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Immunostimulating Activity and Characterization of Polysaccharides from Mycelium of *Phellinus linteus*

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Hot-water extract, Fr. 1, of *Phellinus linteus* mycelium was fractionated into Fr. 2, 3, 4, and 5 by the difference of solubility in ethanol. The polysaccharide fractions were studied for their immunostimulating activity on *in vitro* T-independent polyclonal antibody response to trinitrophenyl-haptened SRBC (sheep red blood cell). The Fr. 4 with the highest immunostimulating activity was subjected to DEAE-cellulose ion exchange chromatography and gave five fractions, 4-I, II, III, IV, and V. The *in vitro* immunostimulating assay of the five fractions showed that 4-I and 4-III had a similar activity to that of LPS but the other fractions had low activity. By analyses of chemical composition and HPLC, all fractions obtained were found to be heteropolysaccharide-protein complex. The molecular weights ranged from 9,000 to 15,000. Sugar analyses showed that glucose, galactose, mannose, arabinose, and xylose were main component. Uronic acid and amino sugar were also detected in the fractions. It should be noted that the molecular weight (15,000) of 4-III was very small and the structure of 4-III may be different from the known immunostimulating branched β-(1→3)-glucan.

Enormous effort has been made in the field of chemotherapeutic treatment of cancer but no decisive cancer chemotherapy has been developed so far. It was known that fungi or their components can induce antigen-specific immune responses as well as stimulate non-specific antigen-independent events (7). Therefore, fungal cell wall was considered and tested as a source of new and potential therapeutic agents against tumor. Numerous polysaccharides from different biological origins, e.g., yeast, algae, bacteria, higher plants and especially fungi have been investigated for anti-tumor and immunomodulation activities (9). Biologically active polysaccharides showed anti-tumor effects against allogenic, syngenic, and even autologous tumors. Most anti-tumor polysaccharides have been isolated from basidiomycetes. β-(1-3)-glucans were known to be the most efficient. Lentinan (4), Schizophyllan (28), Krestin (29), and Meshima (11) are now in clinical use.

Maeda *et al* (21) reported that hot-water extracts of *Phellinus linteus* showed higher levels of inhibition against sarcoma 180. This mushroom has also been a popular folk or oriental medicine to cure stomachache and knee arthritis. The fungus is thus attracting attention as a material for the development of drugs. Previously, we reported that a polysaccharide with a huge molecular weight (153 kDa) stimulated polyclonal antibody production in an *in vitro* culture system (27). In this study, we isolated and characterized five polysaccharides with a small molecular weight. The relationship between the structure of the polysaccharide and its immunostimulating activity was discussed.

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Key words: *Phellinus linteus*, polysaccharide, immunostimulating activity

MATERIALS AND METHODS

Preparation of Polysaccharide and Fractionation

Mycelium of *P. linteus* (KCTC 0173BP) was cultured as previously described (11). Hot-water extract, Fr. 1, from the mycelium was treated with ethanol to final concentration of 80% (v/v) and left for 2 days at 4°C. The precipitated polysaccharide, Fr. 3, was collected by centrifugation (6000× g, 20 min) and further fractionated into Fr. 4 and Fr. 5 by solubility difference in 60% ethanol. All fractions were concentrated, dialyzed, and freeze-dried.

DEAE-cellulose Ion Exchange Chromatography

Two grams of Fr. 4 was subjected to the DEAE-cellulose (Merk Art. 3201) chromatography which was

equilibrated with 5mM sodium phosphate buffer, pH 7.7. The column was washed with the same buffer to remove the nonbinding material. The binding material was removed with a linear gradient of 1.0 M NaCl in the same buffer. Each fraction of 10 ml was collected. The fraction was measured for sugar and protein, and divided into 4-I, II, III, IV, and V.

General Analytical Methods

Total sugar content was measured by the phenol-sulfuric acid method (8) and expressed as the polysaccharide content. Glucose was used as a standard. Protein was measured by the Bradford method (3) with Bio-Rad protein assay reagent (California, U.S.A.) using bovine serum albumin as a standard. Colorimetric measurement of uronic acid was done by meta-hydroxydiphenyl reagent using glucuronic acid as a standard (2). The measurement of amino sugar was made by benzothiazolone hydrazone assay using glucosamine as a standard (26).

