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Structural Characteristics of Immunostimulating Polysaccharides from *Lentinus edodes*

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There is a significant amount of experimental evidence suggesting that polysaccharides from mushrooms enhance the host immune system by activating various mechanisms in immune cells, including macrophages. In this study, polysaccharides from *Lentinus edodes* were found to stimulate the functional activation of macrophages to secrete inflammatory mediators and cytokines and increase the phagocytotic uptake. The chemical properties of the stimulatory polysaccharides, CPFN-G-I, CPBN-G, and CPBA-G, were determined based on their monosaccharide composition, which mainly consisted of glucose and mannose. According to FT–IR and GC/MS, the structure of CPFN-G-I, purified from the fruiting body of *L. edodes*, was found to consist of a β-1,6-branched-β-1,4-glucan, whereas CPBN-G and CPBA-G, purified from the liquid culture broth, were found to be composed of a heteromannan. The configuration of the β-linkage and triple helical conformation of each polysaccharide were confirmed using a Fungi-Fluor kit and Congo red, respectively.

Keywords: *Lentinus edodes*, immunostimulating polysaccharide, mushroom-derived β-glucan, triple helical conformation, Congo red, structural analysis

A number of bioactive molecules, including antitumor agents, have recently been identified in various higher basidiomycete mushrooms. Furthermore, since the polysaccharides of these mushrooms are known to have no toxic side effects, unlike existing anticancer chemical medications, when used as a cancer therapeutic, these polysaccharides are able to prolong the life span of cancer patients [1]. Thus, polysaccharides are the best known and most potent mushroom-derived substances displaying immunomodulating properties [15, 22]. Pharmacological studies are currently being conducted on the antitumor activity of the polysaccharide β-1,6-branched-β-1,3-glucan from hot-water extractions of *L. edodes*, as it was shown to display antitumor activity against Sarcoma 180 in the 1970s [10, 33]. In most cases, the antitumor activity of polysaccharides is exerted by activating the immune responses of the host organism, such as complementary system activation [12], macrophage-dependent immune system responses [17, 23], and upregulation of interferon expression [8]. Polysaccharides have also been reported to decrease asthenia symptoms, such as fever, which is caused by the tumor necrosis factor (TNF) and anemia [5]. The immunomodulating activities of polysaccharides are usually characterized according to their chemical composition, molecular weight, conformation, glycosidic linkage, and degree of branching. [7, 21, 34].

Biologically active polysaccharides are widespread among mushrooms, and most have unique structures in different species. As a result, several studies have already been conducted to determine the precise structures of these different polysaccharides. Accordingly, the primary goal of this study was to determine the immunostimulating activities and structural characteristics of the following polysaccharides from *Lentinus edodes*: the neutral fraction from the fruiting body (CPFN-G-I, 580 kDa), the neutral fraction from the cultured cell-free broth (CPBN-G, 40 kDa), and the acidic fraction from the cultured cell-free broth (CPBA-G, 46 kDa).

Materials and Methods

Materials

The strain used in this study was *Lentinus edodes* KCTC 6734, which was grown in a liquid containing 60 g/l glucose, 10 g/l yeast

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extract, 1.0 g/l $MgSO_4 \cdot 7H_2O$, and 2.0 g/l KH_2PO_4. The water-soluble crude polysaccharides, CPF and CPB, which were obtained from the fruiting body and culture cell-free broth of *L. edodes* by hot-water extraction and ethanol precipitation, were then fractionated using DEAE cellulose and Sepharose CL-6B column chromatography, resulting in three polysaccharide fractions, CPFN-G-I, CPBN-G, and CPBA-G. These fractions 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, U.S.A). The FITC–dextran, lipopolysaccharide (LPS, *E. coli* 0111:B4), and Congo red were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A), the fetal bovine serum and RPMI1640 cells were obtained from GIBCO (Grand Island, NY, U.S.A), and the RAW264.7 was purchased from the American Tissue Culture Center (Rockville, MD, U.S.A). All other chemicals were of Sigma grade.

