B. (B) Molecular weights of standard dextrans and HEB-AP Fr I determined by Sepharose CL-6B gel filtration chromatography.

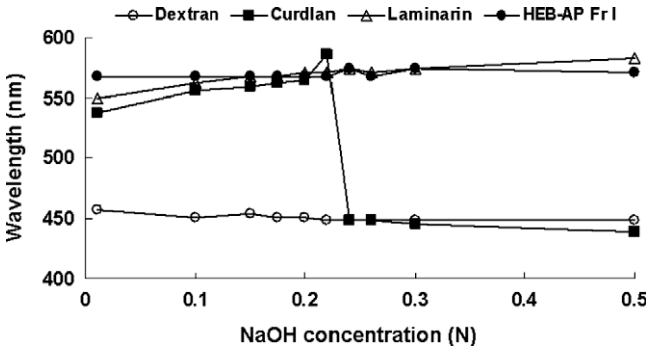

Fig. 5. Helix–coil transition analysis of HEB-AP Fr I and standard polymer according to the absorption maximum of the congo red–polysaccharide complex at various concentrations of NaOH. For more details, see Section 2.

3.8. Glycosidic linkage of the polysaccharide

HEB-AP Fr I 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 HEB-AP Fr I 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} characteristic of a carbohydrate ring (Fig. S2). Following reductive cleavage, HEB-AP Fr I was found to be hydrolyzed to its monosaccharide components, as indicated by comparing the GC traces of

Table 2
Proximate composition and monosaccharide composition of purified polysaccharide, HEB-AP Fr I, from the liquid culture broth of *Hericium erinaceus* (%, dry basis).

Polysaccharide	Protein	Hexosamine	Uronic acid	Total sugar	Component sugar (molar %)			
					Glc	Gal	Man	Fuc
HEB-AP Fr I	N.D.	0.46	2.70	93.50	N.D.	N.D.	100	N.D.

N.D., not detected.