Sugar Composition of Polysaccharides

The polysaccharides (1 mg) were hydrolyzed with 1 ml of 2 M trifluoroacetic acid (TFA) for 2 h at 121°C in a screw-capped tube. Acid was removed by repeated evaporation. Sugars were derivatized as the corresponding alditol acetates conventionally. The sugar alditol acetates were analyzed by Varian gas chromatography (model GC 3400 fitted with a flame-ionization detector) using an SP-2330 fused silica capillary column (0.32 mm i.d.×30 m., 0.2 film thickness) (Supelco, Bellefonte, BA). A column temperature program was used: 200°C for the first 2 min then 4°C/min up to 250°C, which was then held for 10 min. The temperature of both injector and detector was 250°C.

Infrared Spectrum of 4-III

IR spectrum of 4-III was recorded with an FT-IR Spectrometer (Laser precision Analet RFX-65s) on KBr disk.

***In vitro* Immunostimulating Activity**

The plaque forming cell (PFC) response to sheep red blood cell (sRBC) was determined by the previously described method (24). Briefly, spleen cells were suspended in RPMI 1640 with 10% fetal calf serum and adjusted to 5×10^6 cells/ml. Sample or LPS were added to them. The polyclonal antibody response was measured after *in vitro* stimulation for two days. The number of PFC was counted by a haematocytometer.

RESULTS AND DISCUSSION

Hot-water extract, Fr. 1, from mycelium of *P. linteus* was treated with differential concentrations of ethanol to afford Fr. 2, 3, 4, and 5 (Fig. 1). PFC assay of each polysaccharide fraction at 1mg/ml showed that the fractions

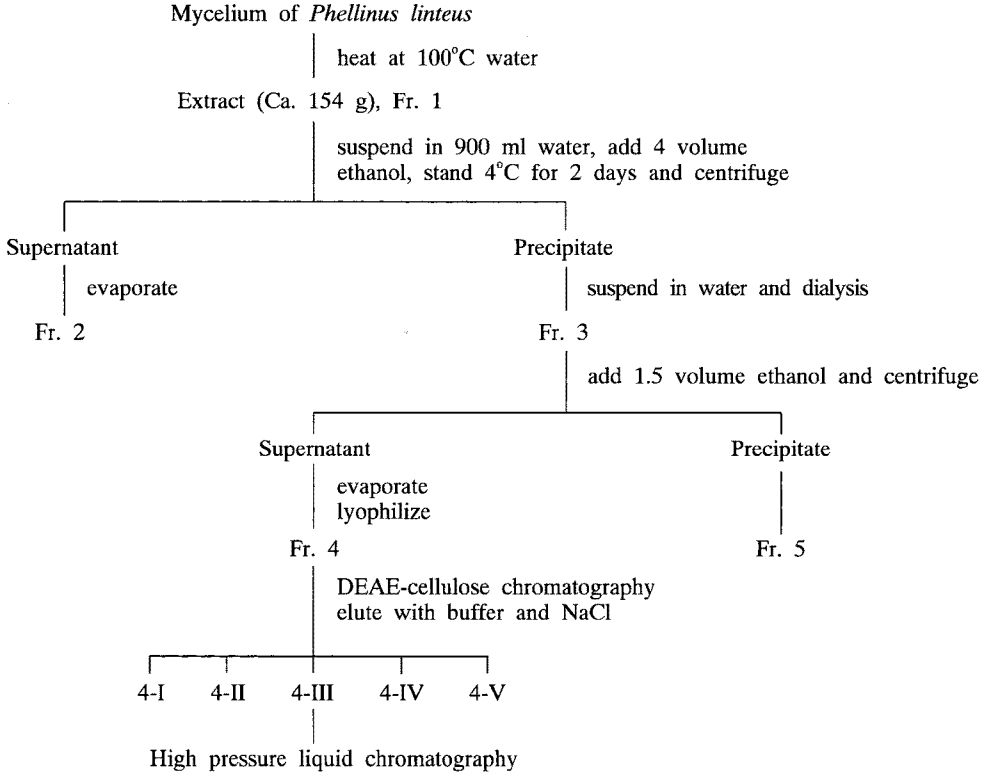

Fig. 1. Purification scheme of polysaccharide with immunostimulating activity from cultured mycelium of *Phellinus linteus*.