Cell Culture

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

Cell Viability

The effect of the polysaccharides on the viability of the RAW264.7 cells was determined using a [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 [4]. After pre-incubating the RAW264.7 cells (1×10^6 cells/ml) for 18 h, CPFN-G-I, CPBN-G, CPBA-G (100 μg/ml), or LPS (2.5 μg/ml) was added and incubated with the cells for an additional 24 h, as reported previously. 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 incubating for 4 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 A540 read using a scanning multi-well spectrophotometer.

Determination of NO Production

After pre-incubating the RAW264.7 cells (1×10^6 cells/ml) for 18 h, CPFN-G-I, CPBN-G, CPBA-G (100 μg/ml), or LPS (2.5 μg/ml) was added and incubated with the cells for an additional 24 h, as reported previously. The nitrite in the culture supernatants was then 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 samples.

TNF-α Production

The induction effect of each purified polysaccharide from *L. edodes*, which was solubilized in the culture medium, on TNF-α production by the LPS-treated RAW264.7 cells was determined as described previously [9]. The supernatants were harvested and the presence of murine TNF-α was determined using an enzyme-linked immunosorbent assay (ELISA).

Determination of Phagocytotic Uptake

A previously described method, with slight modifications, was used to measure the phagocytotic activity of the RAW264.7 cells [14]. The RAW264.7 (2×10^6) cells were resuspended in 1 ml of PBS containing 1% human AB serum, and incubated with FITC–dextran (1 mg/ml) in the presence or absence of LPS (2.5 μg/ml) or each polysaccharide (100 μg/ml) at 37°C for 30 min. The phagocytosis was stopped by adding 2 ml of ice-cold PBS containing 1% human serum and 0.02% sodium azide, and then the cells were washed three times with cold PBS–azide and analyzed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, U.S.A.).

Chemical Properties Analysis

The total sugar content of each polysaccharide was determined using the phenol–sulfuric acid method [6], the total protein concentration determined using the Bradford method [3], the hexosamine content evaluated using the Elson–Morgan method [13], and the uronic acid content assessed using the Blumenkrantz and Asboe-Hansen method [2].

Monosaccharide Composition Analysis

The monosaccharide composition and ratios were determined by first hydrolyzing the polysaccharides with 2 M TFA (trifluorocetic acid) in a sealed tube at 100°C for 4 h. Thereafter, the acid was removed by repeated evaporation using a vacuum distillation device, and then the hydrolysate was dissolved in 1.0 ml of distilled water and filtered using a 0.2 μm PTFE membrane. The aqueous hydrolysate was analyzed by reversed-phase HPLC using a CarboPac PA10 Analytical Column (4 mm×240 mm) with (A) deionized water and (B) 200 mM sodium hydroxide as the solvents based on a program of 0–20 min (B conc. 8%), 20–40 min (B conc. 25%), and 40–70 min (B conc. 8%) at a flow rate of 0.9 ml/min and temperature of 30°C. Glucose, galactose, mannose, and fucose were used as the monosaccharide standards.

Helix–Coil Transition Analysis

The conformational structure of the polysaccharides in the solution was determined by characterizing the Congo red–polysaccharide complexes. The transition from a triple-helical arrangement to a single-stranded conformation was examined by measuring the λ_{max} of the 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 subjected to the different concentrations of NaOH. The visible absorption spectra were recorded using a Genesys 5 UV/visible spectrophotometer at each alkali concentration [26, 27].

Identification of Anomeric Configuration

To ascertain the presence or absence of an α or β configuration in each polysaccharide, a Fungi-Fluor Kit (Polysciences, Warrington, PA, U.S.A.) was used. After the sample was dissolved in distilled water, the solution was placed on a slide and dried in a drying oven. Following the addition of methanol, the slide was dried for an additional 20 min. Thereafter, a few drops of solution A (cellufluor, water, and potassium hydroxide), used as a dye, were added and the mixture was incubated for 3 min. Finally, the slide was washed with distilled water, and the presence or absence of fluorescence confirmed using a UV Illuminator (Vilber Lourmat Inc., France).