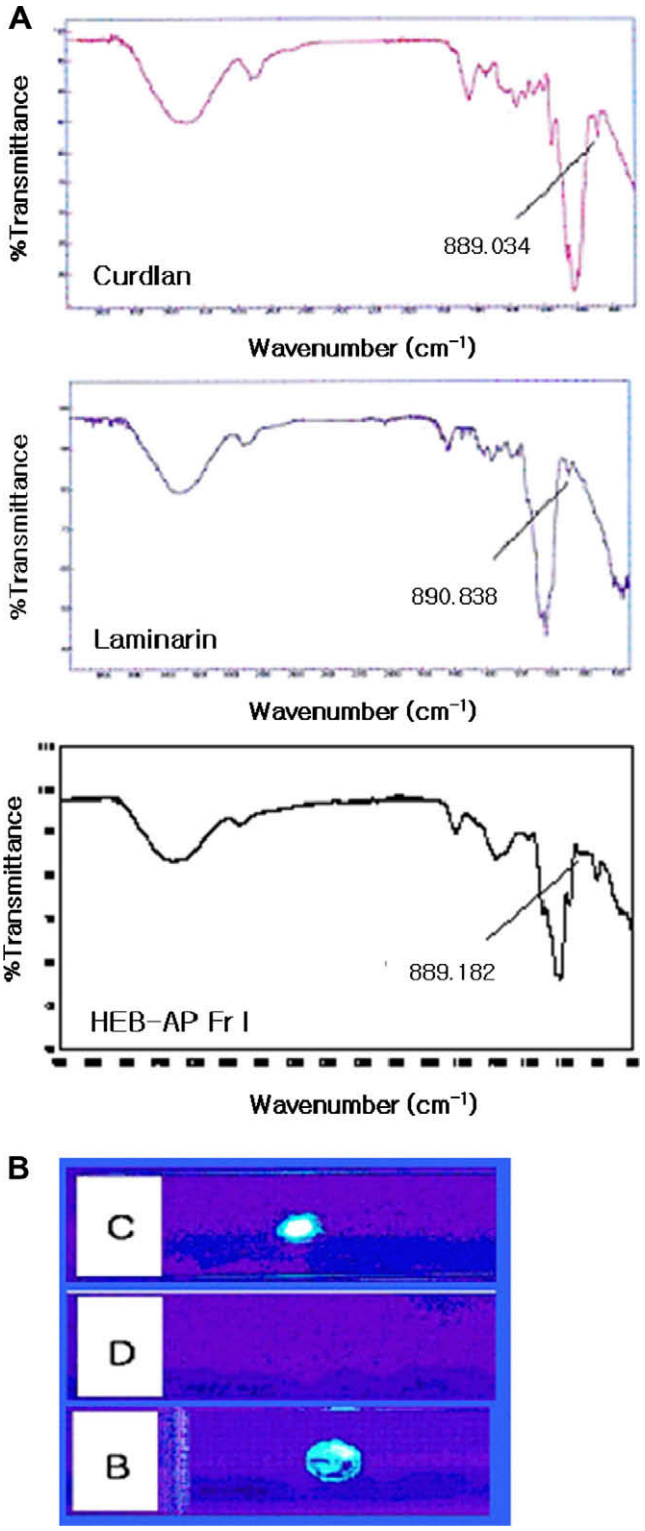

Fig. 6. Identification of the anomeric configuration of HEB-AP Fr I and standard polymers. (A) FT-IR analysis of HEB-AP Fr I and standard polymers showing identical spectra. (B) Visualization of β-linked polysaccharides using the Fungi-Fluor kit. **C**. Curdlan **D**. Dextran **B**. HEB-AP Fr I.

the polysaccharide hydrolysate to those of monosaccharide standards. The data summarized in Table 3 (see also Fig. S3) indicate that the principal component of HEB-AP Fr I is a (1 → 2)-linked mannopyranosyl residue with a (1 → 3)-linked mannopyranosyl side chain, with a degree of branching (DB) of 0.2.

4. Discussion

Polysaccharides obtained from different natural sources represent a structurally diverse class of macromolecules, and are known to affect a variety of biological responses, especially the immune response. Mushroom-derived polysaccharides exert their antitumor action mostly by activating various immune system responses in the host such as complementary system activation (Dennert & Tucker, 1973) and macrophage-dependent immune system responses (Lee et al., 2008). In an indirect manner, activated macrophages play an important role in antitumor activity by secreting secondary compounds, such as tumor necrosis factor-alpha (TNF-α) and nitric oxide (NO), which are harmful to cancer cells, and by regulating the immune system to process and present antigens (Mackay & Pussell, 1986). In the present study, HEB-AP Fr I, which was obtained from the liquid culture broth of *H. erinaceus* by 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. 3). 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 chemical composition, molecular weight, conformation, glycosidic linkage, degree of branching, etc. (Yadomae & Ohno, 1996). In the present study, HEB-AP Fr I, which was found to act as an immunostimulant through the activation of macrophages, was a β-1,3-branched-β-1,2-mannan that had a molecular weight of 46 kDa (Table 3 and Fig. 4). In contrast to the polysaccharide composition of the fruiting body, no glucans have been detected among the antitumor active fractions obtained from the liquid culture of mushrooms. 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). For a long time molecular weight has been recognized as a critical parameter dictating the antigenicity of a molecule (Kabat & Bezer, 1958). Interestingly, a low-molecular-weight (20 kDa) fraction from the fruiting body of *A. 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). In contrast, water-soluble glucan fraction from culture broth of *H. erinaceus*, with molecular weights larger than 100 kDa, exhibited anti-artificial pulmonary metastatic tumor and immunoenhancing effects (Wang, Hu, Su, & Lee, 2001). In this study, HEB-AP Fr I, which was found to act as an immunostimulant through the activation of macrophages, was shown to be a β-mannan with a laminarin-like triple helix conformation (Fig. 5). 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).

In conclusion, HEB-AP Fr I, a small molecular mass polysaccharide with a laminarin-like triple helix conformation of the β-1,3-branched-β-1,2-mannan, was a potent murine macrophage

Table 3
Identification and linkage analysis of partially methylated alditol acetates of the purified polysaccharide, HEB-AP Fr I, isolated from the liquid culture broth of *Hericium erinaceus*.

Polysaccharide	Alditol acetate derivative	Type of linkage	Relative molar ratio
HEB-AP Fr I	1,5-Anhydro-2,3,4,6-tetra-*O*-methyl-D-mannitol	Terminal Man*p*	0.26
	1,5-Anhydro-2-*O*-acetyl-3,4,6-tri-*O*-methyl-D-mannitol	→ 2)-Man*p*-(1 →	1
	1,5-Anhydro-3-*O*-acetyl-2,4,6-tri-*O*-methyl-D-mannitol	→ 3)-Man*p*-(1 →	0.59
	1,5-Anhydro-6-*O*-acetyl-2,3,4-tri-*O*-methyl-D-mannitol	→ 6)-Man*p*-(1 →	0.27
	1,5-Anhydro-2,6-di-*O*-acetyl-3,4-di-*O*-methyl-D-mannitol	→ 2,6)-Man*p*-(1 →	0.40

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.2009.04.036.

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