had different immunostimulating activity (Fig. 2). Fr. 4 showed the highest activity among the tested fractions and had 93% of LPS activity, which was used as a positive control. Fr. 3 and 5 had a relatively higher activity. Fr. 1 and 2 did not significantly increase the number of PFC. To purify the active material, Fr. 4 was subjected to DEAE-cellulose ion exchange chromatography. Sugar and protein were measured for eluate. Non-binding fractions were collected and designated as 4-I and 4-II, and the binding fraction, which was eluted with a linear gradient of 1 M NaCl, was divided into 4-III, IV, and 4-V (Fig. 3). At the concentration of 1 mg/ml, the five fractions were tested for PFC activity. Fraction 4-I and 4-III increased significantly the number of PFC immunoglobulin to sRBC (Fig. 4). Thus 4-III showed a little higher activity (115%) than that of LPS. The activity of Fraction 4-I was 88% of LPS activity. Other fractions showed a relatively low activity. While the activity was lowered at the concentrations of 0.1 mg/ml and 0.01 mg/ml, the activity pattern was similar to that of 1 mg/ml concentration (data not shown).

As all five fractions were considered as polysaccharide but their activity was different to each other, the physicochemical properties of the fractions were investigated in order to study what factors determined the activity. Table 1 showed that all obtained fractions were polysaccharide-protein complexes. Sugar contents in five fractions was measured from 23 to 67% and especially, fraction 4-IV had the highest sugar content being 67%. The protein content also varied from 2 to 10%. In 4-IV and 4-V, a higher amount of protein was detected. Both 4-I and 4-III had a similar amount of sugar (30%) and protein (2%). The contents of sugar and protein were lower than the expected value. Although the phenomenon is generally observed in heteropolysaccharide-protein com-

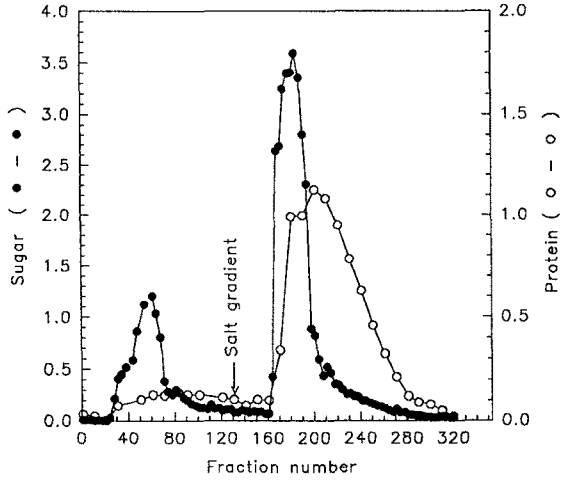

Fig. 3. Elution profile of polysaccharide Fr. 4 on DEAE-cellulose ion exchange chromatography.
Sugar (●—●) and protein (○—○) were measured by phenol-sulfuric acid and Bradford method, respectively. After elution of the column with 5 mM sodium phosphate buffer, pH 7.7, the bound material was eluted with a linear gradient from 0 to 1 M NaCl in the same buffer. Fractions of 22-78, 79-161, 162-198, 199-280, and 281-320 were combined, dialyzed, freeze-dried and afforded 4-I, 4-II, 4-III, 4-IV, and 4-V, respectively.