Methylation of Polysaccharides

Each polysaccharide was methylated according to the method developed by Ciucanu and Kerek [11], using powdered NaOH in Me_2SO–MeI. Methylation was then confirmed based on the FT–IR spectrum (Bio-Rad EXCALIBER Series).

Glycosidic-Linkage Determination

After extracting the permethylated polysaccharides using dichloromethane, reductive cleavage, using a combination of trimethylsilyl methanesulfonate and trifluoride etherate as the catalyst, was performed as previously reported [28]. The reaction was allowed to proceed for 8–12 h at room temperature, and then the products were isolated and acetylated. The reaction was quenched by adding a solution of sodium bicarbonate. The organic layer was separated using a syringe and analyzed by GC–MS using a micromass apparatus equipped with an HP-5MS column and temperature program (120–180°C at 5°C/min and 180–250°C at 2°C/min). The mass spectra were taken at an ion energy of 70 eV, current intensity of 500 μA, and temperature of 250°C.

Statistical Analysis

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

RESULTS AND DISCUSSION

Macrophage Activation by Polysaccharides

The direct cytotoxicity of each polysaccharide from *L. edodes* was tested against the macrophage-like cell line, RAW264.7. From this analysis, treatment with each polysaccharide was not found to have any appreciable effect on cell viability (Fig. 1A). Among the samples, CPFN-G-I, purified from the fruiting body, was shown to