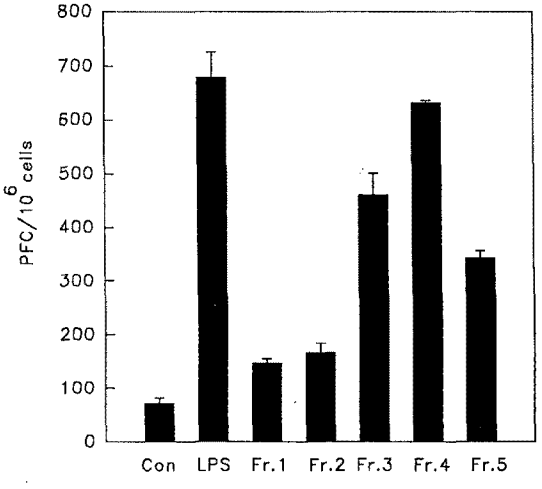

Fig. 2. Immunostimulating activity of polysaccharide fractions from hot water extract of mycelium of *P. linteus*.
Spleen cells were suspended in RPMI 1640 with 10% fetal calf serum and adjusted to 5×10^6 cells/ml. Sample (1 mg/ml) and LPS were added for activation of B cell to the cells and incubated at 37°C for 2 days. The immunized cell suspension was mixed with complement, indicator cell of TNP-sRBC, and agarose. The number of plaque forming cell (PFC) was counted.

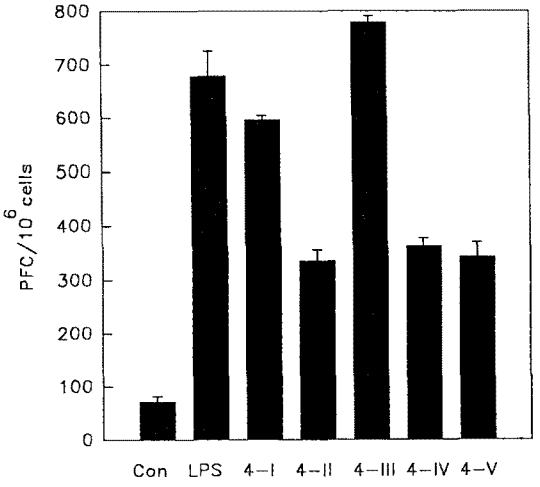

Fig. 4. Immunostimulating activity of polysaccharide fractions from DEAE-cellulose ion exchange chromatography of Fr. 4.
Sample (1 mg/ml) and LPS were added for activation of B cell to spleen cells and incubated at 37°C for 2 days. The immunized cell suspension was mixed with complement, indicator cell of TNP-sRBC, and agarose. The number of plaque forming cell (PFC) was counted.

plexes (5, 13, 20), the reason is not yet known. Analysis of sugar alditol acetate by gas chromatography showed glucose (Glc), galactose (Gal), mannose (Man), xylose (Xyl), and arabinose (Ara) as constituent sugars of the polysaccharides (Table 2). Also a reasonable amount of amino sugar, which has not been identified yet, was detected in all five fractions. Uronic acid was measured in 4-III, IV, and 4-V and identified as glucuronic acid (data not shown). Thus Table 2 indicated that all fractions were heteropolysaccharide and 4-I and 4-III were glucan and mannan, respectively. Many heteropolysaccharide with anti-tumor or immunomodulating activity were isolated from basidiomycetes (5, 6, 18, 23).

Molecular weight of all fractions was estimated by HP-GPC with pullulan as a standard. The elution pattern of fraction 4-III indicated homogeneity and other fractions showed a symmetrical peak similarly (data not shown). Also sugar and protein was eluted at almost the same retention time, which confirmed that the 4-III was a heteropolysaccharide-protein complex. Table 3 showed that the molecular weight of the fractions ranged from 9,000 to 15,000. The molecular weight was relatively small compared to those of the known anti-tumor polysaccharides. Although a polysaccharide from Iceland moss *Cetraria islandica* had a relatively small molecular weight of 18,000, however, it showed a pronounced immunostimulating activity (14). β-(1→3)-glucan with a molecular weight of 21,000, which was obtained by heat treatment of the corresponding intact β-glucan from *Grifola frondosa*, also showed the effect of B-lymphocyte activation. But the β-glucan of 6,400 did not show the effect (1). It has been also suggested that the critical molecular weight of branched β-(1→3)-glucan for activation of the alternative complement pathway is larger than that of linear glucan (12). Our results suggested that the limit of molecular weight of a heteropolymer could be lowered than that of a homopolymer for immunostimulating activity. Therefore, the critical molecular weight for the immunomodulating effect seems to be different for homopolymers and heteropolymers.