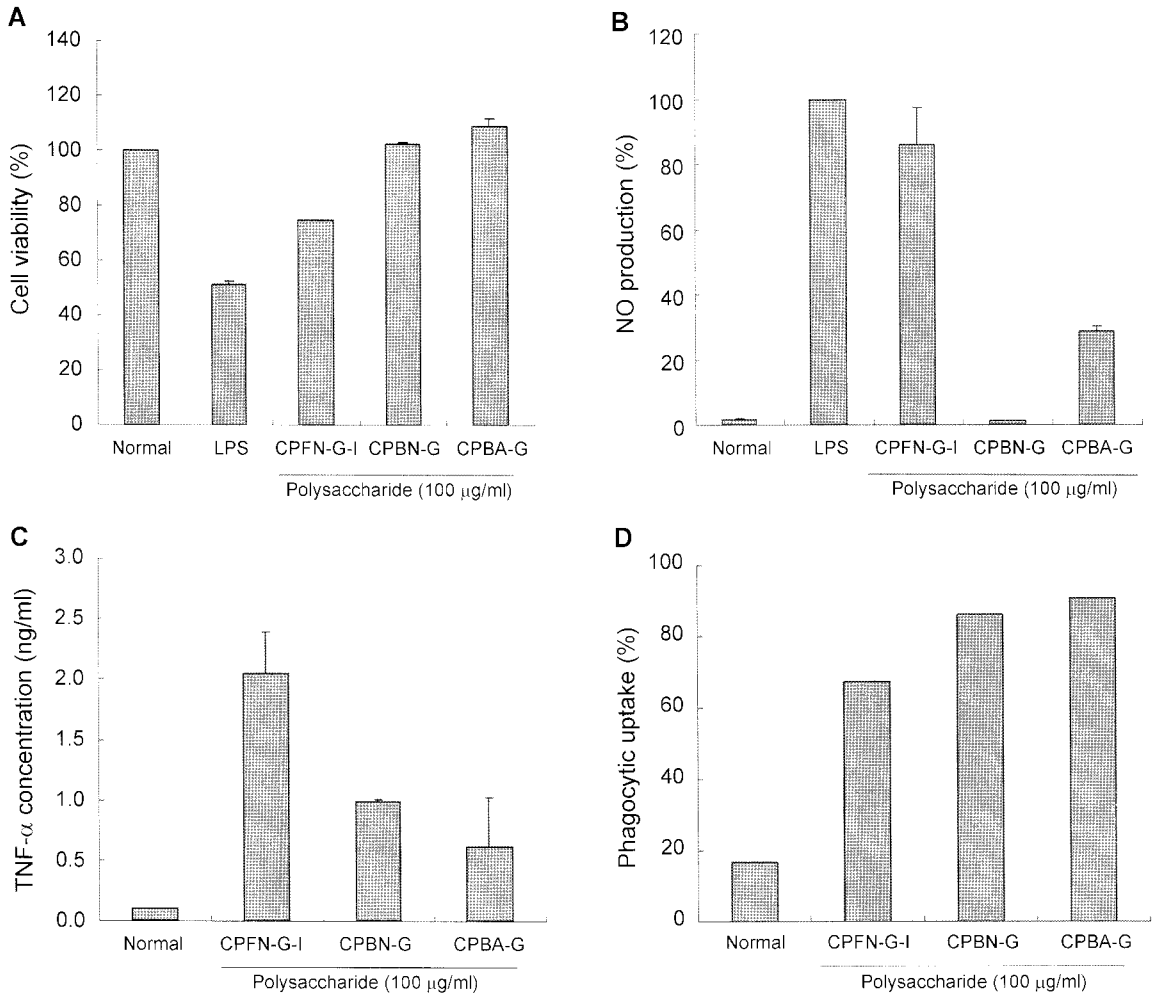

Fig. 1. Immunostimulating effects of purified polysaccharides extracted from cultured cell-free broth and fruiting body of *Letinus edodes*.

A. Effects of polysaccharides from cultured cell-free broth and fruiting body on macrophage cell viability. **B**. Effects of purified polysaccharides from cultured cell-free broth and fruiting body on NO synthesis in murine macrophage cells. **C**. Effects of purified polysaccharides from cultured cell-free broth and fruiting body on TNF-α production in RAW 264.7 cells. **D**. Effects of purified polysaccharides extracted from cultured cell-free broth and fruiting body on phagocytosis of macrophages. The mean fluorescence after drug treatment was compared with the control and represents the phagocytosis index. Each column represents the mean ±SEM of at least three independent experiments.

be slightly more cytotoxic, as the nitric oxide (NO) generated by the sample possibly had an affect on the cell viability. However, since the cell viability was lower than that for LPS, it was difficult to determine whether the cell toxicity was significant.

Macrophages are the first line of defense among innate immune responses against microbial infection [16]. The defense mechanism of macrophages against pathogens includes secreting cytokines, such as the tumor necrosis factor-α (TNF-α), and inflammation mediators, such as NO [24]. The NO produced by macrophages is synthesized enzymatically from L-arginine by NOS. Under strict anaerobic conditions, NO remains stable, yet in the presence of oxygen, NO becomes oxidized, forming higher nitrogen oxides, such as peroxynitrite, and the end products are nitrite and nitrate [31]. Therefore, macrophages were incubated with each polysaccharide or LPS, and the amount of NO production was measured and compared with that produced by the normal control group (Fig. 1B). For all the experimental groups, the amount of NO production was higher than that produced by the control RAW264.7 cells. These results are in agreement with a previous study, where polysaccharides from basidiomycetes were shown to facilitate the secretion of NO by activating macrophages [18]. TNF-α is considered the most important mediator directly involved in killing tumor cells [30]. As shown in Fig. 1C, the amount of TNF-α produced by the macrophages in the presence of each polysaccharide was higher than the amount produced by the untreated cells. Among all the polysaccharides, CPFN-G-I, purified from the fruiting body, exhibited the highest productivity.

Macrophages play a major role in defending the host against infection and cancer [20, 25], as they process and present antigens to the lymphocytes based on engulfing and digesting pathogens that cross the epithelial barrier [32]. Thus, to determine the effect of each polysaccharide on the phagocytotic activity of the macrophages, the uptake of FITC-labeled dextran in the polysaccharide-treated and untreated macrophages was compared (Fig. 1D). From this analysis, it was found that all the polysaccharides increased the phagocytotic uptake of the RAW264.7 cells.

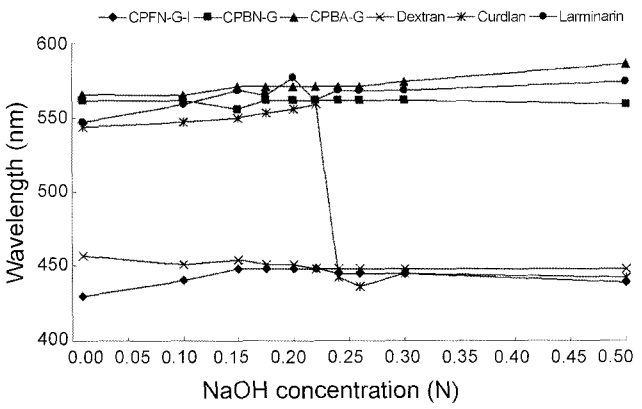

Fig. 2. Helix–coil transition analysis of each polysaccharide and standard polymer according to the absorption maximum of Congo red–polysaccharide complex at various concentrations of NaOH.
For details, see Materials and Methods.

Chemical Properties and Monosaccharide Composition

The total sugar content of CPFN-G-I, CPBN-G, and CPBA-G was 92.37%, 91.75%, and 93.58%, respectively. The sugar composition of CPFN-G-I was mainly glucose, whereas CPBN-G contained mannose (89.23%) and galactose (6.43%) as the major sugar constituents, and CPBA-G was primarily composed of mannose (82.35%) and glucose (16.53%). Han *et al.* [19] reported that during the liquid culture period, the main carbon source was glucose, and when the carbon source ran out, the amount of glucose in the polysaccharide decreased, while the amount of mannose increased. It is also worth noting that the composition of the polysaccharides in the liquid culture may depend on the composition of the nutrient medium used. Protein was not detected in any of the polysaccharides. The hexosamine content in CPFN-G-I, CPBN-G, and CPBA-G was found to be 0.09%, 0.65%, and 0.38%, respectively, whereas the uronic acid content was determined to be 2.24%, 1.64%, and 2.27%, respectively.

Identification of Helix–Coil Transition

A shift in the visible absorption maximum of Congo red, induced by the presence of the polysaccharides, can be used to provide conformational information. The absorption maximum of dextran, a random coil conformation, remained around 450 nm, whereas curdlan exhibited a triple helical

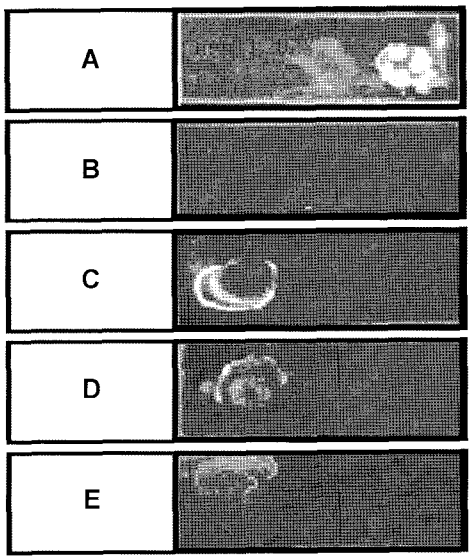

Fig. 3. Visualization of β-linked polysaccharides determined using the Fungi-Fluor Kit.
A. Curdlan; **B.** Dextran; **C.** CPFN-G-I; **D.** CPBN-G; **E.** CPBA-G.