Table 1. Yield and chemical composition of fractions from DEAE-cellulose chromatography.

Fractions	Yield (mg)	Carbohydrate (%)	Protein (%)
4-I	116	31.9	1.53
4-II	90	23.1	2.14
4-III	740	30.7	2.47
4-IV	358	66.2	6.29
4-V	40	35.3	9.63

Table 2. Sugar component of fractions from DEAE-cellulose chromatography.

Fractions	Component sugar						
	molar ratio (%) of neutral sugar					uronic acid (μg/mg)	amino sugar (μg/mg)
	Glc	Gal	Man	Xyl	Ara		
4-I	40.8	14.4	17.8	6.8	16.2	-	45
4-II	15.9	10.4	34.3	16.1	15.8	-	50
4-III	9.3	14.3	35.8	18.3	22.3	52.3	58.4
4-IV	15.8	14.2	33.0	17.5	19.5	4.5	85.0
4-V	50.9	-	13.0	21.2	14.9	5.2	35.1

Table 3. Weight average moecular weight of fractions from DEAE-cellulose chromatography.

Fractions	Molecular weight (Da)
4-I	9,400
4-II	9,200
4-III	15,000
4-IV	13,600
4-V	11,500

Many researches of homopolymer β-glucan suggested that the anti-tumor activity was mediated through immunopotentiation such as macrophage activation, T and B lymphocytes activation. The glucan is thus a broad-spectrum enhancer of host immune systems. But tylopilan from *Tylopilus felleus* fruit bodies showed cytotoxic activity on tumor cell (10). Kobayashi *et al* (17) reported that protein-bound polysaccharide of *Coriolus versicolor* expressed the mimicking activity of superoxide dismutase. Heteropolysaccharide seems to have a similar immunostimulating mechanism to the branched β-(1→3)-glucan (14, 18). Fraction 4-I and 4-III may thus affect other cells such as T cells or alternative complement pathway, and macrophage in addition to B cells. It was reported that Fr. 1 had an activation effect on macrophage and T lymphocytes (30).

Further characterization of 4-III was carried out be-

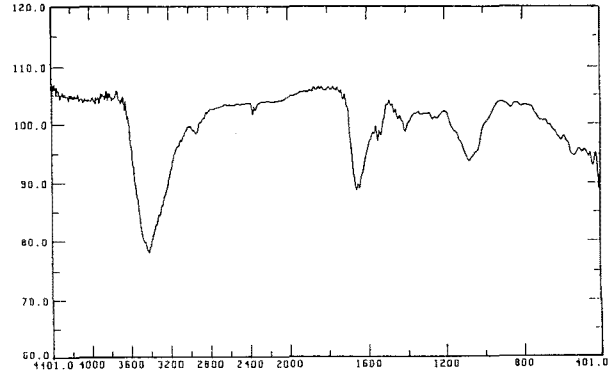

Fig. 5. IR spectrum of 4-III.
The IR spectrum was recorded on a FT-IR spectrometer using a potassium bromide disc.

cause the fraction had the highest activity. The IR spectrum of 4-III showed the typical characteristics of a polysaccharide (Fig. 5). The O-H stretching, C-O stretching, C-H bending and C-O bending frequencies were observed at 3,300-3,400, 1,630, and 1,000-1,100 cm^{-1}, respectively. The product of 4-III with $NaIO_4$ and $NaBH_4$ treatment, 4-III polyol, did not show the activity (data not shown). The branched β-(1→3) glucan with the same treatment, however, increased the activity (16, 22). Linkage type is known as an important factor for the antitumor or immunostimulating activity (19). Thus the structure of 4-III might be different from the β-(1→3)-glucan. The chemical structure of 4-III is being studied.

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