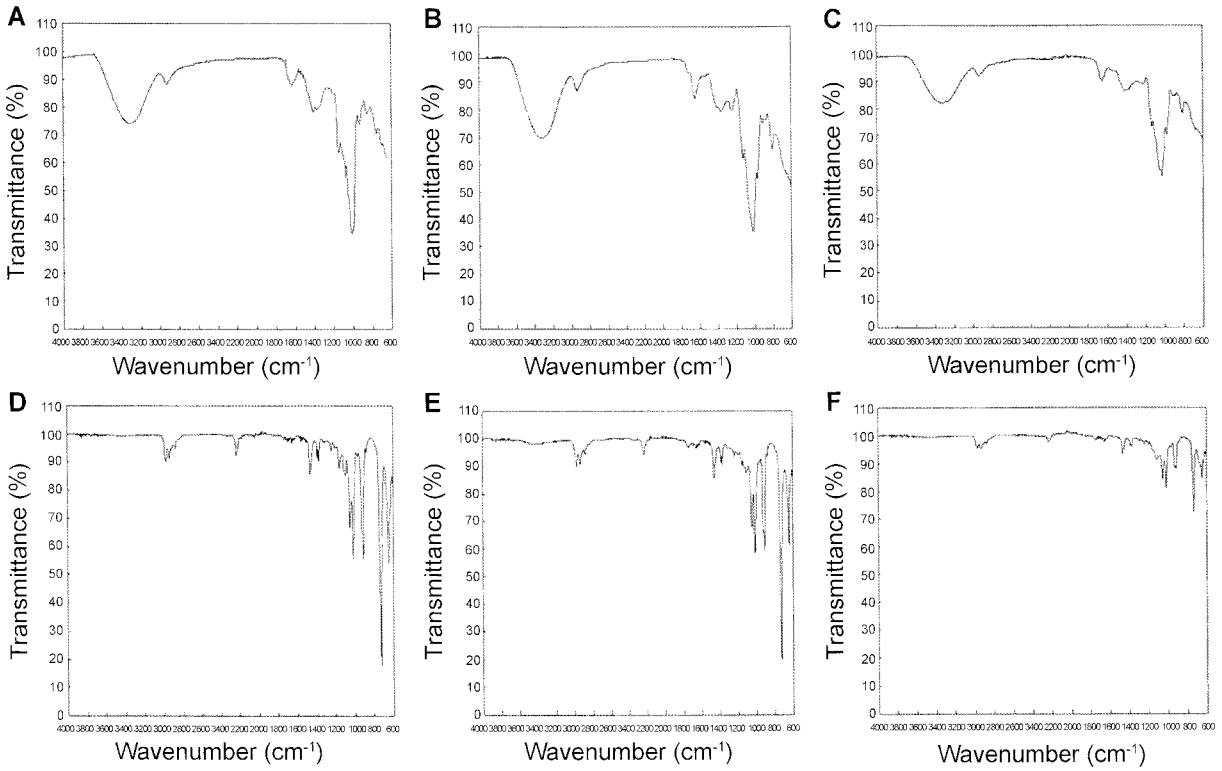

Fig. 4. Identical FT-IR spectra for purified (**A** to **C**) and permethylated polysaccharides (**D** to **F**) derived from fruiting body and cultured cell-free broth.

A. CPFN-G-I; **B**. CPBN-G; **C**. CPBA-G; **D**. permethylated CPFN-G-I; **E**. permetylated CPBN-G; **F**. permethylated CPBA-G.

conformation, as shown by the shift in the absorption maximum at 0.24 M NaOH. However, the adsorption maximum of laminarin, another triple helical conformation, was around 580 nm and did not change. Therefore, based on this analysis, CPFN-G-I exhibited a random coil conformation, whereas CPBN-G and CPBA-G exhibited a triple helical conformation similar to laminarin (Fig. 2).

Identification of Anomeric Configuration

The staining solution in the Fungi-Fluor it consisted of a 0.05% solution of highly pure cellufluor in deionized water with potassium hydroxide added as a clearing agent. Cellufluor can be used for rapid identification by binding nonspecifically to β-linked polysaccharides [29]. As shown in Fig. 3, whereas no fluorescence was observed in the

Table 1. Identification and linkage analysis of partially methylated alditol acetates from purified polysaccharides by GC-MS.

Polysaccharide	Alditol acetate derivative	Type of linkage	Relative molar ratio
CPFN-G-	1,5-Anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol	Terminal Glc*p*	1
	1,5-Anhydro-4-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol	→4)-Glc*p*-(1→	1.75
	1,5-Anhydro-4,6-di-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol	→4,6)-Glc*p*-(1→	1
CPBN-G	1,5-Anhydro-2,3,4,6-tetra-*O*-methyl-D-galactitol	Terminal Gal*p*	1
	1,5-Anhydro-2-*O*-acetyl-3,4,6-tri-*O*-methyl-D-mannitol	→2)-Man*p*-(1→	3
	1,5-Anhydro-3-*O*-acetyl-2,4,6-tri-*O*-methyl-D-mannitol	→3)-Man*p*-(1→	2
	1,5-Anhydro-3,6-di-*O*-acetyl-2,4-di-*O*-methyl-D-mannitol	→3,6)-Man*p*-(1→	1
CPBA-G	1,5-Anhydro-2,3,4,6-tetra-*O*-methyl-D-mannitol	Terminal Man*p*	3
	1,5-Anhydro-2-*O*-acetyl-3,4,6-tri-*O*-methyl-D-mannitol	→2)-Man*p*-(1→	3
	1,5-Anhydro-3-*O*-acetyl-2,4,6-tri-*O*-methyl-D-mannitol	→3)-Man*p*-(1→	3
	1,5-Anhydro-6-*O*-acetyl-2,3,4-tri-*O*-methyl-D-mannitol	→6)-Man*p*-(1→	1
	1,5-Anhydro-2,6-di-*O*-acetyl-3,4-di-*O*-methyl-D-glucitol	→2,6)-Glc*p*-(1→	2
	1,5-Anhydro-3,6-di-*O*-acetyl-2,4-di-*O*-methyl-D-mannitol	→3,6)-Man*p*-(1→	1
	1,5-Anhydro-4,6-di-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol	→4,6)-Glc*p*-(1→	2

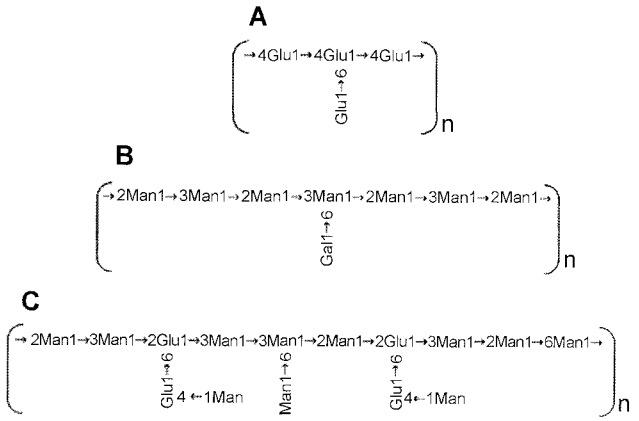

Fig. 5. Repeating unit of purified immunostimulating polysaccharides. **A**. CPFN-G-I; **B**. CPBN-G; **C**. CPBA-G.

dextran samples (known as α-glucan), it was clearly observed in the curdlan samples (known as β-glucan). Thus, when the CPFN-G-I, CPBN-G, and CPBA-G polysaccharides, all β-linked polysaccharides, were subjected to this assay, they all displayed a very similar fluorescence to the curdlan sample.

Glycosidic Linkage of Polysaccharides

The reductive cleavage method was used for the glycosidic linkage analysis of the fully methylated polysaccharides [28]. All the samples exhibited the characteristic IR absorption of polysaccharides at 1,080 cm^{-1} (C=O), 2,800 cm^{-1} to ~2,900 cm^{-1} (C-H), and 3,400 cm^{-1} (O-H). However, after methylation, the characteristic carbohydrate ring at 3,400 cm^{-1} disappeared, confirming that each polysaccharide had been fully methylated (Fig. 4). The monosaccharide component of the polysaccharide hydrolyzates, as determined from the GC traces of the polysaccharide hydrolyzates in comparison with standard monosaccharides, are summarized in Table 1. Therefore, the results indicated that the principal component of CPFN-G-I was a (1→4)-linked glucopyranosyl residue with a (1→6)-linked glucopyranosyl residue as a side chain, and the DB was determined to be 0.33 (Fig. 5A). The composition of CPBN-G was found to be a (1→6)-branched-(1→2,3)-galactomannan with a DB of 0.17 (Fig. 5B). Finally, the GC–MS analysis of CPBA-G revealed the presence of seven components in a molar ratio of 3:3:3:1:2:1:2, as summarized in Table. 1, indicating that CPBA-G consisted of a (1→6)-branched-(1→2,3)-glucomannan with a DB of 0.3 (Fig. 5C).

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